

Efeitos de compostos naturais sobre a atividade peroxidásica de AhpCs e sobrevivência de bactérias patogênicas

Vitória Isabela Montanhero Cabrera

UNIVERSIDADE ESTADUAL PAULISTA

“Júlio de Mesquita Filho”

INSTITUTO DE BIOCIÊNCIAS

CÂMPUS DO LITORAL PAULISTA

Efeitos de compostos naturais sobre a atividade peroxidásica de
AhpCs e sobrevivência de bactérias patogênicas.

Vitória Isabela Montanhero Cabrera

Marcos Antônio de Oliveira

Dissertação apresentada ao Instituto de Biociências,
Campus do Litoral Paulista, UNESP, para obtenção
do título de Mestre no Programa de Pós-Graduação
em Biodiversidade de Ambientes Costeiros.

SÃO VICENTE - SP

2023

UNIVERSIDADE ESTADUAL PAULISTA

“Júlio de Mesquita Filho”

INSTITUTO DE BIOCÊNCIAS

CÂMPUS DO LITORAL PAULISTA

Efeitos de compostos naturais sobre a atividade peroxidásica de AhpCs e sobrevivência de bactérias patogênicas.

Vitória Isabela Montanhero Cabrera

Orientador: Prof. Dr. Marcos Antônio de Oliveira

Dissertação apresentada ao Instituto de Biociências, Campus do Litoral Paulista, UNESP, para obtenção do título de Mestre no Programa de Pós-Graduação em Biodiversidade de Ambientes Costeiros.

DATA: 02 /02 / 2023

Prof. Dr. Marcos Antônio de Oliveira

Prof. Dr. João Henrique Guilhardi Lago

Prof. Dr. Marcos Hikari Toyama

SÃO VICENTE - SP

2023

C117e

Cabrera, Vitória Isabela Montanhero

Efeitos de compostos naturais sobre a atividade peroxidásica de AhpCs e sobrevivência de bactérias patogênicas / Vitória Isabela Montanhero Cabrera. -- , 2023

183 p. : il., tabs.

Dissertação (mestrado) - Universidade Estadual Paulista (Unesp), Instituto de Biociências, São Vicente,

Orientador: Marcos Antonio de Oliveira

1. Peroxirredoxinas. 2. Inibidores. 3. Compostos naturais. 4. Peróxidos orgânicos. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca do Instituto de Biociências, São Vicente. Dados fornecidos pelo autor(a).

Essa ficha não pode ser modificada.

Dedico essa dissertação a todos que me deram apoio durante os momentos mais difíceis da vida, aos que estão nessa jornada desde o meu primeiro ano de graduação e também aos que entraram na minha vida no período de 2020 a 2022.

Dedico a família da senhora Pedrina por me acolherem como uma filha, a família Ralha por me dar ajuda e afeto, aos meus amigos que conheci no LABIMES por me aguentarem em momentos de crise.

Dedico ao meu orientador Marcos Antônio de Oliveira por ter paciência durante todos esses anos que estou como orientada, e de me dar uma oportunidade de encontrar um rumo para minha vida.

AGRADECIMENTOS

Primeiramente agradeço ao meu orientador Professor Dr. Marcos Antônio de Oliveira por além de ter tido paciência comigo desde o meu primeiro ano de faculdade, permitiu que eu tivesse várias oportunidades na minha vida, confiou em meu trabalho desde a minha primeira bolsa de iniciação científica. Tenho grande admiração pelo seu trabalho, e por todos os ensinamentos que foram dados. Muito obrigada por tudo! Por todo o empenho em me auxiliar no trabalho/projeto e por toda preocupação. Serei eternamente grata.

À Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) pela concessão da bolsa, processo nº 2020/02868-1.

À Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) pelo apoio financeiro através do CEPID - REDOXOMA, processo nº 2013/07937-8, e do auxílio regular processo nº 2017/07937-8

Agradeço ao Professor Dr. João G.H. Lago, por fornecer os compostos aqui apresentados no trabalho, e por sempre sanar as dúvidas referentes aos mesmos. Muito obrigada.

Agradeço ao programa de pós graduação que permitiu a minha formação e a toda a equipe acadêmica e principalmente a Supervisora Técnica de Seção da PPG-BAC Fabiana Cecchi Cerqueira, por sempre sanar minhas dúvidas sobre o programa.

Agradeço aos meus amigos do LABIMES, Gabrielle, Sabrina e Ary que fui a responsável em co-orientar durante este ano, me ensinaram a ser uma pessoa mais comunicativa e um pouco menos insegura para ensinar. Agradeço a Gabrielle por me aguentar em momentos de crises de ansiedade, a Sabrina por se preocupar comigo e sempre me fazer rir, ao Ary por me fazer companhia e me escutar em momento difíceis. Sou grata a todos vocês

Agradeço a todos que conheci no LABIMES, alguns não tenho mais contado, mas fizeram sua parte no caminho da minha vida, agradeço imensamente a Melina (Melinissima) por todos esses anos me auxiliando, sempre tirando dúvidas, e além disso, sendo uma ótima amiga que posso contar para tudo. Obrigada por tudo Melina. Agradeço também ao Carlão por ter socorrido em alguns momentos em que estávamos perdidos em como fazer alguns ensaios, e por ter me ajudado a deixar de ser tão nervosa durante apresentação de seminários. Obrigado Carlão!

Agradeço a Thaisa, por ter sido minha melhor amiga nesses últimos 7 anos, por sempre estar por perto quando preciso, você será uma amizade que quero levar para vida toda. Agradeço ao Pedro Henrique por ter me ajudado a arrumar um lugar para ficar, sendo este lugar

a casa da Dona Pedrina, sua avó, e me permitindo ter um ano que pude ter paz na vida e pude viver o que não vive em 23 anos. Agradeço a Dona Pedrina por ter me acolhida em sua casa como uma filha!

Agradeço imensamente a todos que puderem fazer desse período de mestrado, que foi grande parte na pandemia, um dos melhores momentos da minha vida, tanto em questão de liberdade e felicidade, como em questão da formação da minha carreira, onde posso ver que finalmente poderei ter um futuro com importância e alegria.

Muito obrigada a todos.

RESUMO

A resistência de bactérias patogênicas a múltiplos antibióticos aumentou em todo o mundo nos últimos anos, exigindo a busca de novos compostos antibacterianos. Estudos indicam que ação convergente de algumas classes de antibióticos, independente do mecanismo de ação, é a geração de espécies reativas de oxigênio (EROS), o que contribui para a morte de patógenos bacterianos. As bactérias possuem várias enzimas antioxidantes capazes de decompor as EROS, entre elas, as peroxirredoxinas 2-Cys típicas, denominadas de AhpC em bactérias, são peroxidases altamente reativas e extremamente abundantes no ambiente celular, e estudos sugerem que a atividade dessa enzima seria um fator importante para virulência de determinadas cepas bacterianas. A alta reatividade de AhpC está relacionada a uma tríade catalítica, composta por dois resíduos polares (Thr/Ser e Arg) que mantêm o enxofre da cisteína catalítica, denominada de cisteína peroxidásica (C_P) na forma tiolato (S⁻) e estão envolvidos no direcionamento e estabilização do substrato para permitir a substituição nucleofílica bimolecular química (S_N2). Apesar da importância da AhpC em bactérias, até o presente momento nenhum inibidor foi identificado para estas enzimas. Por outro lado, alguns produtos naturais foram identificados como inibidores de isoformas humanas. De forma geral estes compostos apresentam como características comuns um esqueleto hidrofóbico volumoso e um sistema carbonílico capaz de realizar uma adição de tiól-Michael. Neste trabalho, avaliamos a atividade inibitória de compostos naturais oriundos da biota costeira do estado de São Paulo sobre a atividade peroxidase de AhpC de *Pseudomonas aeruginosa* (PaAhpC) e *Staphylococcus epidermidis* (SeAhpC), duas bactérias oportunistas Gram negativas e positivas, respectivamente, responsáveis por diversas infecções hospitalares. A seleção dos compostos foi efetuada com base em características funcionais e estruturais de compostos naturais identificados para isoformas humanas. Através de ensaios bioquímicos identificamos um ácido benzoico prenilado (CN-ABP1) oriundo de *Piper crassinervium* foi capaz de inibir a atividade peroxidásica de PaAhpC, mas não inibiu SeAhpC ou da isoforma humana (HsPrx2). Adicionalmente demonstramos que CN-ABP1 não é capaz de inibir outras tiól enzimas. A determinação do IC₅₀ foi de 20.3 µM e por SDS PAGE mostramos que o composto não foi capaz de realizar uma adição de tiól-Michael, indicando um modo de inibição ainda não descrito para este grupo de proteínas. Resultados de simulações envolvendo *docking* molecular, indicam que o composto é estabilizado no sítio catalítico com aminoácidos da tríade catalítica através de ligações polares e um grande número de interações hidrofóbicas. Neste contexto os resultados obtidos nesta investigação levaram a identificação do primeiro composto natural oriundo da biota costeira brasileira com alta especificidade para AhpC bacteriana. Como consequências diretas da investigação iniciamos procedimentos com uma série de compostos similares a CN-ABP1 visando a identificação de características estruturais/funcionais envolvidas na inibição promovida por CN-ABP1. Ainda baseado na estrutura hidrofóbica alongada do composto hipotetizamos que este poderia mimetizar um substrato biológico como peróxidos derivados de ácidos graxos de cadeia longa e resultados obtidos revelaram que AhpC possui alta afinidade por este tipo de substrato com taxas de hiperoxidação de $1.06 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ indicando que estes possam ser inibidores biológicos de AhpC. Visando aprofundar o conhecimento da biologia estrutural da enzima realizamos ensaios de cristalização e obtivemos cristais passíveis de difração de raios-X. A determinação da estrutura destas proteínas deve auxiliar em um maior entendimento da interação entre CN-ABP1-PaAhpC abrindo a perspectiva da identificação de outros inibidores que guardem semelhança com CN-ABP1 ou hidroperóxidos derivados de lipídeos de cadeia longa, que podem em última instancia auxiliar no combate a bactérias patogênicas resistentes a múltiplos antibióticos.

Palavras-chave: Peroxirredoxinas, Inibidores, Compostos naturais, Peróxidos orgânicos

ABSTRACT

The resistance of pathogenic bacteria to multiple antibiotics has increased worldwide in recent years, requiring the search for new antibacterial compounds. Recently, some studies have shown that distinct antibiotics with different targets have the convergent ability to produce reactive oxygen species (ROS), which contributes to the death of bacterial pathogens. Bacteria possess several antioxidant enzymes capable of breaking down ROS. Among them, the typical 2-Cys peroxiredoxins, called AhpC in bacteria, are highly reactive peroxidases that are extremely abundant in the cellular environment, and studies indicate that AhpC is associated with virulence. The high reactivity of AhpC is related to a catalytic triad, composed of two polar residues (Thr/Ser and Arg) that keep the sulfur of the catalytic cysteine, called cysteine peroxidase (C_P) in the thiolate (S⁻) form and are involved in the targeting and stabilization of the substrate to allow for chemical bimolecular nucleophilic substitution (S_N2). Despite the importance of AhpC in bacteria, to date no inhibitors have been identified for these enzymes. On the other hand, some natural products have been identified as inhibitors of human isoforms. In general, these compounds present as common features a bulky hydrophobic skeleton and a carbonyl system capable of performing a thiol-Michael addition. In this work, we evaluated the inhibitory activity of natural compounds from the coastal biota of São Paulo state on the peroxidase activity of AhpC from *Pseudomonas aeruginosa* (PaAhpC) and *Staphylococcus epidermidis* (SeAhpC), two opportunistic Gram negative and positive bacteria, respectively, responsible for several hospital infections. The selection of compounds was made based on functional and structural characteristics of natural compounds identified for human isoforms. Through biochemical assays we identified a prenylated benzoic acid (CN-ABP1) from *Piper crassinervium* was able to inhibit the peroxidase activity of PaAhpC but did not inhibit SeAhpC or the human isoform (HsPrx2). Additionally we demonstrated that CN-ABP1 is not able to inhibit other thiol enzymes. The IC₅₀ determination was 20.3 μM and by SDS PAGE we showed that the compound was not able to perform a thiol-Michael addition, indicating a mode of inhibition not yet described for this group of proteins. Computational simulations involving molecular docking, indicate that the compound is stabilized in the active site pocket by amino acids of the catalytic triad through polar and a large number of hydrophobic interactions. In this context, the results obtained in this investigation led to the identification of the first natural compound from the Brazilian coastal biota with high specificity for bacterial AhpC. As direct consequences of the investigation we initiated approaches with a series of compounds similar to CN-ABP1 aiming the identification of structural/functional characteristics involved in the inhibition promoted by CN-ABP1. Based on the elongated hydrophobic structure of the compound we hypothesized that it could mimics biological substrates such as long chain fatty acid hydroperoxides and initial results revealed that AhpC has high affinity for this type of substrate with hyperoxidation rates of $1.06 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ indicating that these compounds may act as biological inhibitors of AhpC. In order to deepen the knowledge of the structural biology of the enzyme we performed the enzyme crystallization assays and obtained crystals suitable to X-ray diffraction approaches. The determination of the structure of these proteins may help in a better understanding of the interaction between CN-ABP1- PaAhpC opening the perspective to identify other inhibitors that keep similarity with CN-ABP1 or long-chain hydroperoxides derivatives, which may, ultimately, assist the combat against pathogenic bacteria resistant to multiple antibiotics.

Keywords: Peroxiredoxins, Inhibitors, Natural Compounds, Organic Peroxides

LISTA DE FIGURAS

Figura 1. Representação das reações de Fenton e de Harber-Weiss.	18
Figura 2. Mecanismo comum de morte celular induzido por drogas bactericidas	18
Figura 3. Sistema de <i>turnover</i> das peroxirredoxinas 2-Cys típicas.	22
Figura 4. Aspectos funcionais e estruturais de enzimas da subclasse AhpC-Prx1.	22
Figura 5. Diferenças no microambiente do sítio ativo entre eucariotos e procariotos em razão da cauda C-terminal de eucariotos e da substituição de Thr por Ser na tríade catalítica.	24
Figura 6. Estrutura química inibidores identificados para peroxirredoxinas 2-Cys típicas de eucariotos.	27
Figura 7. Estrutura química e mecanismos de ação de Adn sobre Prx 2-Cys.	28
Figura 8. Estruturas de compostos naturais que atuam como inibidores de Prx e compostos utilizados neste trabalho.	36
Figura 9. Resultado da expressão e purificação por IMAC de PaAhpC, SeAhpC, EcTrx e EctrxR expressas em <i>E. coli</i> BL21 (DE3).	37
Figura 10. Avaliação da atividade peroxidásica pelo ensaio de oxidação do NADPH de amostras de PaAhpC previamente tratadas com os compostos naturais.	38
Figura 11. Avaliação da atividade peroxidásica pelo ensaio de oxidação do NADPH de amostras de SeAhpC previamente tratada com os compostos naturais.	39
Figura 12. Alinhamento das sequências de aminoácidos de AhpCs de <i>S. typhimurium</i> , <i>P. aeruginosa</i> e <i>S. epidermidis</i> .	40
Figura 13. Alinhamento da estrutura cristalográfica de StAhpC com os modelos teóricos de PaAhpC e de SeAhpC e superfície da estrutura decamérica de StAhpC.	41
Figura 14. Resultados de CN-ABP1 e CN-HP1 gerados por <i>docking</i> molecular para StAhpC, e suas <i>best poses</i> .	43
Figura 15. Representações bidimensionais das interações moleculares entre StAhpC e os compostos naturais.	44
Figura 16. Representação dos resultados de CN-ABP1 gerados para PaAhpC, e <i>best pose</i> .	45
Figura 17. Representação dos resultados de CN-ABP1 e CN HP1 gerados para SeAhpC <i>best poses</i> selecionadas.	46
Figura 18. Representações bidimensionais do sítio de ligação de CN-ABP1 em PaAhpC e de CN-ABP1 e CN-HP1 em SeAhpC.	47

Figura 19. Determinação do IC ₅₀ de PaAhpC tratada com CN-ABP1 pelo ensaio acoplado de oxidação de NADPH.	49
Figura 20. Avaliação da atividade peroxidásica pelo ensaio de oxidação do NADPH das enzimas HsPrx2, EcTrx e EcTrxR previamente tratadas com CN-ABP1.	50
Figura 21. Avaliação da formação de dissulfetos PaAhpC após tratamento com CN-ABP1.	52
Figura 22. Características de inibição do composto CN-ABP1 sobre PaAhpC com concentrações crescentes de hidroperóxido orgânico.	53
Figura 23. Estrutura química dos compostos semi-sintéticos contendo características funcionais/estruturais similares a CN-ABP1.	55
Figura 24. Avaliação da atividade peroxidásica pelo ensaio de oxidação do NADPH de PaAhpC previamente tratadas com os compostos semi-sintéticos.	56
Figura 25. Avaliação da inibição de PaAhpC pelos compostos semi-sintéticos comparado com CN-ABP1.	56
Figura 26. Interações moleculares entre resíduos do microambiente do sítio ativo de AhpC hidroperóxidos de lipídeos.	58
Figura 27. Hiperóxidação de C _P de peroxirredoxinas por monoperóxidos e di-peróxidos.	60
Figura 28. Ensaio de oxidação de NADPH para avaliação da hiperóxidação de PaAhpC.	61
Figura 29. Ensaio de oxidação de NADPH para avaliação da hiperóxidação de SeAhpC.	63
Figura 30. Plotagem comparativa das velocidades iniciais × baixas concentrações de peróxido de hidrogênio, e os hidroperóxidos orgânicos de cumeno e oleico para PaAhpC e SeAhpC.	64
Figura 31. Cinética de hiperóxidação de PaAhpC por hidroperóxido de ácido oleico.	65
Figura 32. Resultados de experimentos de cristalização de PaAhpC em diferentes estados oxidativos e contendo ligante.	66

LISTA DE TABELAS

Tabela 1. Coeficiente de extinção molar específico e peso molecular das proteínas utilizadas no trabalho	33
Tabela 2. Valores de v_0 obtidos para SeAhpC tratada com os compostos naturais.	39
Tabela 3. Principais resíduos observados nas <i>best poses</i> dos compostos no sítio de ligação das três estruturas utilizadas.	48
Tabela 4. Determinação do IC ₅₀ de PaAhpC utilizando os valores de v_0 com a proteína tratada com concentrações crescentes de CN-ABP1.	49
Tabela 5. Valores de v_0 obtidos para as enzimas HsPrx2 de humanos (células cinzas), EcTrx e EcTrxR de <i>E. coli</i> (células azuis) tratadas com o composto CN-ABP1.	51
Tabela 6. Parâmetros enzimáticos determinados para o hidroperóxido de (CHP) e PaAhpC pré-tratada ou não com CN-ABP1.	53
Tabela 7. Valores de v_0 obtidos para a enzima PaAhpC tratada com os compostos semi-sintéticos.	57
Tabela 8. Parâmetros cinéticos determinados para os peróxidos com a enzima PaAhpC.	62
Tabela 9. Parâmetros cinéticos determinados para os peróxidos com a enzima SeAhpC.	63

LISTA DE ABREVIATURAS

Adn	Adenantina
AhpC	Alquil hidroperóxido redutase subunidade C
AhpF	Alquil hidroperóxido redutase subunidade F
Cat	Catalase
CHP	Hidroperóxido de cumeno
C _P	Cisteína peroxidásica
C _R	Cisteína de resolução
DTNB	Ácido 5,5'-ditiobis (2-nitrobenzóico)
ERNs	Espécies reativas de nitrogênio
EROs	Espécies reativas de oxigênio
FF	<i>Fully folded</i> (totalmente enovelado)
IPTG	Isopropil β-D-1-tiogalactopiranosídeo
LU	<i>Locally unfolded</i> (parcialmente desenovelado)
MDR	Resistente a múltiplas drogas
MIC	Concentração inibitória mínima
MRSA	<i>Staphylococcus aureus</i> resistente a meticilina
NADH	β-nicotinamida adenina dinucleotídeo reduzido
NADPH	β-nicotinamida adenina dinucleotídeo 2-fosfato reduzido
NTD	Domínio N-terminal
PMBN	Polimixina B nonapeptídeo
Prx	Peroxirredoxina
SDS-PAGE	Eletoforese em gel de poliacrilamida com dodecil- sulfato de sódio
Sod	Superóxido dismutase
TC	Tríade catalítica
TCA	Ciclo dos ácidos tricarboxílicos
Trx	Tiorredoxina
TrxR	Tiorredoxina redutase
Tsa 1/2	<i>Thiol-specific antioxidant 1/2</i>
UFC	Unidades formadoras de colônia

SUMÁRIO

1 INTRODUÇÃO	15
1.1 ANTIBACTERIANOS	15
1.2 A LETALIDADE DE ANTIBACTERIANOS É ACOMPANHADA PELA GERAÇÃO DE ESPÉCIES REATIVAS DE OXIGÊNIO (EROS)	17
1.3 EXPLOSÃO RESPIRATÓRIA DO HOSPEDEIRO E DEFESAS DOS PATÓGENOS.....	18
1.4 DIVERSIDADE DAS PRX 2-CYS TÍPICAS E CARACTERÍSTICAS ESTRUTURAIS	20
1.5 IMPORTÂNCIA DA AHPC PARA BACTÉRIAS PATOGÊNICAS	24
1.6 INIBIDORES DE PRX 2-CYS TÍPICAS	26
1.7 BUSCA POR NOVOS INIBIDORES DE AHPCs DE BACTÉRIAS ORIUNDOS DA BIOTA COSTEIRA DO ESTADO DE SÃO PAULO	29
2 OBJETIVO	30
3 MATERIAIS E MÉTODOS.....	31
3.1 REAGENTES	31
3.2 MOLÉCULAS PARA AVALIAÇÃO DA ATIVIDADE INIBITÓRIA SOBRE PROTEÍNAS BACTERIANAS	31
3.3 MEIOS DE CULTURA PARA BACTÉRIAS E LINHAGENS BACTERIANAS.....	31
3.3.1 Meios de cultura	31
3.3.2 Linhagens de <i>E. coli</i> para propagação de plasmídeos e/ou expressão de proteínas	31
3.4 PLASMÍDEOS DE EXPRESSÃO E GENES DE PROTEÍNA ALVO.....	31
3.5 EXPRESSÃO EM BACTÉRIAS E PURIFICAÇÃO DAS PROTEÍNAS.....	32
3.6 QUANTIFICAÇÃO DE PROTEÍNAS.....	32
3.7 DETERMINAÇÃO DA ATIVIDADE PEROXIDÁSICA ATRAVÉS DE ENSAIO DE OXIDAÇÃO DE NADPH	32
3.8 AVALIAÇÃO DA ATIVIDADE INIBITÓRIA DOS COMPOSTOS SOBRE A ATIVIDADE DAS ENZIMAS	33
3.9 ANÁLISE DE ESTRUTURAS DE PROTEÍNAS.....	33
3.10 DOCKING MOLECULAR DOS COMPOSTOS NATURAIS COM AHPC.....	33
3.11 DETERMINAÇÃO DE CONSTANTES CINÉTICAS POR FLUORESCÊNCIA POR STOPPED-FLOW.....	34
3.12 ENSAIOS DE CRISTALIZAÇÕES DE PAAHPC	34

4 RESULTADOS E DISCUSSÃO	35
4.1 OBTENÇÃO COMPOSTOS NATURAIS ORIUNDOS DA BIOTA DO BRASIL.....	35
4.2 EXPRESSÃO, PURIFICAÇÃO E QUANTIFICAÇÃO DE PROTEÍNAS RECOMBINANTES.	36
4.3 AVALIAÇÃO DE INIBIÇÃO DE COMPOSTO NATURAIS ORIUNDOS DA FLORA	
BRASILEIRA SOBRE AS ENZIMAS PAAHPC E SEAHPC PELO ENSAIO ACOPLADO DE OXIDAÇÃO	
DE NADPH.....	37
4.4 CONSTRUÇÃO DOS MODELOS TEÓRICOS DE PAAHPC E SEAHPC	39
4.5 ANÁLISE DAS INTERAÇÕES DOS COMPOSTOS COM AHPC POR DOCKING	
MOLECULAR	41
4.6 DETERMINAÇÃO DO IC₅₀ DO COMPOSTO CN-ABP1 PARA PAAHPC	47
4.7 AVALIAÇÃO DE INIBIÇÃO PELO COMPOSTO CN-ABP1 DA ATIVIDADE	
PEROXIDÁSICA DA PRX2 HUMANA E ENZIMÁTICA DO SISTEMA TRX DE E. COLI	48
4.8 AVALIAÇÃO DE ADIÇÃO DE TIÓL-MICHAEL POR CN-ABP1 SOBRE CISTEÍNAS DE	
PAAHPC	50
4.9 AVALIAÇÃO DO EFEITO DE COMPOSTOS SEMI-SINTÉTICOS SIMILARES ACN-ABP1	
SOBRE A ATIVIDADE ENZIMÁTICA DE PAAHPC	53
4.10 AVALIAÇÃO DA AFINIDADE DE PAAHPC PARA PERÓXIDOS DERIVADOS DE	
LIPÍDEOS DE CADEIA LONGA POR MEIO DE ENSAIO DO NADPH E FLUORESCÊNCIA	
INTRÍNSECA DO TRP	56
4.11 CRISTALIZAÇÃO DE PAAHPC EM DIFERENTES ESTADOS OXIDATIVOS E	
CONTENDO LIGANTE.....	63
5 CONCLUSÕES E CONSIDERAÇÕES FINAIS	65
6 PERSPECTIVAS	67
REFERÊNCIAS	68
APÊNDICES	
1 CABRERA, V.I.M., SIVIDANES, G.N., QUINTILIANO, N.F., TOYAMA, M.H., LAGO,	
J.H.G., DE OLIVEIRA, M.A. EXPLORING FUNCTIONAL AND STRUCTURAL FEATURES OF	
CHEMICALLY RELATED NATURAL PRENYLATED HYDROQUINONE AND BENZOIC ACID FROM <i>PIPER</i>	
<i>CRASSINERVIVUM</i> (PIPERACEAE) ON BACTERIAL PEROXIREDOXIN INHIBITION. MANUSCRITO ACEITO	
PARA PUBLICAÇÃO NO PERIÓDICO PLoS ONE.....	77
2 TAIRUM, C.A., SANTOS, M.C., BREYER, C.A., OLIVEIRA, A.L.P., CABRERA, V.I.M.,	
TOLEDO-SILVA, G., MORI, G.M., TOYAMA, M.H., NETTO, L.E.S., DE OLIVEIRA, M.A. EFFECTS	

OF SERINE OR THREONINE IN THE ACTIVE SITE OF TYPICAL 2-CYS PRX ON HYPEROXIDATION SUSCEPTIBILITY AND ON CHAPERONE ACTIVITY. ANTIOXIDANTS (BASEL). 2021. 7: 1032.....	129
SANTOS, M.C., TAIRUM, C.A., CABRERA, V.I.M., CAUZ, A.C., RIBEIRO, L.F., TOLEDO-JUNIOR J.C., TOYAMA, M.H., LAGO, J.H.G., BROCCHI, M., NETTO, L.E.S., DE OLIVEIRA, M.A. ADENANTHIN IS AN EFFICIENT INHIBITOR OF PEROXIREDOXINS FROM PATHOGENS, INHIBITS BACTERIAL GROWTH, AND POTENTIATES ANTIBIOTIC ACTIVITIES. CHEM. RES. TOXICOL. 2022. EPUB AHEAD OF PRINT.....	151
4 DE OLIVEIRA, M.A., TAIRUM, C.A., NETTO, L.E.S., OLIVEIRA, A.L.P., ALEIXO-SILVA, R.L., CABRERA, V.I.M., BREYER, C.A., DOS SANTOS, M.C. RELEVANCE OF PEROXIREDOXINS IN PATHOGENIC MICROORGANISMS. APPL. MICROBIOL. BIOTECHNOL. 2021. 105: 5701-5717.....	164

1 INTRODUÇÃO

1.1 Antibacterianos

Os antibacterianos são os principais compostos utilizados para o tratamento de infecções ocasionadas por bactérias patogênicas. Sua descoberta foi realizada por Sir Alexander Fleming, em 1928, responsável pelas primeiras pesquisas com penicilina, o que transformou de forma significativa a medicina moderna (Ventola 2015; Prasad *et al.*, 2022). O primeiro uso em larga escala desse tipo de medicamento foi durante a segunda guerra mundial para o tratamento de infecções de combatentes e com o passar dos anos houve avanço significativo das pesquisas nessa área, resultando na descoberta de novos antibacterianos de diferentes classes como aminoglicosídeos, quinilonas, β lactâmicos, dentre outros, o que tornou possível curar ou prevenir infecções geradas em cirurgias, transplantes, quimioterapia e doenças crônicas (Centers for Disease Control and Prevention, 2019; Ventola, 2015).

No entanto, em 1962 no Reino Unido, foi relatado o primeiro caso de *Staphylococcus aureus* resistente a meticilina (MRSA) e em 1972 começou a se utilizar vancomicina para o tratamento de infecções causadas por *S. aureus* (Ventola, 2015). O aumento de casos de resistência à vancomicina, levou a pesquisa e caracterização de novos antibacterianos, mas conforme passaram as décadas, ocorreu o decaimento da caracterização de novos medicamentos, como também o surgimento de bactérias super-resistentes aos mais diversos antibacterianos (Edwards *et al.*, 2019). Essa resistência pode ser gerada devido à mutações, modificações enzimáticas (como exemplo a metilação do resíduo de adenina no RNA 23S em algumas bactérias), a existência de uma parede celular extra (caso das Gram⁻), mutações pontuais, transferência de genes, dentre outras razões (Aslam *et al.*, 2018; Ventola, 2015).

Atualmente a ocorrência de infecções ocasionadas por bactérias resistentes a múltiplas drogas (conhecidas como MDR – *Multi Drug Resistance*) tem se tornado uma ameaça global crescente, pois vem mitigando o uso terapêutico dos antibióticos existentes, criando a necessidade da busca por novas drogas que sejam eficazes no combate as MDR (Antimicrobial Resistance Collaborators 2022; Chang *et al.*, 2015; Chopra *et al.*, 2002; Docquier e Mangani, 2018). Dentre as causas para o aumento da ineficiência do tratamento com antibacterianos estão: o seu uso excessivo, o aumento das viagens e relações comerciais internacionais, além da falta de saneamento básico em muitos países, onde são liberados de forma incorreta antibacterianos ou seus resíduos no meio ambiente.

Outro fator que leva a resistência é o uso de antibacterianos na agropecuária, pois leva a contaminação da microbiota ambiental. Somente nos Estados Unidos, cerca de 45.000 kg de tetraciclina e estreptomicina são utilizados como pesticidas em árvores frutíferas no oeste e sul do país (Golkar *et al.*, 2014). Também existe a utilização de antibacterianos na criação de animais para a alimentação humana, onde são usados para tratamento de infecções ou mesmo como promotor de crescimento no manejo de animais criados em confinamento. Quando utilizados em excesso, os compostos podem ser excretados nas fezes e urina desses animais, expondo microrganismos a doses diárias desses medicamentos, o que leva a uma seleção de bactérias resistentes (Aslam *et al.*, 2018; Ventola *et al.*, 2015).

Em um estudo recente foram analisados dados de 204 países e constataram que 1.27 milhões de óbitos ocorridos em 2019, eram devido a bactérias resistentes a múltiplos antibióticos, sendo os principais organismos responsáveis pelas mortes as bactérias *Escherichia coli*, *S. aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii* e *Pseudomonas aeruginosa* que juntos causaram mais de 900.000 óbitos (Murray *et al.*, 2022).

O custo resultante da resistência aos tratamentos com antibacterianos vem se tornando um desafio global. Nos EUA, foi registrado cerca de US\$ 20 bilhões em gastos, sendo que ainda existe ~ US\$ 35 bilhões, em despesas anuais, para produzir antibacterianos para o sistema de saúde (Aslam *et al.*, 2018; Ventola, 2015). Somente para o tratamento de seis espécies de bactérias MDR as despesas anuais se aproximam de 2 bilhões de dólares (Ventola *et al.*, 2015). Para a União Europeia (UE) é observada uma situação semelhante. A cada ano ~25.000 pacientes morrem devido a infecções causadas por bactérias multirresistentes, custando à sociedade aproximadamente € 1.5 bilhão. Infelizmente no Brasil, estes dados não são claros, mas é crescente a preocupação com o aumento de casos de infecções com bactérias super-resistentes, o que levou ao controle da venda deste tipo de fármaco (Mattos *et al.*, 2017).

Portanto, de forma global, esta situação é altamente preocupante, e de acordo com a *Research and Development Corporation*, o cenário pode piorar, e caso ocorra, há a previsão de ~ 400 milhões de mortes devido às infecções causadas por bactérias resistentes, até 2050 (Aslam *et al.*, 2018). De fato, segundo a organização mundial da saúde (OMS) até 2050, as perdas acumuladas esperadas devido à multirresistência poderão atingir 2.9 trilhões de dólares (Machowska *et al.*, 2018).

Por outro lado, é importante mencionar que a administração de antibacterianos largamente utilizados (como os β -lactâmicos, aminoglicosídeos e fluoroquinolonas) também pode ser danosa para os hospedeiros pois já foram relatadas complicações renais, hepáticas e

até mesmo encefalopatia em fetos (Kalghatgi *et al.*, 2013; Rehman *et al.*, 2020; Xiao *et al.*, 2018). Estes aspectos revelam a importância de um tratamento cuidadoso com antibacterianos e a necessidade de descobertas de novos compostos que apresentem menor toxicidade ou que quando associados a antibacterianos permitam a redução de suas doses e que principalmente possam ser biodegradados.

1.2 A letalidade de antibacterianos é acompanhada pela geração de espécies reativas de oxigênio (EROs)

Estudos recentes indicam que diferentes classes de antibacterianos geram níveis variáveis de espécies reativas de oxigênio (EROs) que contribuem significativamente para a morte celular dos patógenos (Blázquez *et al.* 2018; Grant *et al.*, 2012; Kohanski *et al.*, 2010; Li *et al.*, 2021). Dentre as EROs de maior importância biológica encontramos o peróxido de hidrogênio (H_2O_2), o radical ânion superóxido ($O_2^{\bullet-}$) e o radical hidroxila ($HO^{\bullet}$) (Halliwell & Gutteridge, 2015). Apesar de existir ainda grande debate sobre a relação de antibacterianos, geração de EROs e letalidade em patógenos, trabalhos recentes utilizando as mais distintas abordagens por diferentes grupos de pesquisa reforçam esta relação. Neste contexto, foi demonstrado que três das principais classes de drogas bactericidas (β -lactâmicos, aminoglicosídeos e fluoroquinolonas) utilizam um mecanismo comum que estimula a produção de $HO^{\bullet}$ por meio de reações químicas como as de Haber-Weiss e Fenton (Figura. 1) (Ibacache-Quiroga *et al.*, 2018; Li *et al.*, 2012). O H_2O_2 juntamente com o $O_2^{\bullet-}$ é capaz de produzir $HO^{\bullet}$ por meio da reação de Harber-Weiss (Figura 1A), um potente oxidante capaz de danificar qualquer tipo de biomolécula como DNA, RNA, proteínas e lipídeos (Halliwell & Gutteridge, 2015). Ainda, na presença de metais bivalentes como o ferro (Fe^{+2}) pode ocorrer as reações químicas de Fenton, que também geram $HO^{\bullet}$ (Figura 1B).

A) Reação de Haber-Weiss:	$H_2O_2 + O_2^{\bullet-} \rightarrow O_2 + HO^{\bullet} + OH^-$
B) Reações de Fenton:	$Fe^{3+} O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$ (Reação 1)
	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\bullet}$ (Reação 2)

Figura 1. A) Representação da reação de Haber-Weiss, originando radical hidroxila a partir da reação entre peróxido de hidrogênio e radical ânion superóxido; B) Reações de Fenton, em que a reação entre uma molécula de Fe^{3+} com uma do radical ânion superóxido (reação 1) resultando na formação de O_2 e Fe^{2+} , o último ao reagir com peróxido de hidrogênio, produz o radical hidroxila (reação 2).

Ponto de importância reside no fato que apesar dos diferentes fármacos bactericidas terem alvos biológicos distintos, seus efeitos convergem para uma *via* comum (Kohanski *et al.*, 2010). Essa *via* é baseada na produção de oxidantes através de resposta metabólica envolvendo o ciclo do ácido tricarboxílico (CAT) que induz a rápida depleção de nicotinamida adenina dinucleotídeo (NADH), resultando em uma hiperativação da cadeia de transporte de elétrons e

aumento da produção de produtos secundários como o $O_2^{\cdot-}$ e o H_2O_2 (Kohanski *et al.*, 2010; Shatalin *et al.*, 2011). O $O_2^{\cdot-}$ pode atacar grupamentos ferro-enxofre (Fe-S) presente em proteínas da cadeia transportadora de elétrons levando a disponibilização de Fe ferroso (Fe^{2+}), que participa da reação de Fenton, resultando na formação de $HO^{\cdot}$ que auxilia na morte celular (Kohanski *et al.*, 2010; Shatalin *et al.*, 2011). Cabe ressaltar que o H_2O_2 também é capaz de danificar grupos Fe-S levando a disponibilização de Fe^{2+} (Jang & Imlay, 2010) ocasionando mais danos celulares (Figura 2).

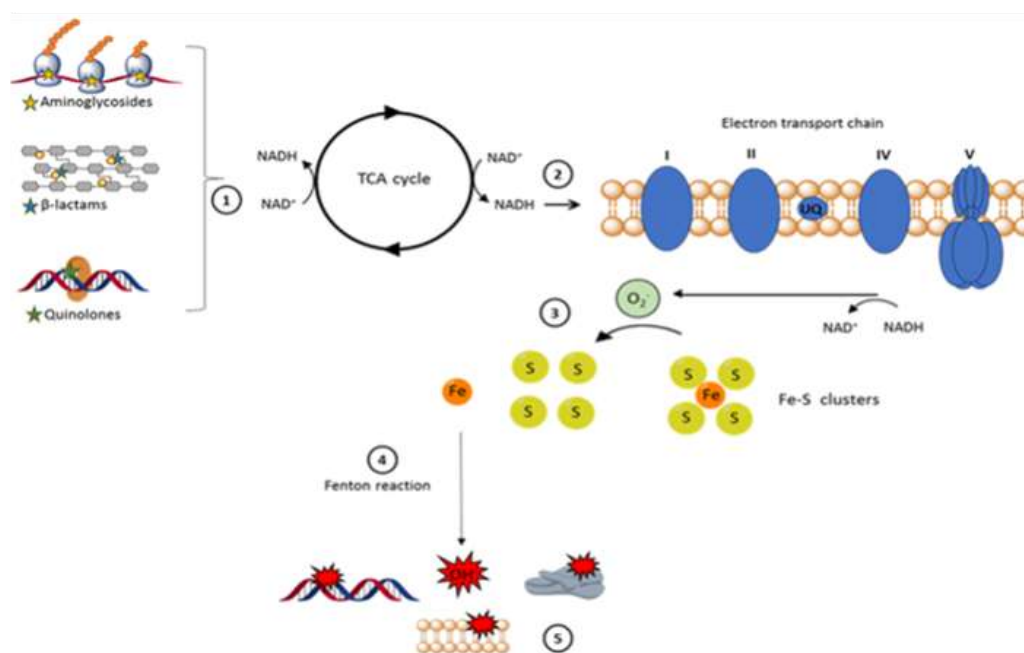


Figura 2. Mecanismo comum de morte celular induzido por antibacterianos bactericidas. As interações primárias fármaco-alvo biológico (aminoglicosídeo com o ribossomo, quinolona com topoisomerase e β-lactâmicos com proteínas de ligação à penicilina (PBPs)) estimulam a oxidação do NADH através da cadeia de transporte de elétrons, que é dependente do ciclo do ácido tricarboxílico. A hiperativação da cadeia de transporte de elétrons estimula a formação do radical ânion superóxido ($O_2^{\cdot-}$), o qual danifica grupamentos Fe-S, tornando o ferro ferroso disponível para oxidação pela reação de Fenton. A reação de Fenton produz radicais hidroxila ($HO^{\cdot}$), que danificam DNA, lipídios e proteínas. Isto contribui para a morte celular induzida por antibacterianos.

É importante salientar que apesar de altas concentrações ou concentrações apropriadas de antibacterianos levam a sérios danos oxidativos em biomoléculas do patógeno, que inviabilizam sua sobrevivência, baixas doses de antibacterianos estão associadas ao aparecimento de linhagens resistentes em função de mutações provocadas no DNA por EROs, que aceleram as taxas de evolução (Kampf, 2019; Ter Kuile *et al.*, 2016).

1.3 Explosão respiratória do hospedeiro e defesas dos patógenos

Uma das principais linhas de defesa do hospedeiro contra o ataque de patógenos é a explosão respiratória (*respiratory burst*), caracterizada pela elevada produção de EROs e espécies reativas de nitrogênio (ERNs). Esse processo se dá pela ativação da NADPH oxidase,

também conhecida como fagócito oxidase, responsável por oxidar o NADPH, reduzindo o oxigênio molecular (O_2) a $O_2^{\bullet-}$, que sofre dismutação, produzindo H_2O_2 , molécula conhecida pela sua capacidade de difusão em membranas biológicas, além de ser capaz de desencadear mecanismos tóxicos para as células como a produção de espécies radicalares altamente deletérias como o $HO^{\bullet}$ (Halliwell & Gutteridge, 2015; Rocha *et al.*, 2018).

Outro modo do organismo eliminar o patógeno é a produção do radical óxido nítrico ($NO^{\bullet}$), gerado a partir da oxidação da arginina na presença de O_2 em uma reação catalisada pela enzima óxido nítrico sintetase (NOS). Quando em excesso, o $NO^{\bullet}$ leva a origem de diversos compostos reativos, denominados como espécies reativas de nitrogênio (ERNs), sendo uma delas o peroxinitrito ($ONOO^-$), e ele ou seus derivados podem causar danos a qualquer tipo de biomolécula (Calcerrada *et al.*, 2011; Saini *et al.*, 2019).

Além disso, as EROs e ERNs também podem reagir diretamente com ácidos graxos insaturados e iniciar a peroxidação lipídica, atacando as membranas biológicas, ocasionando perda de fluidez e aumento da permeabilidade ao H^+ , que podem resultar na ruptura e perda do conteúdo celular (Alegria *et al.*, 2017; Halliwell & Gutteridge, 2015).

Para ultrapassar a defesa gerada pelo hospedeiro, os organismos patogênicos apresentam enzimas com alta eficiência para decomposição destas moléculas, portando, denominadas de enzimas antioxidantes, que contribuem para a infecção do hospedeiro e o crescimento/manutenção do patógeno, como as superóxido dismutases (Sod), as catalases (Cat), as peroxirredoxinas (Prx) dentre outras (Oliveira *et al.*, 2021; Rocha *et al.*, 2018). Dentre essas três enzimas, as Prx são as que apresentam maior abundância, estando presente em diversos organismos, possuindo múltiplas isoformas e elevada eficiência catalítica (Kim *et al.*, 2019; Oliveira *et al.*, 2021; Rocha *et al.*, 2018). É importante ressaltar que as Prx são capazes de decompor diversos tipos de hidroperóxidos como peróxido de hidrogênio (H_2O_2), peroxinitrito ($ONOO^-$) e diversos peróxidos orgânicos como hidroperóxidos de lipídeos (Lp-OOH), hidroperóxido de urato (UOOH), peróxido de monocarbonato (HCO_4^-), peróxidos de aminoácidos e proteínas, dentre outros, com taxas de decomposição que variam entre 10^4 - 10^8 $M^{-1} s^{-1}$ (Baker *et al.*, 2001; Carvalho *et al.*, 2017; Cox *et al.*, 2009; Jacobson *et al.*, 1989; Longo *et al.*, 2020; Peskin *et al.*, 2010; Reyes *et al.*, 2011; Truzzi *et al.*, 2019; Zeida *et al.*, 2015). Um ponto interessante é que os orgânicos possuem uma estrutura mais volumosa do que os outros hidroperóxidos citados e são acomodados na cavidade hidrofóbica do sítio ativo das Prx, sendo decompostos eficientemente pelas Prx. Já foi demonstrado que a isoforma de Prx1 de *Paracoccidioides brasiliensis* é capaz de decompor com eficiência de 12 vezes maior para

hidroperóxido de ácido linoleico quando comparado aos peróxidos orgânicos sintéticos, como por exemplo hidroperóxido de cumeno (CHP) (Longo *et al.*, 2020).

Diversas pesquisas indicam que o bloqueio das enzimas antioxidantes por compostos químicos pode acarretar a morte das células patogênicas quando submetidas a estresse oxidativo. Logo, uma estratégia de combate promissora seria utilizar inibidores de enzimas antioxidantes para tratamento de infecções causadas por microrganismos patogênicos (Novaes *et al.*, 2019). Contudo, os hospedeiros apresentam isoformas dessas enzimas que possuem alta semelhança com as dos patógenos, principalmente a Sod e Cat, tornando assim os inibidores tóxicos para o hospedeiro (Brennan *et al.*, 2015; Mishra & Imlay, 2012). Neste contexto, as Prx se tornam um alvo interessante, uma vez que apresentam diferenças funcionais e estruturais entre as isoformas de patógenos e hospedeiro e que serão discutidas a seguir.

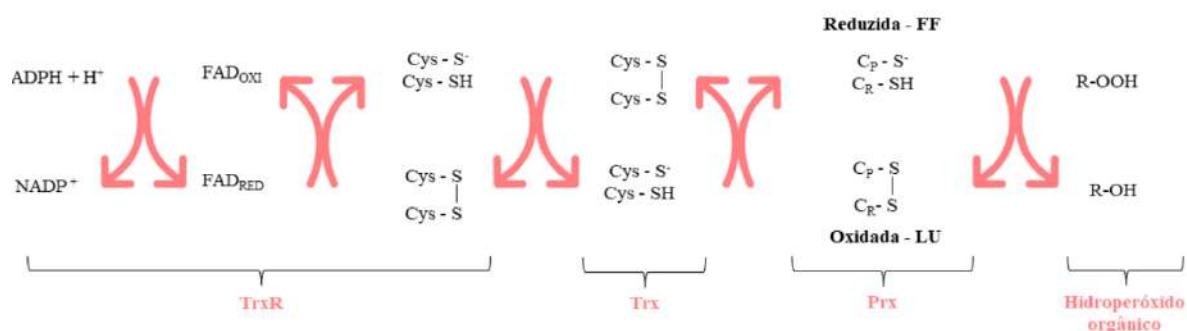
1.4 Diversidade das Prx 2-Cys típicas e características estruturais

As Prx são largamente distribuídas entre eucariotos e procariotos. Em eucariotos, isoformas destas enzimas estão presentes em diversos compartimentos da célula, a exemplo de mamíferos, que apresentam seis isoformas presentes no citosol (Prx1, Prx2 e Prx6), endossomo e mitocôndria (Prx3), retículo endoplasmático (Prx4), peroxissomo, mitocôndria e núcleo (Prx5) sendo que a Prx2 é a isoforma com maior abundância. Já nas bactérias foram descritas seis isoformas distintas (AhpC, AhpE, Bcp, Tpx, LsfA e Prx5) que são encontradas citosol, associadas as membranas e mesmo exportada para o espaço extracelular. Em bactérias a isoforma mais é denominada de AhpC, a qual é homóloga a Prx1/Prx2 de mamíferos (Kaiahami *et al.*, 2014; Oliveira *et al.*, 2021; Wood *et al.*, 2003a).

Para decomposição dos hidroperóxidos as Prx utilizam um resíduo reativo, denominado de cisteína peroxidásica (C_P). Ao se analisar a estrutura quaternária, é possível constatar que a C_P se encontra próxima a um resíduo treonina (Thr), em alguns casos serina (Ser), e a uma arginina (Arg), sendo que estes aminoácidos são totalmente conservados em todas as Prx. Esses três aminoácidos formam a denominada tríade catalítica (TC), sendo que Thr/Ser e Arg são responsáveis pela desprotonação e manutenção da C_P em forma de tiolato (S^-), aumentando sua reatividade (Flohé *et al.*, 2011; Tairum *et al.*, 2016; Tairum *et al.*, 2021).

As Prx são heterogêneas, podendo ser divididas em três classes: Prx 1-Cys, Prx 2-Cys típicas e Prx 2-Cys atípicas. As 1-Cys utilizam apenas uma Cys (C_P) para decomposição do hidroperóxido, enquanto as 2-Cys apresentam um segundo resíduo, denominado de cisteína de resolução (C_R), que é apto para formar um dissulfeto C_P . Adicionalmente, as 2-Cys são subdivididas em atípicas e típicas em relação ao dissulfeto formado, sendo intramolecular nas

2-Cys atípicas. Por sua vez, as Prx 2-Cys típicas são homodímeros obrigatórios e o dissulfeto gerado é intermolecular (os resíduos de cisteína se encontram em monômeros distintos) (Wood *et al.*, 2003a). Dentre as três classes citadas acima, as Prx 2-Cys típicas são as mais abundantes e as mais estudadas (Rhee & Kil, 2017). Utilizando como exemplo o H_2O_2 (H-O-O-H), o ciclo catalítico inicia-se com o ataque da C_P a um oxigênio do H_2O_2 , resultando na clivagem da ligação O-O, liberando uma molécula de água e oxidando a C_P a cisteína ácido sulfênico (C_P - SOH), sendo seguido pela condensação com a C_R -SH, resultando no dissulfeto (C_P -S-S- C_R) e liberação de uma segunda molécula de água (Flohé *et al.*, 2011; Tairum *et al.*, 2016).



De forma geral, quando aptas para reduzir seus substratos, as Prx 2-Cys típicas são encontradas como decâmeros do tipo $\alpha 2[5]$ formados por cinco homodímeros ($\alpha 2$) em uma conformação denominada *fully folded* (FF). (Figura 4A). Após a redução do peróxido para que ocorra a formação do dissulfeto ocorre um remodelamento de elementos da estrutura secundária (conformação *locally unfolded* = LU) que resulta na dissociação do decâmero em Prx contendo Thr na tríade catalítica (Figura 4B) (Pastor-Flores *et al.*, 2020; Parsonage *et al.*, 2005; Tairum *et al.*, 2012). Quando em baixas quantidades de peróxidos, o decâmero é restabelecido após a redução pelo sistema redutor (Figura 4A, B).

2004). Esta transição funcional já foi observada tanto para as 2-Cys Prx típicas de eucariotos (Jang *et al.*, 2004; Pastor-Flores *et al.*, 2020) quanto de procariotos (Huang *et al.*, 2010; Kamariah *et al.*, 2018a; Tairum *et al.*, 2021). A hiperoxidação de C_P não pode ser revertida pelo sistema Trx, porém a enzima sulfirredoxina (Srx), pode reduzir C_P-SO₂H em C_P-SOH as custas de ATP (Forshaw *et al.*, 2021; Jang *et al.*, 2004).

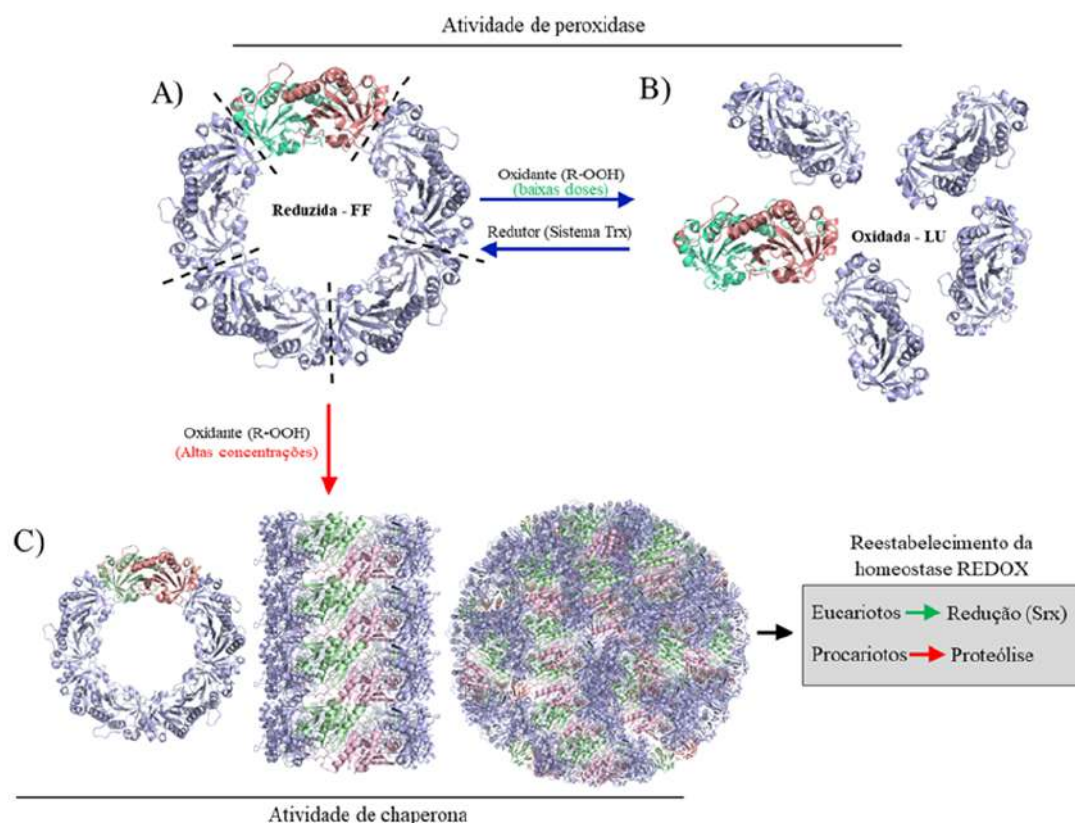


Figura 4. Aspectos funcionais e estruturais de enzimas da subclasse Prx 2-Cys típicas. (A) Quando reduzidas as enzimas da subclasse Prx 2-Cys típicas encontram-se como decâmeros e aptas a reduzir os substratos (conformação FF). (B) A decomposição de hidroperóxidos resulta na formação de dissulfetos (conformação LU) o que leva a dissociação dos decâmeros em dímeros. (C) Em situações de elevadas concentrações de hidroperóxidos ocorre a hiperoxidação de C_P em cisteína ácido sulfinico (C_P-SO₂H), com concomitante perda da atividade peroxidásica e com ganho da atividade de chaperona. Quando hiperoxidada as Prx 2-Cys típicas podem formar complexos de alto peso molecular através de intensa oligomerização. O restabelecimento da homeostase redox permite a redução de C_P-SO₂H pela enzima sulfirredoxina. Bactérias não possuem Srx e as enzimas hiperoxidadas são degradadas e necessitam ser ressintetizadas.

Cabe ressaltar que existem duas diferenças marcantes entre enzimas de eucariotos e de bactérias. De fato, Wood e colaboradores (2003b) demonstraram que nas enzimas de eucariotos existe um prolongamento na região C-terminal, representado por uma α hélice adicional e que a ausência desta porção em procariotos está relacionada a uma maior resistência das Prx 2-Cys típicas à hiperoxidação da cisteína peroxidásica, sendo que para eucariotos esse prolongamento serviria para favorecer a superoxidação da C_P, que impede a formação do dissulfeto, resultando na inativação da atividade peroxidásica das Prx 2-Cys típicas e consequente ativação da função de chaperona molecular com implicações na transdução de sinal (Jang *et al.*, 2004; Netto e

Antunes, 2016; Wood *et al.*, 2003a). Análises realizadas por nosso grupo de pesquisa revelam que a extensão C-terminal presente em proteínas eucarióticas, além de estar relacionada com as diferenças funcionais descritas acima, também levam a diferenças significativas no microambiente do sítio ativo. Quando é gerada a superfície molecular de Prx 2-Cys de eucariotos (como exemplo a Tsa1 de levedura) é possível constatar que a porção C-terminal constitui parte do microambiente do sítio ativo e fica em grande proximidade a tríade catalítica (Figura 5A), o que leva a uma diferença significativa do microambiente quando comparada a isoforma de bactérias (Figura 5B).

Nosso grupo de pesquisa também explorou outra diferença importante. Dos três aminoácidos da tríade catalítica (TC) das Prx 2-Cys típicas dois são estritamente conservados (C_P e Arg), ao passo que o terceiro aminoácido representado por uma Thr pode ser substituído por uma Ser em alguns organismos. Durante muito tempo esta substituição foi considerada sem importância, pois a ausência de um grupo metila (CH_3) nas Prx 2-Cys típicas que possuem Ser, não afetaria o seu papel na desprotonação de C_P . Entretanto, utilizando isoformas de Prx 2-Cys típicas de leveduras, Tsa1 (a qual possui naturalmente Thr como parte da TC) e Tsa2 (a qual possui Ser na TC), demonstramos pela primeira vez que a presença de Ser confere a proteína diferenças estruturais marcantes de acordo com estado redox (Tairum *et al.*, 2016). De fato, enzimas que possuem Thr como Tsa1, quando reduzidas se apresentam como decâmero, ao passo que quando as cisteínas estão oxidadas em dissulfeto estas enzimas se tornam dímeros (Figura 4). No caso de enzimas que possuem Ser a dissociação do decâmero não ocorre e estas enzimas são decâmeros constitutivos independentes do estado redox (Tairum *et al.*, 2016; Tairum *et al.*, 2021). Análises adicionais do sítio ativo de enzimas com Thr revelam que o acesso a C_P é bastante restrito (Figura 5C) enquanto as Prx 2-Cys que contém Ser possuem uma maior liberdade espacial de C_P (Figura 5D). Estas características nos parecem importantes, pois tanto a ausência da cauda C-terminal quanto a substituição de Thr por Ser, levam a diferenças no microambiente do sítio ativo o que pode indicar diferenças na utilização de substratos e, em última instância, a afinidade por substratos/inibidores.

Recentemente, também demonstramos que essa substituição, Thr por Ser, é capaz de conferir elevada resistência/suscetibilidade à hiperoxidação de CP, além de influenciar na afinidade por substratos, como por exemplo o H_2O_2 e peróxido de cumeno (CHP) (Tairum *et al.*, 2021). Adicionalmente, estudos envolvendo análises filogenéticas revelaram que enquanto Prx 2-Cys típicas que possuem Thr são largamente distribuídas entre archaea, bactéria e eucariotos, as Prx 2-Cys contendo Ser são majoritariamente detectadas em bactérias, sendo diversas delas patógenos de animais e plantas. É importante ressaltar que no caso dos

vertebrados, apenas peixes primitivos (Chondrichthyes) foram detectados com Ser na TC (Tairum *et al.*, 2021). Para os outros cordados (anfíbios, répteis, aves e mamíferos), bem como plantas vasculares, todas as enzimas detectadas em nossa análise possuem Thr na tríade catalítica, o que reforça que esta diferença possa ser explorada para a busca de quimioterápicos antibacterianos.

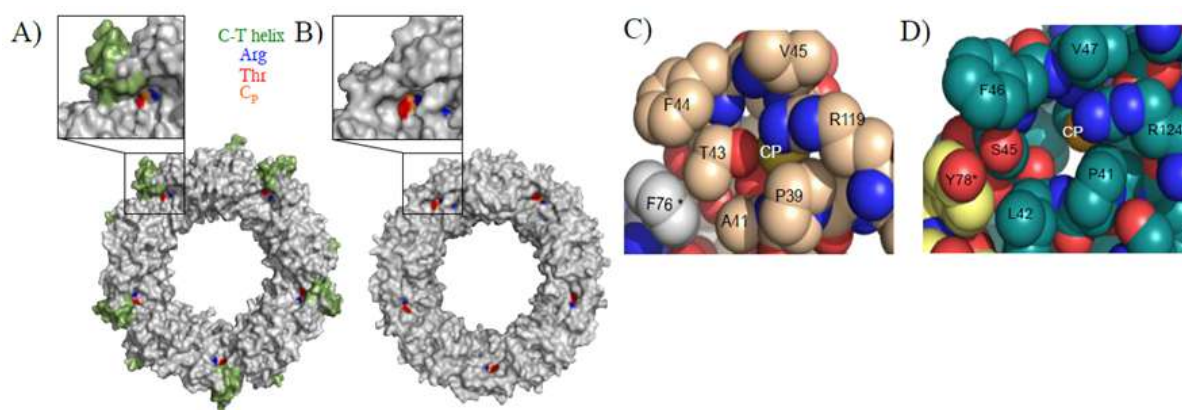


Figura 5. Diferenças no microambiente do sítio ativo entre eucariotos e procariotos em razão da cauda C-terminal de eucariotos e da substituição de Thr por Ser na tríade catalítica. Superfície molecular de Prx de eucarioto (A) e de bactéria (B). As estruturas estão coloridas em cinza, os aminoácidos da tríade catalítica estão coloridos em laranja (Cp), azul (Arg) e vermelho (Thr/Ser) e a cauda C-terminal presente em eucariotos em verde escuro. Representação em esferas de estruturas cristalográficas dos aminoácidos do microambiente do sítio ativo que circundam a cisteína peroxidásica (Cp) em 5 Å, em proteína de eucarioto que possui Thr (Tsa1 de *S. cerevisiae*; PDB: 3SBC) (C) e de bactéria contendo Ser (AhpC de *Enterococcus faecalis*; PDB: 5Y63) (D). Em C os átomos de carbono estão representados em bege (no dímero adjacente do decâmero em cinza) e em D em verde escuro (no dímero adjacente do decâmero em amarelo claro). Os demais átomos estão em CPK (O = vermelho, N = azul e S = laranja claro). As representações foram geradas utilizando PyMol (www.pymol.org).

1.5 Importância da AhpC para bactérias patogênicas

Como mencionado anteriormente, as Prx 2-Cys típicas de bactérias são denominadas de alquil hidropéroxido redutase C (AhpC) e são altamente abundantes em bactérias, estando entre as dez mais expressas em *E. coli* (Link *et al.*, 1997), como também estão entre as mais expressas em *Bacillus anthracis* (Muntel *et al.*, 2014), *K. pneumoniae* (Khan *et al.*, 2017), *P. aeruginosa* (Hanna *et al.*, 2000), *Salmonella typhimurium* (Rollenhagen e Bumann, 2006), *S. aureus* (Enany *et al.*, 2014), dentre outras. A AhpC de todas as bactérias citadas acima contrasta com as Prx de eucariotos por não possuírem o prolongamento C-terminal, entretanto algumas possuem Thr como parte da tríade catalítica (*E. coli*, *P. aeruginosa*, *S. typhimurium*) e outras Ser (*B. subtilis*, *K. pneumoniae*, *S. aureus* e *Staphylococcus epidermidis*).

Estudos utilizando diferentes abordagens indicam que a AhpC de bactérias está relacionada com o processo de infecção e sobrevivência no hospedeiro (Alharbi *et al.*, 2019; Chaves-moreno *et al.*, 2016). Como exemplo, *P. aeruginosa* é um organismo patogênico que

afeta normalmente indivíduos com sistema imunológico comprometido, como no caso de AIDS, câncer e fibrose cística. Esta bactéria é responsável por causar infecções no trato respiratório, trato urinário e outros tecidos, além de ser um frequente colonizador de equipamentos médicos em hospitais (Wongsaroj *et al.*, 2018). As infecções por *P. aeruginosa* multirresistentes são uma ameaça mundial, sendo responsável por aproximadamente 10% de todas as infecções hospitalares em todo o mundo, com alta taxa de mortalidade (Rocha *et al.*, 2021).

Agências europeias demonstraram que 13.7% dos isolados de *P. aeruginosa* são resistentes a pelo menos três grupos de antibacterianos, enquanto 5.5% são resistentes a todos os cinco grupos de antimicrobianos mais utilizados (Rello *et al.*, 2019). De fato, somente nos EUA ocorrem gastos expressivos para pacientes diagnosticados com *P. aeruginosa* (Blanchette *et al.*, 2017). Neste organismo, a AhpC (PaAhpC, que possui Thr como parte da TC) apresenta atividade de peroxidase sobre vários substratos, já foi identificada tanto no meio intracelular quanto no extracelular, é capaz de desempenhar a função de chaperona (Ouidir *et al.*, 2014) e recentemente foi caracterizada como um fator de virulência deste organismo (Rocha *et al.*, 2021). Curiosamente, apesar do mecanismo ainda não ser compreendido, a administração de azitromicina em pacientes com fibrose cística é benéfica ao paciente e estudos de expressão global indicam que enzimas antioxidantes tem sua expressão diminuída no patógeno, dentre elas a superóxido dismutase B (SodB), TsaA, a chaperonina GroEL e PaAhpC, sugerindo que não só o papel antioxidante de AhpC, mas também o de chaperona pode ter importância nas infecções causadas por esta bactéria (Nalca *et al.*, 2006).

Também foi demonstrado que PaAhpC está envolvida na resposta ao estresse oxidativo do hospedeiro e na resistência ao antibacteriano Meropenem (β -lactâmico) (Maura *et al.*, 2016). Ainda, estudos envolvendo ratos com fibrose cística que apresentam variações na população de *P. aeruginosa* incluindo as SCV (*Small Colony Variants*) em infecções crônicas, revelou que entre as SCV, ocorre re-arranjo cromossômico que leva ao aumento da expressão de diversas enzimas antioxidantes, dentre elas a AhpC, o que está relacionado com a maior resistência a antibacterianos (Irvine *et al.*, 2018).

Como exemplo de bactérias que possuem Ser como parte da tríade catalítica de AhpC encontramos *S. epidermidis*, um organismo comensal, que representa a bactéria mais abundante encontrada na pele humana, mas que em algumas situações (pacientes imunocomprometidos, idosos e neonatos) se porta como um patógeno oportunista responsável por infecções hospitalares em razão de sua capacidade de contaminar cateteres e dispositivos implantados (Dong *et al.*, 2018). Uma vez que estes tipos de materiais têm aumentado drasticamente na

prática médica, este microrganismo está entre os cinco mais prevalentes nas infecções nosocomiais (originadas em hospitais). Também é o agente etiológico de infecções no trato respiratório, endocardites e sepses que podem levar ao óbito (Otto, 2009) e trabalhos recentes também indicam que *S. epidermidis* está fortemente envolvido em morbidades neonatais (Dong *et al.*, 2018).

Estudos metatranscriptômicos desta bactéria revelaram que o gene *ahpc* é expresso em altas taxas no trato respiratório e possui a expressão três vezes maior que em células cultivadas em meio de cultura (Chaves-moreno *et al.*, 2016). No que concerne à resistência a antibacterianos, linhagens de *S. epidermidis* são frequentemente resistentes a múltiplos antibacterianos como rifamicina, fluoroquinolonas, gentamicina, tetraciclina, clindamicina e sulfonamidas (Otto, 2009). A resistência à meticilina é particularmente difundida, com 75 a 90% dos isolados hospitalares resistentes ao medicamento, levando ao risco de infecções potencialmente incuráveis (Lee *et al.*, 2018). Curiosamente o tratamento com NO[•], é capaz de sensibilizar cepas resistentes ao tratamento com antibacterianos (Lee *et al.*, 2018), cabe ressaltar que antibacterianos levam a produção de O₂^{•-} o qual pode gerar ONOO⁻, substrato de AhpC. Recentemente demonstramos que esta enzima é inativada por meio de hiperoxidação de C_P por peróxido orgânico de forma muito mais eficiente eu o H₂O₂, indicando que esta enzima possa ter um papel de relevância na decomposição de peróxidos orgânicos (Tairum *et al.*, 2021).

1.6 Inibidores de Prx 2-Cys típicas

A expressão acentuada de Prx 2-Cys típicas está relacionada com doenças genéticas e infecciosas em mamíferos e trabalhos recentes buscaram por inibidores deste grupo de proteínas de mamíferos. Até o presente momento foram descritas doze moléculas, entre compostos naturais e sintéticos (Brindisi *et al.*, 2015; Lin *et al.*, 2019; Liu *et al.*, 2012; Muchowicz *et al.*, 2014; Shi *et al.*, 2017; Ye *et al.*, 2019; Wei *et al.*, 2016; Zhu *et al.*, 2019). É importante ressaltar que todos os compostos identificados possuem em comum um extenso esqueleto hidrofóbico indicando que esta característica é importante para interação do inibidor com o microambiente do sítio ativo da enzima (Figura 6).

Dentre estas moléculas a mais estudada é a adenantina (Adn), um *ent*-caurânio (diterpenóide) extraído das folhas de *Isodon adenanthus* e foi descoberta como um inibidor específico para as isoformas de Prx 2-Cys típicas de humanos HsPrx1/HsPrx2, através de análises envolvendo *pull down* e cromatografia de afinidade de extrato total de células humanas NB40 (Bajor *et al.*, 2018; Fidy *et al.*, 2019; Hou *et al.*, 2014; Hu *et al.*, 2019; Liu *et al.*, 2012;

Liu *et al.*, 2013; Soethoudt *et al.*, 2014). Esta molécula tem despertado interesse em função de suas propriedades antitumorais, uma vez que possui a capacidade de induzir a apoptose ou diferenciação de células tumorais linfocíticas, e apoptose hepatocarcinomas e tumores de mama (Bajor *et al.*, 2018; Fidy *et al.*, 2019; Hou *et al.*, 2014; Hu *et al.*, 2019; Liu *et al.*, 2012; Liu *et al.*, 2013; Mo *et al.*, 2019).

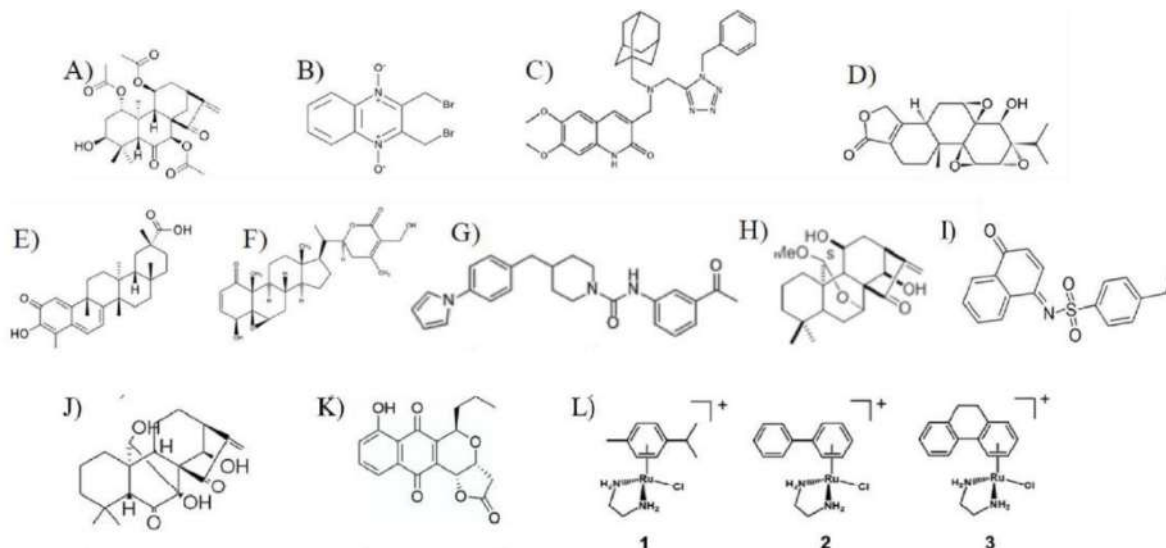


Figura 6. Estrutura química inibidores identificados para peroxirredoxinas 2-Cys típicas de eucariotos. A) Adenantina, **B)** Conoidina A, **C)** 3-aminometil quinolona N,N-bisubstituída, **D)** Triptolide, **E)** Celastrol, **F)** Withaferina A, **G)** AMRI-59, **H)** JDA-202, **I)** H7, **J)** ParvifolinaAA, **K)** Frenolicina B e **L)** Rutênio organometálico.

Outro composto bastante estudado foi o JDA-202, diterpenóide natural isolado de *Isodon rubescens*. O JDA-202 possui forte atividade antiproliferativa em linhagens tumorais de células de esôfago, e foi capaz de inibir a atividade de HsPrx1, *in vitro*, resultando na morte celular. Este composto também foi capaz de inibir o crescimento de xenoinxerto em camundongos, e no final do tratamento com o composto foi possível observar que não houve perda significativa da massa corporal e nem toxicidade aos órgãos (Shi *et al.*, 2017; Sarvar 2020).

O mecanismo de ação também foi determinado, tanto a Adn quanto o JDA-202, se ligam as cisteínas das Prx 2-Cys humanas atuando como um aceptor de Michael em uma reação de adição entre um sistema carbonílico α , β -insaturado dos compostos e o $S\gamma$ da cisteína de HsPrx1 e HsPrx2, aniquilando a atividade peroxidásica, devido ao impedimento da formação do dissulfeto intermolecular (Liu *et al.*, 2012; Liu *et al.*, 2013; Shi *et al.*, 2017; Soethoudt *et al.*, 2014). Na Figura 7 é representando o mecanismo de ação para Adn. Adicionalmente, abordagens envolvendo *docking* molecular indicaram que o esqueleto hidrofóbico de Adn é capaz de estabelecer interações de caráter hidrofóbico com aminoácidos na região do sítio ativo, incluindo os resíduos da tríade catalítica Thr⁴⁹ e Arg¹²⁸ (Liu *et al.*, 2012; Liu *et al.*, 2013).

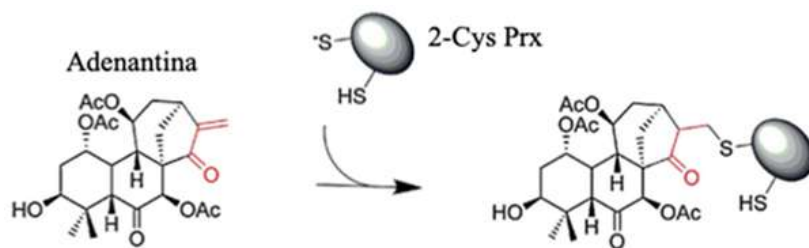


Figura 7. Estrutura química e mecanismos de ação de Adn sobre Prx 2-Cys. A adenantina contém sistema carbonílico α , β -insaturado que serve como aceptor de Michael (vermelho), que pode reagir com tióis de peroxirredoxinas, em especial a forma reativa da cisteína peroxidásica (tiolato, S-) (Figura adaptada de Muchowicz *et al.*, 2014).

A ParvifolinaAA (PAA), é um composto natural que também pertencente à classe dos *ent*-caurânos, isolado da mesma planta do composto JDA-202, *Isodon rubescens*. Foi demonstrado que a PAA estimula a expressão de ligantes NKG2D, que são receptores expressos por células *Natural Killer* (NK), em células de carcinoma hepatocelular, o que resulta no aumento e reconhecimento dessas células e lise pelas células NK. Adicionalmente, a PAA é capaz de se ligar covalentemente a cisteína das enzimas HsPrx1/HsPrx2 de humano, inibindo sua atividade. Em seguida, é ativada a via ROS/ERK, aumentando a lise tumoral mediada por células NK, devido a regulação positiva dos ligantes NKG2D nas células de carcinoma hepatocelular (Zhu *et al.*, 2019). Os ensaios foram realizados *in vivo*, e cabe ressaltar que não foram observados níveis aumentados de NKG2D em células saudáveis do fígado, o que mostra a especificidade de PAA com as células tumorais (Zhu *et al.*, 2019).

No entanto, era inexistente na literatura a avaliação de inibidores sobre AhpC de bactérias. Em trabalho desenvolvido pela doutoranda Melina C. Santos demonstramos que Adn é capaz de inibir AhpC bacteriana de forma altamente eficiente com IC_{50} aparente 0.481 ± 0.024 μ M, cerca de $30 \times$ menor do que o determinado para Prx2 humana e $3 \times$ menor que o determinado para Prx1 utilizando método semelhante ao descrito para as enzimas humanas (Liu *et al.*, 2012). Ainda, demonstramos que Adn é capaz de aniquilar células de patógenos Gram+ (*B. subtilis*, *S. aureus* e *S. epidermidis*) apresentando MIC_{50} da ordem de ~ 110 - 170 μ M. Estes foram os primeiros resultados gerados para um inibidor de AhpC. Ainda, os resultados foram compilados em uma solicitação de propriedade intelectual obtido por nosso grupo de pesquisa (BR 10 2020 016245 4). Recentemente, publicamos um artigo científico dos nossos resultados que contou com minha co-autoria (Santos *et al.*, 2022).

1.7 Busca por novos inibidores de AhpCs de bactérias oriundos da biota costeira do estado de São Paulo

A diversidade biológica da flora brasileira é extremamente rica e um grande número de compostos que apresentam atividade biológica das mais diversas como hipotensivos, antitumorais, anti-microbianos, antioxidantes dentre outros já foram isolados (Brito *et al.*, 2022; Rosa *et al.*, 2021; Diniz do Nascimento, 2022). Neste contexto, a busca por compostos que sejam oriundos da biota costeira de São Paulo nos parece um campo altamente promissor de investigação. De fato, a maioria dos compostos naturais identificados para as Prx de humanos são oriundos da biota chinesa (Brindisi *et al.*, 2015; Lin *et al.*, 2019; Liu *et al.*, 2012; Muchowicz *et al.*, 2014; Shi *et al.*, 2017; Ye *et al.*, 2019; Wei *et al.*, 2016; Zhu *et al.*, 2019) e nenhum trabalho até o presente momento atentou para a identificação de inibidores de Prx, em especial de AhpC de bactérias. Desta forma, essa é uma área que carece de estudos e representa uma oportunidade para investigações que explorem a ação de novos compostos.

Cabe ressaltar que todos os inibidores de Prx 2-Cys típicas identificados possuem uma extensa porção hidrofóbica o que indica uma característica aparentemente fundamental para a interação com o sítio ativo e que pode ser explorada de forma racional, bem como os grupos reativos a tióis. Portanto, compostos naturais como lignanas, hidroquinonas, derivados de ácidos, que também apresentem uma porção hidrofóbica em sua estrutura e possuem grupos reativos capazes de efetuar a adição de Michael, podem representar potenciais inibidores de AhpC. De fato, já foi demonstrado que compostos destas classes podem ser efetivos para o tratamento de infecções causadas por microrganismos, entretanto, seus alvos biológicos não foram identificados (Chen *et al.*, 2018). Neste contexto, estudos que busquem novas classes químicas de inibidores podem não somente identificar moléculas com potencial inibitório mais acentuado e/ou específico, mas também aumentar entendimento de características estruturais e funcionais de inibidores deste grupo de enzimas.

2 OBJETIVO

Os objetivos deste trabalho residiram na avaliação de compostos naturais oriundos da biota do Brasil pertencentes a diferentes classes químicas (sesquiterpêno, hidroquinona, ácidos benzóico prenilado e lignana), mas que preservam em comum um esqueleto hidrofóbico (de tamanho variável) e grupo reativo (para adição de Michael) visando a identificação de inibidor(es) de AhpCs bacterianas por meio de ensaios bioquímicos e análises estruturais. Como modelo dos estudos foram utilizadas as enzimas recombinantes AhpC de *P. aeruginosa* (bactéria Gram⁻, contendo Thr na TC) e *S. epidermidis* (Gram⁺, contendo Ser na TC).

3 MATERIAIS E MÉTODOS.

3.1 Reagentes

Os reagentes utilizados no presente trabalho foram adquiridos junto a Merck Millipore, Sigma Aldrich, Thermo Scientific e Cytiva Life Sciences.

3.2 Moléculas para avaliação da atividade inibitória sobre proteínas bacterianas

Para os produtos naturais os procedimentos de obtenção foram efetuados pelo grupo de pesquisa do Prof. Dr. João G.H. Lago (CCNH da UFABC). Brevemente: após coleta e processamento do material vegetal, as substâncias foram purificadas através de técnicas cromatográficas e suas estruturas definidas através de análise espectral envolvendo RMN (ressonância magnética nuclear) e EM (espectrometria de massas). Foram utilizados somente compostos que apresentem similaridades estruturais com compostos já identificados como inibidores de Prx 2-Cys, como a presença de regiões hidrofóbicas extensas e grupo(s) aptos a efetuar adição de tiól-Michael.

3.3 Meios de cultura para bactérias e linhagens bacterianas

3.3.1 Meios de cultura

Luria-Bertani (LB): (1% triptona; 0,5% extrato de levedura (YE); 0,5% NaCl); meio de cultura Super Optimal Broth (SOC): (2% triptona; 0,5% YE; 0,058% NaCl; 0,018% KCl; [glicose] final de 20mM; pH7); ampicilina 100 µg/ml; Meio de cultura Mueller Hinton (Difco).

3.3.2 Linhagens de E coli para propagação de plasmídeos e/ou expressão de proteínas

Propagação de plasmídeos: DH5αF - F' (Z80dlacZ_(lacZ)Adn5)_(lacZYA-argF)U169 recA1 endA1 hsdR17 (rk-,mk+); DH10B – F-mcrA Δ (mrr-hsdRMS-mcrBC) f80dlacZΔAdn5 ΔlacX74 deoR recA1 endA1 araΔ139 D (ara, leu) 7697 galK1- rpsLnupG; Expressão de proteínas recombinantes: BL21(DE3) - [F-, amp T, hsdSb (rB-mb-), gal, dcm (DE3) (Novagen); B84 DE3 [F-, metamp T-, hsdSb (rB-mb-), gal, dcm (DE3).

3.4 Plasmídeos de expressão e genes de proteína alvo

Os genes *ahpc* das bactérias *P. aeruginosa* (AAG03529.1) e *S. epidermidis* (P0A0B7), além dos genes Trx e TrxR (YP_002406546.1 e YP_002406546.1) de *E. coli* foram adquiridos junto a empresa GenScript (www.genscript.com) e clonados em plasmídeos pET15b entre os sítios para enzimas de restrição *Bam* HI e *Nde* I.

3.5 Expressão em bactérias e purificação das proteínas

Os procedimentos para expressão e purificação de proteínas recombinantes seguiram em linhas gerais os procedimentos descritos por Tairum e colaboradores (2016).

3.6 Quantificação de proteínas

As proteínas purificadas foram quantificadas espectrofotometricamente, a 280 nm, utilizando o coeficiente de extinção molar específico de cada uma (Tabela 1) obtidos através da ferramenta ProtParam (<http://web.expasy.org/protparam/>).

Tabela 1. Coeficiente de extinção molar específico e peso molecular das proteínas utilizadas no trabalho.

Enzima	ϵ 280 nm ($M^{-1} cm^{-1}$)	Peso molecular (kDa)	Código Uniprot
PaAhpC	21095	20.54	Q9I6Z3
SeAhpC	25556	21.08	Q8CMQ2
EcTrx	14105	12.63	P0AA25
EcTrxR	19160	35.45	P0A9P4
HsPrx2	21555	21.89	P32119
ScTrx1	10095	11.23	P22217
ScTrxR1	27640	34.23	P29509

3.7 Determinação da atividade peroxidásica através de ensaio de oxidação de NADPH

A atividade peroxidásica de AhpC utilizando o sistema Trx, foi avaliada pelo ensaio acoplado de oxidação do NADPH (Tairum *et al.*, 2016; Oliveira *et al.*, 2010). Para isso, 3 μ M de PaAhpC ou Se AhpC foram adicionados a um *mix* contendo 6 μ M de EcTrx, 0.9 μ M de EcTrxR, 150 μ M de NADPH, em tampão 50 mM HEPES pH 7,4, 1mM DTPA e 100 μ M de azida sódica, sendo então incubado por 5 minutos a 37°C. As reações então foram iniciadas pela adição de 500 μ M de H₂O₂ ou hidroperóxido de cumeno (CHP) e monitoradas espectrofotometricamente a 340nm. Para ensaios de avaliação da afinidade da enzima por substrato, foi utilizado o mesmo procedimento, apenas alterando as concentrações de peróxido utilizado de forma crescente. Para o ensaio de oxidação de NADPH com a enzima HsPrx2, foi utilizado 5 μ M HsPrx2, 10 μ M ScTrx1 e 0.2 μ M de ScTrxR1.

3.8 Avaliação da atividade inibitória dos compostos sobre a atividade das enzimas

Inicialmente, a PaAhpC e SeAhpC foram reduzidas com 5 mM de DTT por 1 hora, em temperatura ambiente, e em seguida dessalinizadas utilizando colunas PD10 (Cytiva Life Sciences) para retirada do excesso de composto, e tratadas com N-etilmaleimida (NEM) (40 μ M), controle de inibição, por mais 1 hora, também em temperatura ambiente. As amostras foram dessalinizadas novamente para a retirada do NEM e quantificadas. Para tratamento da enzima com os compostos, foi seguido os mesmos passos utilizados no tratamento com NEM. Após serem tratadas com o controle e com os compostos, as proteínas foram utilizadas nos ensaios de atividade peroxidásica pelo ensaio acoplado de oxidação de NADPH. No caso dos compostos eles foram diluídos em DMSO e a concentração foi ajustada para 40mM. Para as enzimas HsPrx2, EcTrx e EcTrxR foi utilizado o mesmo protocolo de tratamento.

3.9 Análise de estruturas de proteínas

Investigações estruturais para a racionalização dos dados obtidos foram efetuados por meio da análise de sequências proteicas depositadas no Uniprot (www.uniprot.org) e alinhamento utilizando o Clustal Ω e geração de gráficos com o Jalview (<http://www.jalview.org>). No caso de estruturas tridimensionais, efetuamos análises utilizando estruturas obtidas por cristalografia de raios-X presentes no banco de dados PDB (<https://www.rcsb.org>) e modelos construídos utilizando o Modeller (<https://salilab.org/modeller/>). Representações gráficas foram efetuadas utilizando o programa Pymol (<https://pymol.org/2/>).

3.10 Docking molecular dos compostos naturais com Ahpc

As análises da ligação dos compostos às estruturas de AhpC de *S. typhimurium*, *P. aureginosa* e *S. epidermidis* foram executadas por meio de *docking* molecular utilizando a ferramenta Autodock Vina (Trott e Olson, 2010). A estrutura cristalográfica da primeira enzima utilizada como modelo de estudo neste trabalho já foi determinada e a coordenada cristalográfica foi obtida junto ao Protein Data Bank (códigos de PDB: 1N8J). Já para as outras duas enzimas, foi necessário a criação de modelos teóricos através do Modeller, utilizando as coordenadas 1N8J. As coordenadas dos compostos naturais e hidroperóxidos orgânicos foram construídas utilizando a ferramenta MolView (<http://molview.org>). Em razão de limitações computacionais devido ao tamanho das proteínas (os decâmeros possuem cerca de 2.000 resíduos de AA), foram submetidos ao *docking* as proteínas na forma de tetrâmeros, uma vez

que estudos estruturais demonstram que o sítio ativo das Prx 2-Cys típicas se localiza na interface dos dímeros do decâmero (Schröder *et al.*, 2000; Tairum *et al.*, 2012; Tairum *et al.*, 2016), selecionando-se a região da C_P para que o programa gerasse as *poses*. Para os procedimentos de *docking*, os tetrâmeros foram previamente preparados no Chimera e para análise das interações foram utilizados os programas PyMOL (www.pymol.org) e LigPlot (<https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/>).

3.11 Determinação de constantes cinéticas por fluorescência por stopped-flow

A determinação das constantes por meio da fluorescência intrínseca do triptofano foi efetuada conforme descrito anteriormente com modificações (Tairum *et al.*, 2016). Brevemente: amostras de PaAhpC foram reduzidas com 10 mM de DTT por 1 hora, o qual foi depois removido por dois procedimentos de gel filtração utilizando colunas PD10 (Cytiva Life Sciences). As enzimas em tampão 5 mM Tris-HCl (pH 7.4) foram rapidamente misturadas com concentrações crescentes de H₂O₂, CHP ou Ol-OOH, 25 °C, em espectrofluorímetro *Applied Photophysics SX 17MV* acoplado a *stopped flow* com tempo de mistura menor que 2 ms. Os comprimentos de onda utilizados foram de 280 nm para excitação e 340 nm para emissão. A constante de hiperoxidação de PaAhpC pelos diferentes peróxidos foram calculadas através do decaimento/aumento da fluorescência intrínseca do triptofano após a reação com o hidroperóxido. Estes procedimentos foram efetuados com a colaboração grupo de pesquisa da Profa. Dra. Daniela Truzzi (IQ-USP, SP).

3.12 Ensaios de cristalizações de PaAhpC

Após a purificação de PaAhpC, foram realizados ensaios de crescimento de cristais utilizando os kits BCS Screen, Morpheus, PACT, ShotGun 1 da Molecular Dimensions, JCSG da Jena Biosciences e Salt Rx da Hampton Research seguindo as orientações dos fabricantes, pelo método de gota sentada a 20°C. Inicialmente a concentração da proteína foi de 10 mg/ml em tampão Tris-HCl 5mM. Condições de cristalização foram determinadas por meio de colaboração com o grupo de pesquisa do Prof. Dr. Otávio Henrique Thieman e do pesquisador Dr. Humberto Muniz Pereira (IF, USP-SC).

4 RESULTADOS E DISCUSSÃO

4.1 Obtenção compostos naturais oriundos da biota do Brasil

Neste trabalho, utilizamos quatro compostos naturais extraídos, purificados e selecionados por meio de busca racional baseada em inibidores naturais conhecidos desta classe de enzimas em eucariotos, como esqueleto hidrofóbico volumoso, que pode interagir com resíduos de aminoácidos hidrofóbicos no microambiente do sítio ativo, e a presença de um sistema carbonílico α , β -insaturado capaz de efetuar uma adição de tiól-Michael com o Sy de Cys, como descrito para inibidores identificados para Prx humanas como os *ent*-caurânos adenantina, JDA 202 e parvifolina (Liu *et al.*, 2012, Shi *et al.*, 2012; Zhu *et al.*, 2019) (Fig. 8A-8C) e a lactona sesquiterpênica CN LS3 (Figura 8D) a qual foi caracterizada como um inibidor de AhpC junto ao nosso grupo de pesquisa pela Dra. Melina C. Santos. Os compostos naturais foram extraídos de plantas pertencentes a região costeira do estado de São Paulo (ver legenda figura 8), purificados e selecionados pelo grupo de pesquisa do Prof. Dr. João H. G. Lago do CCNH da Universidade Federal do ABC (Figura 8 E-H). Os compostos pertencem às classes químicas: lactona sesquiterpênica, hidroquinona prenilada, ácido benzóico prenilado e lignana. Neste estudo os compostos foram denominados como CN-LS5, CN-HP1, CN-ABP1 e CN-LG1, respectivamente.

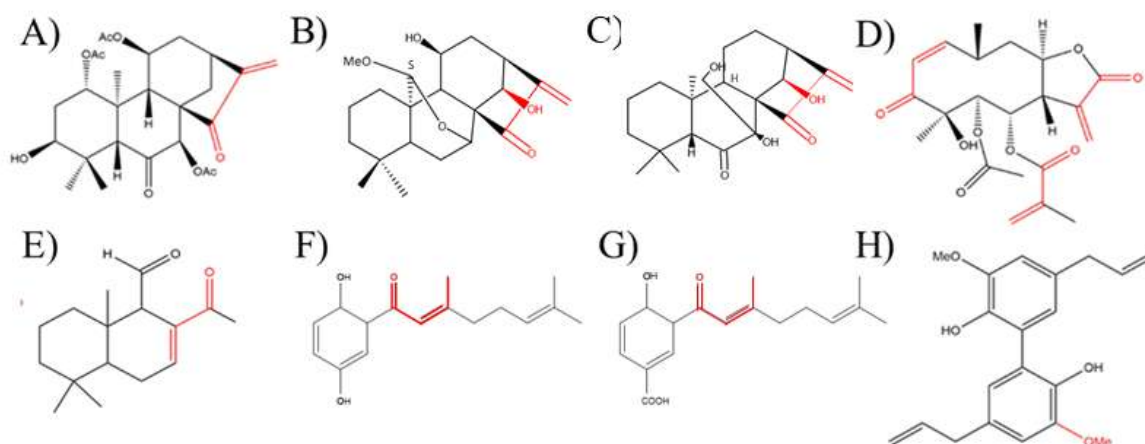


Figura 8. Representação das estruturas de compostos naturais que atuam como inibidores de Prx e compostos utilizados neste trabalho. A figura apresenta as estruturas bidimensionais de compostos naturais de diferentes classes. Nas figuras **A)**, **B)** e **C)** estão representadas as estruturas dos inibidores adenantina, JDA 202 e parvifolina (*ent*-caurânos), que apresentaram atividade inibitória em Prx humanas, e a Adn, em estudos realizados pelo grupo, mostrou-se como um potente inibidor de AhpCs bacterianas. Em **D)** está representada a estrutura de uma lactona sesquiterpênica de *Calea pinnatifida* (denominada de CN-LS3) que apresentou forte atividade inibitória sobre AhpCs bacterianas. Já em **E - H)**, estão representadas as estruturas dos compostos extraídos, purificados e selecionados pelo Prof. João H.G. Lago e Prof. Dr. Rodrigo L.O.R. Cunha (UFABC) para este estudo: CN-LS5 (lactona sesquiterpênica) de *Pentacalia desiderabilis*, CN-HP1 (hidroquinona prenilada) e CN-ABP1 (ácido benzóico prenilado), ambos de *Piper crassinervium*, e CN-LG1 (lignana) de *Nectandra cupidata*, respectivamente. Os grupos em vermelho representam sistemas contendo aceptores de Michael.

4.2 Expressão, purificação e quantificação de proteínas recombinantes

Uma vez que os genes foram clonados em plasmídeos pET15b, o qual insere uma cauda de histidinas em fusão com AhpC de *P. aeruginosa* (PaAhpC) ou de *S. epidermidis* (SeAhpC), as enzimas foram expressas e purificadas por cromatografia de afinidade por íons metálicos imobilizados (IMAC) em gradiente de imidazol (5mM – 500mM). Ambas as enzimas eluíram, majoritariamente em todos os procedimentos efetuados, na concentração de 100mM de imidazol (Fig. 9). Em média, a partir de um litro de cultura de células, foi possível obter ~ 8.0 mg para PaAhpC e ~ 10.0 mg para SeAhpC, todas com alto grau de pureza (> 95%).

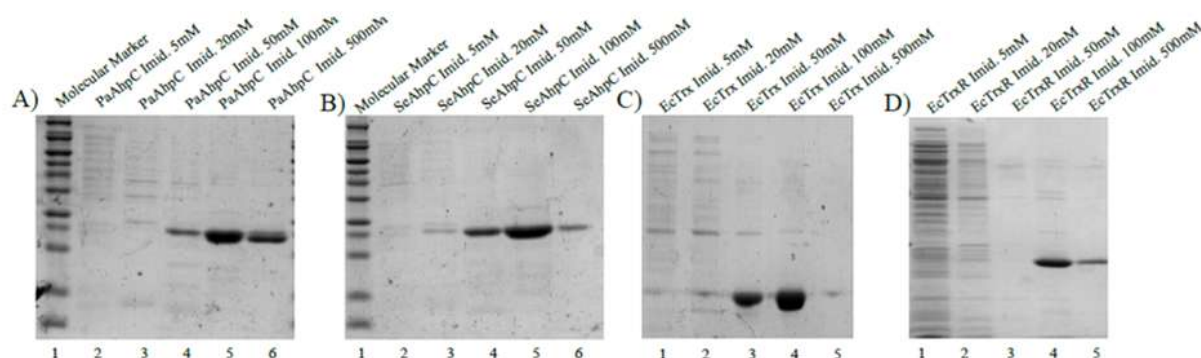


Figura 9. Resultado da expressão e purificação por IMAC de PaAhpC, SeAhpC, EcTrx e EcTrxR expressas em *E. coli* BL21 (DE3). SDS-PAGE redutor (200mM de β -mercaptoetanol), contendo 12% de poliacrilamida, representativo dos resultados de purificações de proteínas recombinantes contendo His-tag purificadas por IMAC utilizando a coluna HiTrap TALON cruda (Cytiva Life Sciences). Resultados de PaAhpC (A) e SeAhpC (B). Na primeira lane foi aplicado o marcador de peso molecular Sigma Marker (Sigma-Aldrich) e nas demais as frações eluídas em diferentes concentrações de imidazol (5mM, 20mM, 50mM, 100mM e 500mM). Resultados representativos da expressão e purificação de EcTrx (C) e EcTrxR (D). Nas lanes 1-5 foram aplicadas as frações eluídas em concentrações crescentes de imidazol (5-500 mM).

Apesar do sistema redutor de AhpC ser representado pela enzima AhpF (Alquil hidroperóxido redutase subunidade F) não é possível realizar ensaios enzimáticos com AhpF em anaerobiose, em razão de limitações laboratoriais. Em aerobiose, além de transferir elétrons provenientes do NADH *via* uma molécula de FAD para as AhpC oxidadas, a enzima AhpF também apresenta um papel de NADH oxidase, gerando H_2O_2 a partir da redução direta do O_2 pela molécula de flavina (Arai *et al.*, 2015). Esse processo ocorre às custas de NADH, o que gera um artefato importante para a avaliação da atividade de AhpC. Neste contexto, optamos por expressar e purificar o sistema Trx heterólogo de *E. coli*, o qual também pode ser utilizado como redutor de AhpC, como nosso grupo e outros já demonstraram (Arai *et al.*, 2015; Tairum *et al.*, 2021). Para tanto as construções em pET15b efetuadas pela pesquisadora Melina C. Santos foram gentilmente cedidas para esta finalidade. Um resultado representativo dos procedimentos de expressão e purificação de Trx e TrxR é mostrado nas Fig. 9C e 9D. Foi possível obter, em média a partir de um litro de cultura de células, ~ 15.0 mg para EcTrx e ~ 7.0 mg para EcTrxR.

4.3 Avaliação de inibição de composto naturais oriundos da flora brasileira sobre as enzimas PaAhpC e SeAhpC pelo ensaio acoplado de oxidação de NADPH

Para avaliação de possíveis propriedades inibitórias dos compostos naturais oriundos da biota florística de São Paulo, sobre a PaAhpC e SeAhpC, foi aplicado o protocolo similar ao utilizado para as Prx humanas descrito por Liu e colegas (2012), sendo utilizado para os ensaios de oxidação de NADPH o sistema redutor Trx de *E.coli* e tendo como controle interno de inibição a proteína PaAhpC ou SeAhpC tratadas com NEM, um conhecido alquilante de cisteínas.

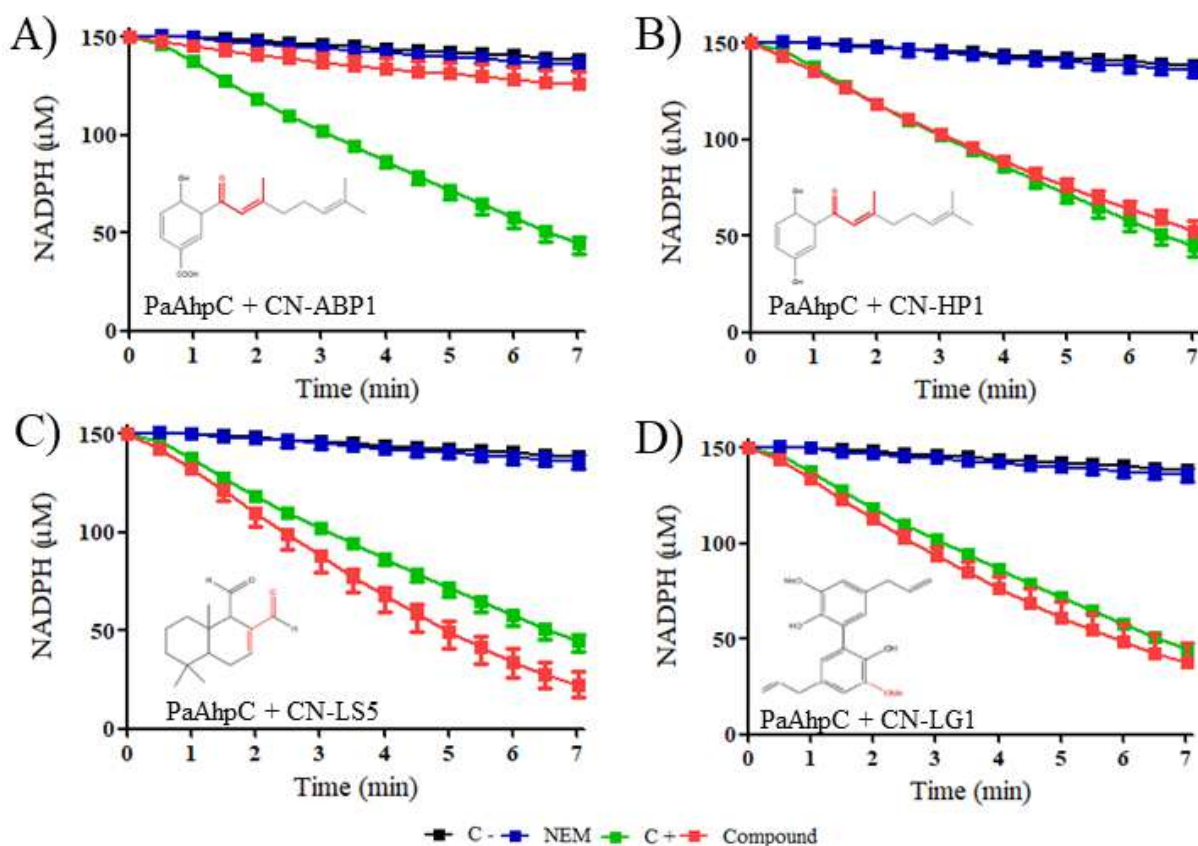


Figura 10. Avaliação da atividade peroxidásica pelo ensaio de oxidação do NADPH de PaAhpC previamente tratadas com os compostos naturais. Amostras de PaAhpC foram reduzidas, dessalinizadas e tratadas com 40 equivalentes dos compostos naturais (CN-ABP1 (A), CN-HP1 (B), CN-LS5 (C) ou CN-LG1 (D)) por 1 hora em temperatura ambiente e dessalinizadas novamente. Os ensaios foram realizados utilizando PaAhpC (3 μM), Trx (12 μM), TrxR (0.9 μM), NADPH (150 μM), HEPES (50 mM, pH 7.0), DTPA (100 μM) e azida sódica (1 mM). As reações foram iniciadas pela adição de 500 μM de H₂O₂ e monitoradas espectrofotometricamente a 340 nm, 37°C, por 7 minutos. Nos gráficos acima são apresentadas quatro curvas, sendo ■ = PaAhpC (controle positivo), ■ = NEM (controle positivo de inibição), ■ = PaAhpC + composto e ■ = Controle negativo (sem enzima). Os ensaios foram feitos em triplicata e repetidos ao menos três vezes.

Os resultados demonstraram, como esperado, que o NEM é capaz de aniquilar a atividade da enzima de *P. aeruginosa* quando comparado com a enzima sem tratamento (Figura 10). Dos quatro compostos testados, o CN-LS5 foi capaz de acelerar a velocidade inicial da reação quando comparado à enzima sem nenhum tratamento (Figura 10C, Tabela 2). Entretanto os motivos destes resultados ainda carecem de investigação. Para os compostos CN-HP1 e CN-

LG1 não ocorreram diferenças significativas da v0 (Figura 10B, 10D, Tabela 2). No entanto, CN-ABP1 apresentou forte efeito inibitório, o que o torna um promissor inibidor de Prx bacterianas (Figura 10, Tabela 2).

Quando o mesmo procedimento foi efetuado para SeAhpC, a qual contém a substituição natural do resíduo de Thr da tríade catalítica por Ser. Os resultados foram bastante contrastantes, uma vez que nenhum dos compostos foi capaz de inibir de forma significativa SeAhpC, como é possível observar na figura 11. Apesar disso, o resultado é relevante, pois indica que o composto tem maior afinidade para PaAhpC uma vez que a presença de Thr como parte do sítio catalítico de PaAhpC (substituído por Ser em SeAhpC) leva a diferenças funcionais e estruturais entre as duas enzimas incluindo a afinidade por substratos distintos, como demonstrado previamente por nosso grupo de pesquisa (Tairum *et al.*, 2016; Tairum *et al.*, 2021). A tabela 2 sumariza as diferenças nas velocidades iniciais de PaAhpC e SeAhpC, tratadas ou não com os compostos.

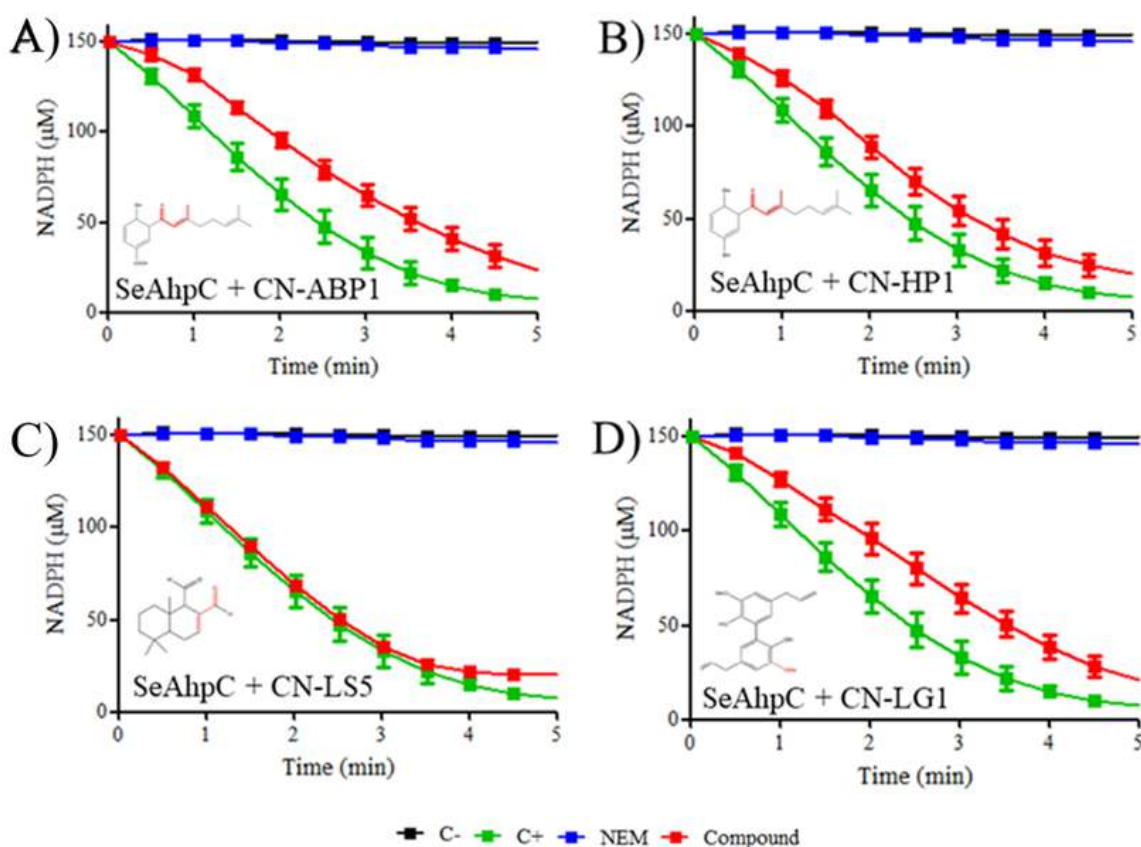


Figura 11. Avaliação da atividade peroxidásica pelo ensaio de oxidação do NADPH de SeAhpC previamente tratada com os compostos naturais. Amostras de SeAhpC foram reduzidas, dessalinizadas e tratadas com 40 equivalentes dos compostos naturais CN-LS5 (A), CN-HP1 (B), CN-ABP1 (C) e CN-LG1 (D) por 1 hora em temperatura ambiente e posteriormente dessalinizadas novamente. Os ensaios foram realizados com 3 μM SeAhpC, 6 μM EcTrx, 0.9 μM EcTrxR, 150 μM NADPH, 50 mM HEPES pH 7.4, 100 μM DTPA e 1 mM azida sódica. As reações foram iniciadas pela adição de 500 μM de H₂O₂ e monitoradas espectrofotometricamente a 340 nm, 37°C, por 5 minutos. Nos gráficos acima são apresentadas quatro curvas, sendo ■ = SeAhpC (controle positivo), ■ = NEM (enzima tratada com NEM - controle positivo de inibição), ■ = SeAhpC + composto e ■ = sem adição de enzima (controle negativo). Os ensaios foram feitos em triplicata e repetidos ao menos três vezes.

Tabela 2. Valores de v_0 obtidos para SeAhpC tratada com os compostos naturais.

Amostra	v_0 ($\mu\text{M/s}$)	Decréscimo de v_0 (%)
Controle (sem SeAhpC)	0.02 (± 0.01)	-
SeAhpC	0.70 (± 0.05)	-
SeAhpC + NEM	0.03 (± 0.01)	95.72 (± 0.50)
SeAhpC + CN-LS5	0.68 (± 0.03)	2.86 (± 2.14)
SeAhpC + CN-HP1	0.69 (± 0.05)	1.43 (± 1.58)
SeAhpC + CN-ABP1	0.64 (± 0.03)	8.57 (± 2.12)
SeAhpC + CN-LG1	0.63 (± 0.04)	10.00 (± 3.91)
Controle (sem PaAhpC)	0.02 (± 0.01)	-
PaAhpC	0.26 (± 0.01)	-
PaAhpC + NEM	0.03 (± 0.01)	88.46 (± 3.14)
PaAhpC + CN-LS5	0.35 (± 0.01)	0.00 (± 3.14)
PaAhpC + CN-HP1	0.24 (± 0.02)	7.69 (± 6.56)
PaAhpC + CN-ABP1	0.05 (± 0.02)	80.76 (± 4.96)
PaAhpC + CN-LG1	0.23 (± 0.03)	11.53 (± 8.30)

Como mencionado anteriormente, os únicos compostos descritos até o presente momento capazes de inibir Prx 2-Cys, através da adição de tiól-Michael, são *ent*-caurânos como a adentanina, JD-202 e Frenolicina B (Liu *et al.*, 20102; Shi *et al.*, 2017; Ye *et al.*, 2019), e a lactona sesquiterpênica CN-LS3 identificada por nosso grupo de pesquisa. Neste contexto, CN-ABP1, o qual é um ácido benzóico prenilado, representa uma nova classe de compostos capaz de inibir as Prx 2-Cys. No entanto, um fato curioso que não escapou a nossa atenção que apesar de extremamente similares os compostos CN-ABP1 e CN-HP1 (Figura 8F e 8G, respectivamente), somente o primeiro foi capaz de exercer atividade inibitória sobre PaAhpC. Para investigar possíveis aspectos moleculares envolvidos nesta diferença investigamos a interação entre as proteínas e ligantes por meio de simulações computacionais envolvendo *docking* molecular.

4.4 Construção dos modelos teóricos de PaAhpC e SeAhpC

Uma vez que as estruturas tridimensionais de PaAhpC e SeAhpC ainda não foram determinadas experimentalmente, utilizamos a estrutura cristalográfica de AhpC de *S. typhimurium* (StAhpC, PDB = 1N8J) para a construção dos modelos teóricos em razão da alta homologia com as proteínas de PaAhpC (60% de similaridade e 75% de identidade) e SeAhpC (63% de similaridade e 79% de identidade) (Figura 12).

StAhpC/1-187	1	MSLINTKIKPKNQAFK--NGEFIEVTEKDTEGRWSVFFYPADTFVCPTELGDVADHYEEL	61
PaAhpC/1-187	1	MSLINTQVQPKVNAFH--NGKFI EVTEESLKKWSVLIMPAAATFNCPTEIEDAANNYGEF	61
SeAhpC/1-189	1	MSLINKEILPETAQAYDPKKDEFKEVTQEDFKCSNVVCYPADSFVCPTELEDLQNQYAKL	63
StAhpC/1-187	62	QKLCVDVYSVSTDTHTTHKAWHSSSETIAKIKYAMIGDPTGALTNRFDNMREDEGLADRATFV	124
PaAhpC/1-187	62	QKAGAEVYIVTDTHTSHKVVHETS PAVGKAQFPLIGDPTHQLTNAFGVHIPEEGLALRGTFV	124
SeAhpC/1-189	64	QELCVNVYSVSTDTHTVHKAWHDHSDAISKLEYSMIGDPSQTI TRNFDVLDEESGLAQRGTFI	126
StAhpC/1-187	125	VDPQCIIQAIEVTAEGIGRDASDLLRKIKAAQYVAAHPGEVCPAKWKEGEATLAPSLDLVGKI	187
PaAhpC/1-187	125	INPEGVIKTVEIHSNEIARDVGETVRKLKAAQYTAAPHPGEVCPAKWKEGEKTLAPSLDLVGKI	187
SeAhpC/1-189	127	IDPDGVVQAAEINADGIGRDASTLVNLIKAAQYVRQHPGEVCPAKWEEGSESLQPGLDLVGKI	189

Figura 12. Alinhamento das seqüências de aminoácidos de AhpCs de *S. typhimurium*, *P. aeruginosa* e *S. epidermidis*. O alinhamento das seqüências de AA foi executado utilizando o Clustal Ω e a figura foi gerada com o programa Jalview. As abreviações utilizadas e códigos de acesso ao Uniprot (www.uniprot.org) são: AhpC de *S. typhimurium* = StAhpC (Uniprot: P0A251), AhpC de *P. aeruginosa* = PaAhpC (Q02UU0) e AhpC de *S. epidermidis* = SeAhpC (Q8CMQ2).

A ferramenta Modeller (Eswar *et al.*, 2006) foi utilizada para gerar modelos monoméricos das enzimas. StAhpC cobre 100% dos resíduos de aminoácidos de PaAhpC (187 AA) e 98.9% de SeAhpC (189 AA), apresentando 2 resíduos de *gap* na região N-terminal da proteína e distante de resíduos envolvidos na catálise. Os dois modelos apresentaram bons parâmetros de qualidade global, com o GA341 de ambos igual à 1.00, e valor de zDOPE (*Discrete Optimized Protein Energy*) de -0.98 para PaAhpC e -0.95 para SeAhpC, indicando que a confiabilidade dos modelos é > 95%. O desvio quadrático médio (*Root Mean Square Deviation*, r.m.s.d) entre os C α dos modelos gerados foi determinado em 0.335 Å e 0.226 Å, para PaAhpC e SeAhpC, respectivamente, representando alta concordância dos modelos gerados com a estrutura utilizada de StAhpC, aspecto esperado em razão da grande semelhança entre as enzimas. A partir dos monômeros foram geradas as estruturas decaméricas das duas enzimas utilizando o software PyMol (Figura 13A). É importante também mencionar que os resíduos da tríade catalítica (Arg, Thr/Ser e CP) ocupam posições espaciais equivalentes, mas não idênticas na estrutura cristalográfica de StAhpC e nos modelos de PaAhpC e SeAhpC (Figura 13B).

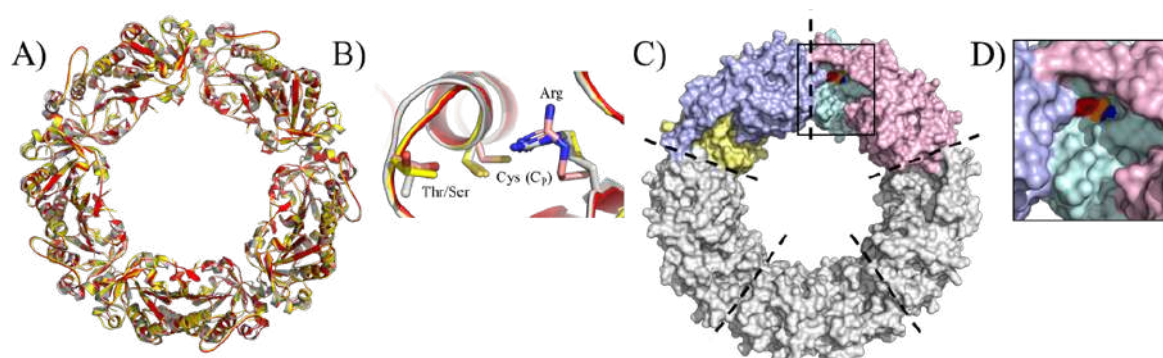


Figura 13. Alinhamento da estrutura cristalográfica de StAhpC com os modelos teóricos de PaAhpC e de SeAhpC e superfície molecular da estrutura decamérica de StAhpC. A) Alinhamento da estrutura cristalográfica de StAhpC (em cinza, 1N8J) com os modelos teóricos de PaAhpC (em amarelo) e SeAhpC (em vermelho). B) Representação da tríade catalítica, em *sticks*, das três estruturas, indicando posição similar dos resíduos. Os carbonos dos resíduos de StAhpC estão representados em cinza, de PaAhpC em amarelo e de SeAhpC em salmão, os nitrogênios, oxigênios e enxofre, são apresentados em azul, vermelho e amarelo. C) StAhpC em sua conformação decamérica representada em superfície colorizada em branco, com exceção de um tetrâmero

(conformação utilizada para os procedimentos de *docking* molecular) onde os monômeros estão colorizados diferencialmente em amarelo, azul, ciano e rosa. As linhas tracejadas destacam as interfaces D dos cinco homodímeros que compõem a AhpC. **D)** Representação do microambiente do sítio ativo da enzima, com os resíduos integrantes da tríade catalítica, Thr⁴⁴, C_P e Arg¹²⁰, em vermelho, laranja e azul respectivamente. Todas as figuras foram geradas utilizando o PyMol.

4.5 Análise das interações dos compostos com AhpC por *docking* molecular

Para as análises de *docking* (acoplamento) molecular se evitou a utilização de dímeros uma vez que diversos estudos indicam que quando as Prx 2-Cys típicas, como AhpC, estão aptas para decompor hidroperóxidos elas se encontram na forma decamérica (Tairum *et al.*, 2012; Wood *et al.*, 2003). De fato, a análise estrutural de enzimas desta classe também revela que os resíduos da tríade catalítica se encontram muito próximo a interface dos homodímeros do decâmero (interface D) (Figura 13C). Já foi demonstrado que um resíduo hidrofóbico conservado entre as Prx 2- Cys (Phe⁷⁶ em *S. typhimurium*) do dímero adjacente é capaz de interagir intimamente com a Thr ou Ser da tríade catalítica por meio de uma interação do tipo CH- π (Tairum *et al.*, 2016; Tairum *et al.*, 2021). Uma vez que o microambiente do sítio ativo possui diversos resíduos do dímero adjacente (Figura 13D) é fundamental a utilização de oligômeros contendo as interfaces diméricas (interface D) nas simulações. Neste contexto, nossas simulações contrastam com outros trabalhos que utilizaram dímeros para simulações de *docking* (Liu *et al.*, 2012; Shi *et al.*, 2017).

Para efetuar as análises, utilizamos a estrutura cristalográfica da enzima StAhpC (PDB = 1N8J) como controle nas análises de *docking* molecular para PaAhpC e SeAhpC. Inicialmente foram efetuados os procedimentos de ancoragem molecular com a StAhpC e depois os resultados foram comparados aos obtidos para os modelos construídos neste projeto. Os modelos tridimensionais dos compostos foram gerados utilizando a ferramenta MolView (Smith, 1995) e efetuamos as simulações de *docking* utilizando o AutoDock Vina (Trott & Olson, 2010). Utilizando uma *grid box* de dimensões 20 × 20 × 20 Å envolvendo ambos os resíduos de cisteína (C_P e C_R), foram geradas no total 150 soluções (*poses*) para todos os compostos. Utilizamos inicialmente dois critérios para seleção das melhores soluções: compostos que estivessem a uma distância máxima de 5 Å do S γ da C_P ou C_R e o C α - β insaturado voltado para o S γ . Dos quatro compostos utilizados, apenas dois apresentaram soluções compatíveis de *docking* para que StAhpC possa efetuar uma adição de tiól-Michael com o S γ das cisteínas: o CN-ABP1 e CN-HP1.

O composto CN-ABP1 o qual apresentou 26 *poses* (Figura 14A), onde todas ficaram voltadas para a C_P, com afinidades variando entre -7.6 e -6.4 kcal/mol. Os principais resíduos que aparecem em mais de 50% dos sítios de ligação de CN-ABP1 são: Pro⁴⁰, Ala⁴¹, Thr⁴⁴, Phe⁴⁵,

Val⁴⁶, Cys⁴⁷, Leu¹¹⁷, Arg¹²⁰, Glu¹³⁹ do monômero de C_P; Val¹⁶⁵ e Pro¹⁷⁹ do monômero complementar; Phe²¹, His⁷⁶, Phe⁷⁷ do dímero adjacente. Além disso, notou-se o predomínio de interações hidrofóbicas e grande parte destas interações são oriundas das cadeias laterais dos resíduos.

Para CN-HP1, dos 150 resultados totais gerados pelo *docking*, somente 14 *poses* se posicionaram próximo a C_P, com afinidades variando de -7.3 e -6.2 kcal/mol (Figura 14D). Os principais resíduos que aparecem em mais de 50% dos sítios de ligação de CN-HP1 são: Pro⁴⁰, Thr⁴⁴, Phe⁴⁵, Val⁴⁶, Cys⁴⁷, Leu¹¹⁷, Arg¹²⁰, Glu¹³⁹ do monômero de C_P; Pro¹⁷⁹ do monômero complementar; His⁷⁶ e Phe⁷⁷ do dímero adjacente.

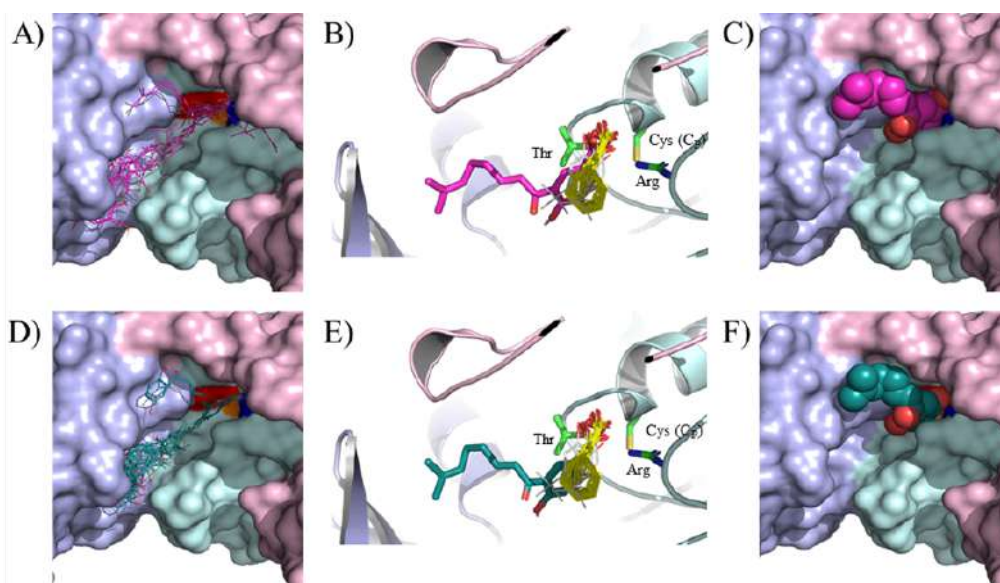


Figura 14. Resultados de CN-ABP1 e CN-HP1 gerados por *docking* molecular para StAhpC e suas *best poses*. Todos os resultados gerados para os tetrâmeros que apresentaram as melhores soluções iniciais de CN-ABP1 e CN-HP1 em StAhpC são mostrados nas figuras **A)** e **D)**, respectivamente, onde cada monômero é representado por uma cor (amarelo, azul, ciano e rosa). Os melhores *hits* do *docking* de StAhpC com CN-ABP1 e CN-HP1 foram selecionados e alinhados aos ligantes H₂O₂ (PDB = 3A2V), benzoato (PDB = 2V32; 1HD2; 1OC3; 2V41; 1H4O) e tert-butilbenzenodiol (PDB = 4K7O). Com este alinhamento foi possível selecionar as *best poses* para cada um dos compostos (*sticks*) que estão representadas em **B)** e **E)** juntamente com os outros ligantes, representados em *lines*, e os carbonos de cada um estão representados nas seguintes cores: CN-ABP1 = rosa; CN-HP1 = ciano; benzoato = amarelo; tert-butilbenzenodiol = branco. Os oxigênios das moléculas estão em vermelho. Na figura **C)** e **F)** estão as *best pose* de CN-ABP1 e CN-HP1 no sítio ativo, respectivamente, e a proteína é representada em superfície molecular, e os aminoácidos do sítio ativo são coloridos, com Thr = vermelho, C_P = laranja, Arg = azul.

Com o intuito de selecionar a melhor solução para cada composto, seguimos observações previamente descritas por Hall e colaboradores (2011). Estes pesquisadores demonstraram que compostos encontrados no sítio ativo de Prx determinadas por cristalografia, normalmente oriundos das soluções de cristalização, muitas vezes adotam conformações estruturais que mimetizam os substratos biológicos. Neste contexto, para seleção da *best pose* dos compostos envolvidos neste estudo, realizamos o alinhamento dos resultados selecionados

com estruturas de Prx depositadas no banco de dados de estruturas de proteínas (PDB: <https://www.rcsb.org>) contendo ligantes no sítio ativo. Foram selecionadas sete estruturas de Prx que apresentavam ligantes: Prx6 de *Arenicola marina* (PDB = 2V32) e Prx 5 de *Homo sapiens* (PDB = 1HD2; 1OC3; 2V41; 1H4O), as quais contêm moléculas de benzoato em seus sítios ativos (Declercq *et al.*, 2001; Evrard *et al.*, 2004, Smeets *et al.*, 2008); Prx 5 de *Homo sapiens*, que conta com a presença do ligante tert-butylbenzene-diol (PDB = 4K7O) (Aguirre *et al.*, 2014); Tpx de *Aquifex aeolicus* que apresenta moléculas de peróxido de hidrogênio como ligante (PDB = 3A2V) (Nakamura *et al.*, 2010). A escolha desses quatro ligantes foi devido a dois deles, o benzoato e o tert-butilbenzenediol apresentarem o anel benzênico. Já o H₂O₂ foi utilizado para avaliar a posição do carbono reativo presente nos compostos naturais em relação ao S_γ de C_p. Levando em conta o alinhamento com os compostos co-cristalizados com Prx foram determinadas as *best poses* para CN-ABP1 e CN HP1 para StAhpC (Figura 14B/14C e 14E/14F, respectivamente).

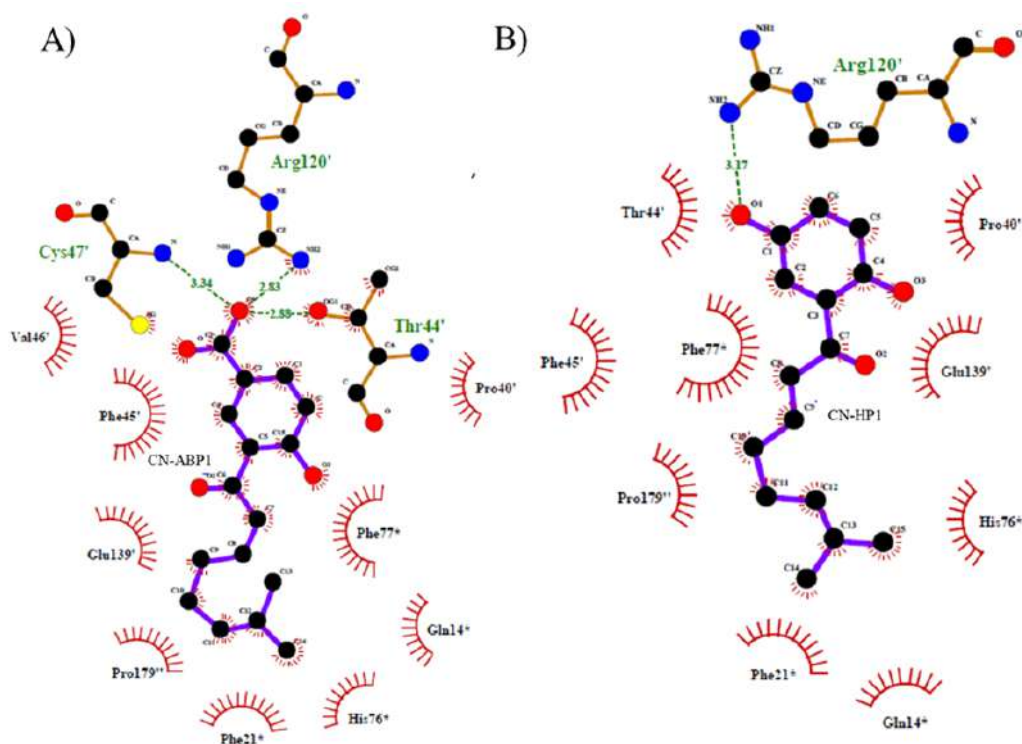


Figura 15. Representações bidimensionais das interações moleculares entre StAhpC e os compostos naturais. As figuras acima mostram as possíveis interações que ocorrem entre os ligantes CN-ABP1 (A) e CN-HP1 (B) com StAhpC. Os resíduos com caracteres em verde e com linhas tracejadas verdes são os que realizaram interações polares com o composto. Os aminoácidos contidos em semicírculos vermelhos e com os caracteres em preto são aqueles envolvidos em interações hidrofóbicas com os ligantes. Resíduos como símbolo linha (') e aspas (") são de diferentes subunidades do homodímero obrigatório, enquanto aqueles com asterisco (*) pertencem ao dímero adjacente. As figuras foram geradas através do programa LigPlot⁺.

De forma geral para ambos os compostos, a maioria das interações moleculares são de caráter hidrofóbico e ocorre com cadeia lateral dos resíduos de aminoácidos (Figura 15A e 15B;

Tabela 3). No entanto, uma diferença bastante importante reside justamente no grupo que diferencia as duas moléculas. Em CN-ABP1 o anel benzênico possui um grupamento ácido carboxílico o qual é capaz de estabelecer interações polares (pontes de hidrogênio) com os três aminoácidos da tríade catalítica (Thr⁴⁴, C_P⁴⁷ e Arg¹²⁰) (Figura 15A). Este grupo é substituído por um grupo hidroxila em CN-HP1, o qual estabelece interação polar somente com a Arg da tríade catalítica. Especulamos que este aspecto pode estar envolvido nas diferenças de um composto se portar como inibidor ou não e será discutido posteriormente.

Após os procedimentos de *docking* para a estrutura cristalográfica de AhpC de *S. typhimurium* nos concentramos nas simulações utilizando os modelos teóricos de AhpC de *P. aeruginosa* (PaAhpC) e *S. epidermidis* (SeAhpC). Para PaAhpC, apenas o composto CN-ABP1 apresentou resultados positivos. Este ponto nos parece importante, uma vez que convergem com os resultados dos ensaios bioquímicos em que o composto CN-HP1, não foi capaz de inibir a enzima PaAhpC.

No que tange aos resultados de CN-ABP1 das 150 soluções obtidas, foi possível selecionar 26 *poses*, novamente todas próximas a C_P com energia livre de Gibbs variando entre -8.3 e -6.0 kcal/mol (Figura 16A). Os resíduos mais frequentemente detectados nas soluções foram: Pro⁴⁰, Thr⁴⁴, Phe⁴⁵, Asn⁴⁶, Cys⁴⁷, Leu¹¹⁷, Arg¹²⁰, Asn¹³⁹ do monômero onde se encontra C_P; Val¹⁶⁷ e Pro¹⁸¹ do monômero complementar do dímero íntimo; Asn¹⁴, Phe²¹, His⁷⁶ e Phe⁷⁷ do dímero adjacente. A escolha da *best pose* foi realizada seguindo o mesmo processo mencionado anteriormente. Para PaAhpC foi possível selecionar 3 soluções, que melhor se posicionaram em relação aos quatro ligantes utilizados (Figura 16B e 16C).

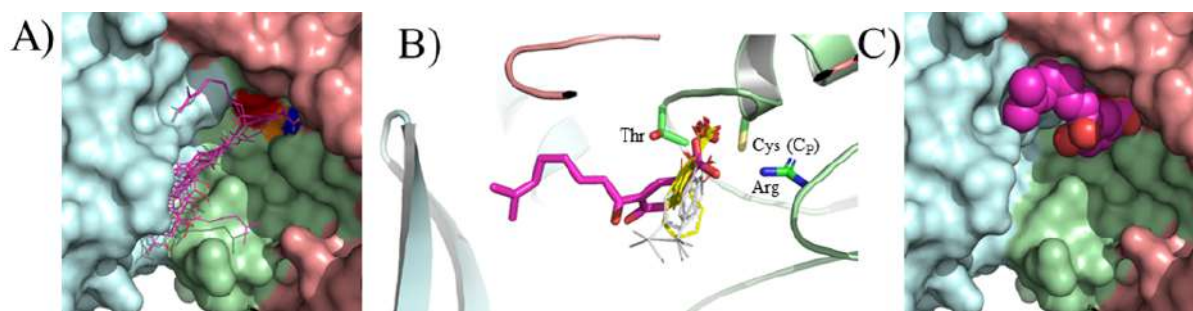


Figura 16. Representação dos resultados de CN-ABP1 gerados para PaAhpC e sua *best pose*. Todos os resultados gerados para o tetrâmero que apresentou a *best pose* de CN-ABP1 em PaAhpC são mostrados na figura A) onde cada monômero é representado por uma cor (laranja, ciano, verde, salmão). Os melhores *hits* do *docking* de PaAhpC com CN-ABP1 foram selecionados e alinhados aos ligantes H₂O₂ (PDB = 3A2V), benzoato (PDB = 2V32; 1HD2; 1OC3; 2V41; 1H4O) e tert-butilbenzeno-diol (PDB = 4K7O). Com este alinhamento foi possível selecionar a *best pose*, que está representada na figura B) como *sticks*, juntamente com os outros ligantes, representados em *lines*, e os carbonos de cada um estão representados nas seguintes cores: CN-ABP1 = rosa; benzoato = amarelo; tert-butilbenzeno-diol = branco. Os oxigênios das moléculas estão em vermelho. Na figura C) está a *best pose* de CN-ABP1 no sítio ativo, a proteína é representada em superfície molecular, e os aminoácidos do sítio ativo são coloridos da seguinte forma: Thr = vermelho, C_P = laranja, Arg = azul.

No caso de SeAhpC, foram obtidas 29 *poses* de CN-ABP1 e 13 para CN-HP1, dos 150 resultados gerados para cada, com afinidades variando entre -8.9 e -6.2 kcal/mol para o primeiro e -8.3 e -5.7 kcal/mol para o segundo (Figura 17A e 17D, respectivamente).

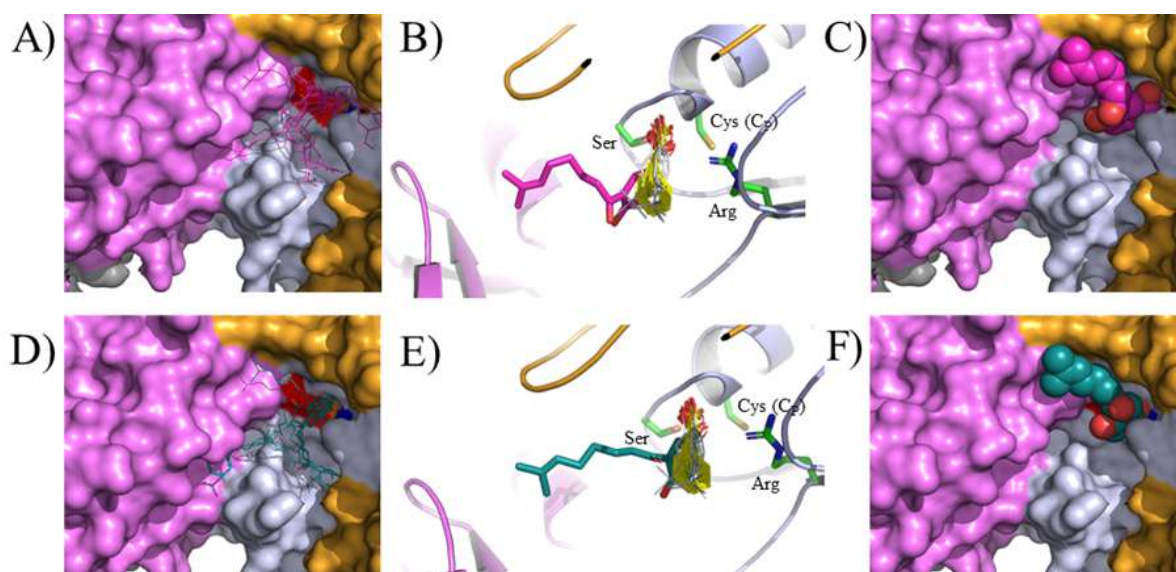


Figura 17. Representação dos resultados de CN-ABP1 e CN HP1 gerados para SeAhpC *best poses* selecionadas. Detalhe do sítio ativo contendo resultados selecionados para CN-ABP1 e CN-HP1 em SeAhpC (A e D, respectivamente). Cada monômero está representado por uma cor distinta (cinza, rosa escuro, azul claro, laranja). Os melhores *hits* do *docking* de SeAhpC com CN-ABP1 e CN-HP1 foram separados e alinhados aos ligantes H₂O₂ (PDB = 3A2V), benzoato (PDB = 2V32; 1HD2; 1OC3; 2V41; 1H4O) e tert-butylbenzene-diol (PDB = 4K7O). *Best poses* de CN-ABP1 e CN HP1 selecionadas com base no alinhamento com ligantes (B e E). Os compostos naturais estão representados por *sticks* e os ligantes, representados em *lines*. Os carbonos de cada composto estão representados nas seguintes cores: CN-ABP1 = rosa; CN-HP1 = ciano; benzoato = amarelo; tert-butylbenzene-diol = branco. Os oxigênios das moléculas estão em vermelho. Na figura C) e F) estão as *best pose* de CN-ABP1 e CN-HP1 no sítio ativo, respectivamente, e a proteína é representada em superfície molecular, e os aminoácidos do sítio ativo são coloridos da seguinte forma: Thr = vermelho, C_P = laranja e Arg = azul.

Para CN-ABP1 os resíduos observados em mais de 50% das *poses* foram: Pro⁴², Ser⁴⁶, Phe⁴⁷, Val⁴⁸, Cys⁴⁹, Asp¹¹⁴, Thr¹¹⁷, Leu¹¹⁹, Arg¹²², Asp¹⁴¹ do monômero onde se encontra C_P; Val¹⁶⁷ e Pro¹⁸¹ do monômero complementar do dímero íntimo; His⁷⁸ e Phe⁷⁹ do dímero adjacente. Para o composto CN HP1 os resíduos foram: Pro⁴², Ser⁴⁶, Phe⁴⁷, Val⁴⁸, Cys⁴⁹, Asp¹¹⁴, Leu¹¹⁹, Arg¹²², Asp¹⁴¹ do monômero onde se encontra C_P; Val¹⁶⁷ e Pro¹⁸¹ do monômero complementar do dímero íntimo; Phe⁷⁹ do dímero adjacente. Em seguida, foram selecionadas as *best poses*, para ambos compostos, que melhor se alinharam aos ligantes presentes em estruturas cristalográficas (Figura 17B, 17C, 17E e F).

As análises das interações dos ligantes revelam que assim como observado nas simulações utilizando a estrutura cristalográfica de AhpC de *S. typhimurium*, a estabilização dos compostos nos sítios ativos de AhpC de *P. aeruginosa* e de *S. epidermidis* ocorreram majoritariamente por interações de caráter hidrofóbico (Figura 18A-C, respectivamente). No

entanto existem contribuições significativas de ligações de hidrogênio, algo também proposto para substratos biológicos de Prx (Hall *et al.*, 2011; Ferrer-Suetta *et al.*, 2011).

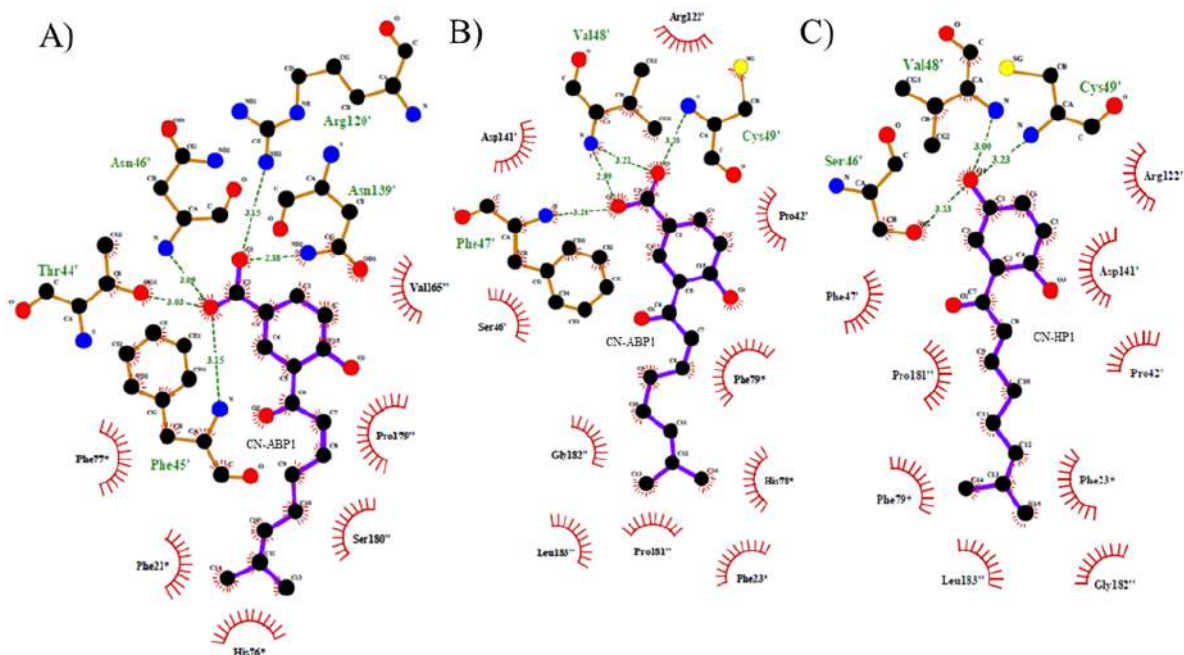


Figura 18. Representações bidimensionais do sítio de ligação de CN-ABP1 em PaAhpC e de CN-ABP1 e CN-HP1 em SeAhpC. As figuras acima mostram as possíveis interações que ocorrem entre os ligantes CN-ABP1 em PaAhpC (A) e em SeAhpC (B) e CN-HP1 (C) com SeAhpC. As figuras foram geradas através do programa LigPlot⁺. Os aminoácidos contidos em semicírculos vermelhos e com os caracteres em preto são aqueles envolvidos em interações hidrofóbicas com os ligantes. Os resíduos com caracteres em verde com linhas tracejadas verdes são os que realizaram interações polares com o composto. Resíduos como símbolo linha (') e aspas (") são de diferentes subunidades do homodímero obrigatório, enquanto aqueles com asterisco (*) pertencem ao dímero adjacente.

Quando se avalia os resultados obtidos para StAhpC e para os modelos de PaAhpC e SeAhpC de forma comparativa, um primeiro ponto que merece destaque é que para todas as soluções, independente da estrutura ou do ligante, os resíduos responsáveis pela estabilização dos ligantes são oriundos não só do dímero obrigatório, mas também do dímero vizinho. Este ponto merece destaque pois alguns trabalhos da literatura utilizam somente o dímero em simulações de *docking* envolvendo 2-Cys Prx (Liu *et al.*, 2012; Shi *et al.*, 2017; Ye *et al.*, 2019), mas o microambiente do sítio ativo compreende a área de junção dos dímeros (Figura 7C).

No que tange a qualidade dos modelos, os resultados comparativos indicam que os resíduos observados nas soluções para as diferentes proteínas são em sua maioria equivalentes, o que reforça que os modelos gerados são adequados para as análises. De fato, foram observados resíduos envolvidos na catálise ou estabilização de ligantes como os do motivo P-X-X-X-(T/S)-X-X-C_P, também descrito como C_P loop, que envolvem a cisteína peroxidásica (Hall *et al.*, 2011; Kamariah *et al.*, 2018), a Phe⁷⁷ (em StAhpC e PaAhpC; Phe⁷⁹ em SeAhpC) e também do dímero adjacente, e o resíduo de Glu¹³⁸ (em StAhpC; substituído por Asn¹³⁹ em PaAhpC e

Asp¹⁴¹ em SeAhpC) que estão relacionados com transições estruturais durante o ciclo catalítico (Perkins *et al.*, 2013; Tairum *et al.*, 2016), nos resultados para as três enzimas (Tabela 3).

Tabela 3: Principais resíduos observados nas *best poses* dos compostos no sítio de ligação das três estruturas utilizadas. Os círculos cheios denotam se a interação ocorreu por meio da cadeia principal (●) ou lateral do resíduo (◐). Os símbolos ', ' e * indicam o monômero que o resíduo pertence, quando vermelho indica que ocorreu interação do caráter polar.

StAhpC CN-ABP1	StAhpC CN-HP1	PaAhpC CN-ABP1	SeAhpC CN-ABP1	SeAhpC CN-HP1
Gln ^{14●*}	Gln ^{14●*}	-	-	-
Phe ^{21●*}	Phe ^{21●*}	Phe ^{21●*}	Phe ^{23●*}	Phe ^{23●*}
Pro ^{40●'}	Pro ^{40●'}		Pro ^{42●'}	Pro ^{42●'}
Thr ^{44●'}	Thr ^{44●'}	Thr ^{44●'}	Ser ^{46●'}	Ser ^{46●'}
Phe ^{45●'}	Phe ^{45●'}	Phe ^{45●'}	Phe ^{47●'}	Phe ^{47●'}
Val ^{46●'}	-	Asn ^{46●'}	Val ^{48●'}	Val ^{48●'}
Cys ^{47●'}	-	Cys ^{47●'}	Cys ^{49●'}	Cys ^{49●'}
His ^{76●*}	His ^{76●*}	His ^{76●*}	His ^{78●*}	-
Phe ^{77●*}	Phe ^{77●*}	Phe ^{77●*}	Phe ^{79●*}	Phe ^{79●*}
Arg ^{120●'}	Arg ^{120●'}	Arg ^{120●'}	Arg ^{122●'}	Arg ^{122●'}
Glu ^{139●'}	Glu ^{139●'}	Asn ^{139●'}	Asp ^{141●'}	Asp ^{141●'}
		Val ^{165●''}		
Pro ^{179●''}	Pro ^{179●''}	Pro ^{179●''}	Pro ^{181●''}	Pro ^{181●''}
		Ser ^{180●''}	Gly ^{182●''}	Gly ^{182●''}
			Leu ^{183●''}	Leu ^{183●''}

Resíduos com os símbolos linha (') ou aspas (") pertencem a diferentes subunidades do homodímero obrigatório, enquanto aqueles com asterisco (*) pertencem a um monômero ao dímero adjacente.

No entanto é importante salientar que apesar de ter apresentados *hits* positivos entre CN-ABP1 e SeAhpC, os dados bioquímicos revelam que este composto não é capaz de exercer atividade inibitória sobre a enzima de *S. epidermidis*, neste contexto, vale pontuar que apesar das simulações computacionais serem valiosas, visto que excluíram a possibilidade de interação dos compostos CN-LS5 e CN-LG1 para ambas as enzimas, as simulações não foram capazes de excluir a possibilidade de interação entre SeAhpC e CN-ABP1.

4.6 Determinação do IC₅₀ do composto CN-ABP1 para PaAhpC

Como o composto CN-ABP1 foi capaz de inibir a atividade da PaAhpC, é importante a determinação do IC₅₀ para obtenção de dados quantitativos. Para a realização do experimento, seguimos protocolos determinados previamente por nosso grupo de pesquisa (dos Santos *et al.*, 2021). Nossos resultados revelaram que a quantidade de CN-ABP1 necessária para inibir o PaAhpC é de 20.3 (±0.4) μM (Figura 19B e Tabela 4), um valor próximo ao obtido para Adn utilizada na inibição da isoforma HsPrx2 de humanos (IC₅₀ = ~15 μM) (Liu *et al.*, 2012).

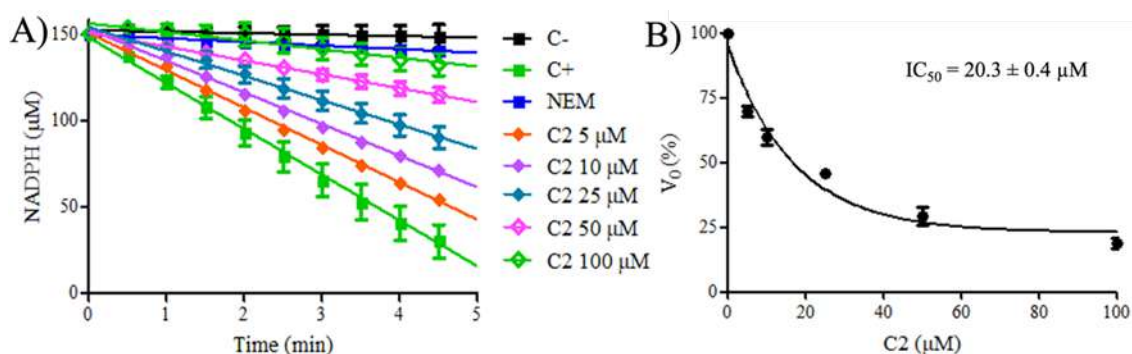


Figura 19. Determinação do IC_{50} de PaAhpC tratada com CN-ABP1 pelo ensaio acoplado de oxidação de NADPH. A) Aliquotas da enzima PaAhpC foram reduzidas com DTT 5 mM por 1 hora e então dessalinizadas para remover o excesso de redutor. Amostras contendo 10 μM das enzimas reduzidas e dessalinizadas foram tratadas variando a concentração de CN-ABP1 (5, 10, 25, 50 e 100 μM) por 1 hora à temperatura ambiente e posteriormente dessalinizadas novamente. Em seguida, 3 μM das proteínas tratadas foram adicionados às reações contendo 6 μM de EcTrx, 0.9 μM de EcTrxR, 150 μM de NADPH, 50 mM de HEPES (pH 7.4), 100 μM de DTPA e 1 mM de azida de sódio. As reações foram incubadas a 37°C por 5 minutos antes de serem iniciadas pela adição de 500 μM H_2O_2 e monitoradas espectrofotometricamente a 340 nm, 37°C, por 5 minutos. Reações sem tratamento com CN-ABP1 foram usadas como controles positivos (■) e reações sem adição de AhpC foram usadas como controles negativos (■), e amostras tratadas com NEM foram utilizadas como controle positivo de inibição (■). B) Os valores de velocidade inicial (v_0) de cada concentração de CN-ABP1 foram calculados e transformados em valores percentuais, e os resultados obtidos foram plotados para calcular o valor de IC_{50} de CN-ABP1 para PaAhpC. Todos os experimentos foram realizados pelo menos três vezes em triplicata

Tabela 4. Determinação do IC_{50} de PaAhpC utilizando os valores de v_0 com a proteína tratada com concentrações crescentes de CN-ABP1.

Amostras	v_0 (μM/s)	Decréscimo de v_0 (%)
Controle (sem PaAhpC)	0.01 (±0.01)	-
PaAhpC	0.51 (±0.01)	0
PaAhpC + NEM	0.03 (±0.01)	94.20 (±0.97)
PaAhpC + CN-ABP1 1 μM	0.36 (±0.02)	29.43 (±2.70)
PaAhpC + CN-ABP1 10 μM	0.31 (±0.01)	39.27 (±1.05)
PaAhpC + CN-ABP1 25 μM	0.22 (±0.01)	56.82 (±1.38)
PaAhpC + CN-ABP1 50 μM	0.15 (±0.03)	70.54 (±2.77)
PaAhpC + CN-ABP1 100 μM	0.09 (±0.02)	82.48 (±1.40)

4.7 Avaliação de inibição pelo composto CN-ABP1 da atividade peroxidásica da Prx2 humana e enzimática do sistema Trx de *E. coli*

CN-ABP1 apresentou atividade inibitória sobre PaAhpC, mas não sobre a enzima de *S. epidermidis*, o que aponta especificidade para a enzima de *P. aeruginosa*. Neste contexto, nos pareceu relevante avaliar se esta especificidade se estende também para outras enzimas em especial a isoforma humana Prx2, uma das peroxirredoxinas mais abundantes em células do hospedeiro (humano). Para contemplar este aspecto expressamos e purificamos a Prx2 de humanos (HsPrx2), e o sistema Trx de leveduras (ScTrx1 e ScTrxR1) para efetuar ensaios de oxidação de NADPH. A necessidade de utilização do sistema heterólogo ocorre em função que a TrxR de humanos é uma selenoproteína capaz de reduzir diretamente hidroperóxidos e muito sensível ao oxigênio molecular. Para transpor esta limitação se utiliza o sistema de levedura em ensaios enzimáticos que são realizados de forma comparativa (Truzzi *et al.*, 2017). Sendo assim, a enzima HsPrx2, previamente reduzida, foi tratada com 40 equivalente molares de CN-

ABP1 ou NEM (controle de inibição), seguindo o mesmo procedimento que as enzimas bacterianas. Os resultados demonstraram que não houve variação significativa da atividade entre a enzima tratada com CN-ABP1 e a sem tratamento (C+), revelando que este composto não é capaz de inibir HsPrx2 (Figura 20A e Tabela 5). Estes resultados são relevantes uma vez que o composto identificado como inibidor de PaAhpC apresenta seletividade sobre a enzima deste microrganismo e não interfere na atividade da enzima do hospedeiro. Estes procedimentos experimentais foram executados em conjunto com a aluna de IC, Gabrielle do Nascimento Sividanes, a qual respondo pela co-orientação.

A atividade inibitória de CN-ABP1 para PaAhpC mas não para SeAhpC e HsPrx2, nos pareceu bastante intrigante uma vez que CN-ABP1 possui um grupo α - β insaturado que, a princípio, é capaz de efetuar a adição de tiól-Michael em resíduos de Cys. De fato, inibidores identificados para Prx bacterianas e de humanos que possuem este grupo químico também são capazes de inibir outras peroxirredoxinas e mesmo outras tiól proteínas (Liu *et al.*, 2012; Muchowicz *et al.*, 2014; dos Santos *et al.*, 2022; Soethoudt *et al.*, 2014). Para lançar luz nesta questão avaliamos a atividade inibitória de CN-ABP1 sobre o sistema Trx de *E. coli* (EcTrx e EcTrxR). Para o procedimento enzimas previamente reduzidas com DTT foram tratadas com 40 equivalentes molares de CN-ABP1 durante 1 hora à temperatura ambiente e avaliou-se a atividade das proteínas por meio do ensaio de oxidação do NADPH.

Como pode ser observado nas figuras 20B e 20C, o composto também não foi capaz de afetar significativamente a atividade de EcTrx ou EcTrxR. De fato, foi detectado apenas um decaimento muito leve da atividade de EcTrx e EcTrxR (Tabela 5). Esses resultados parecem ser importantes, pois indicam seletividade de CN-ABP1 para PaAhpC quando comparado a outras proteínas tiólicas. Neste contexto, nos parece que mesmo possuindo um sistema carbonílico α - β insaturado, a inibição efetuada por CN-ABP1 sobre PaAhpC não deve ser por meio da alquilação das Cys envolvidas na catálise, uma vez que as outras tiól proteínas, mesmo a EcTrx que possui as Cys reativas expostas em sua superfície (Oliveira *et al.*, 2010), não foi inibida de forma significativa pelo composto.

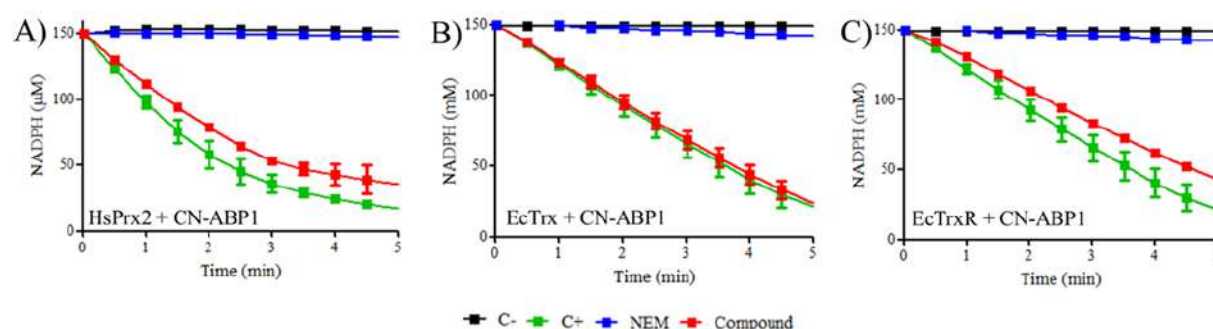


Figura 20. Avaliação da atividade peroxidásica pelo ensaio de oxidação do NADPH das enzimas HsPrx2, EcTrx e EcTrxR previamente tratadas com CN-ABP1. As enzimas HsPrx2, EcTrx e EcTrxR foram reduzidas com DTT e dessalinizadas, em seguida foram tratadas com 40 equivalentes molares de CN-ABP1 por uma hora. As proteínas foram dessalinizadas novamente para remover o excesso de composto e sua atividade foi analisada por ensaio de oxidação de NADPH. **A)** Para HsPrx2 foram feitas reações contendo 5 μM de Prx2, 10 μM de ScTrx1, 0.2 μM de ScTrxR1, 150 μM de NADPH, 50 mM de HEPES (pH = 7.0), 100 μM de DTPA e 1 mM de azida de sódio. Para EcTrx **(B)** e EcTrxR **(C)** foram utilizadas reações com 3 μM de PaAhpC, 6 μM de EcTrx, 0.9 μM de EcTrxR, 150 μM de NADPH, 50 mM de HEPES (pH = 7.4), 100 μM de DTPA e 1 mM de azida sódica.

As reações foram incubadas a 37°C/5 min, iniciadas pela adição de H₂O₂ (500 µM) e monitoradas espectrofotometricamente a 340 nm, 37°C, por 5 minutos. Nos gráficos acima os símbolos representam: ■ = Prx2 (A) e PaAhpC (B e C) com o sistema Trx sem tratamento (controle positivo), ■ = Prx2 tratada com CN-ABP1 (A), EcTrx tratada com CN-ABP1 (B) e EcTrxR tratada com CN-ABP1 (C) e ■ = Controle negativo. Os ensaios foram feitos em triplicata e repetidos ao menos três vezes.

Tabela 5. Valores de v_0 obtidos para as enzimas HsPrx2 de humanos (células cinzas), EcTrx e EcTrxR de *E. coli* (células azuis) tratadas com o composto CN-ABP1.

Amostra	v_0 (µM/s)	Decréscimo v_0 (%)	Amostra	v_0 (µM/s)	Decréscimo v_0 (%)
Controle negativo (sem HsPrx2)	0.01 (±0.02)	-	Controle negativo (sem PaAhpC)	0.01 (±0.01)	-
Controle positivo (com HsPrx2)	0.62 (±0.03)	-	Controle positivo (com PaAhpC)	0.51 (±0.01)	-
Prx2 + NEM	0.03 (±0.02)	95.17 (±0.60)	EcTrx + CN-ABP1	0.50 (±0.2)	2.00 (±0.91)
Prx2 + CN-ABP1	0.59 (±0.01)	4.84 (±1.45)	EcTrxR + CN-ABP1	0.49 (±0.01)	3.93 (±1.10)

4.8 Avaliação de adição de tiól-Michael por CN-ABP1 sobre cisteínas de PaAhpC

A adição de tiól-Michael ocorre de forma irreversível pela ligação covalente do composto à cisteínas envolvidas na catálise. Neste contexto, uma vez que durante o ciclo catalítico das Prx 2-Cys típicas ocorre a formação de um dissulfeto intermolecular é possível avaliar se ocorre a alquilação das cisteínas por meio de SDS-PAGE não redutor pois existe diferença na migração da proteína em seus diferentes estados de oxidação. Brevemente: apesar de AhpC ser uma enzima decamérica formada por cinco homodímeros o SDS leva a desnaturação da enzima, e em um SDS-PAGE as 2-Cys Prx típicas reduzidas são detectadas como monômeros (~ 22 kDa). Por outro lado, quando a enzima se encontra no estado oxidado, ocorre a formação de um dissulfeto intermolecular entre os homodímeros, o qual é uma ligação covalente que não é desfeita pelo SDS e a proteína é detectada com o dobro da massa (~ 44 kDa) (Tairum *et al.*, 2012). Neste contexto é possível avaliar se o inibidor foi capaz de reagir com um dos resíduos de cisteínas: se caso ocorrer a alquilação das Cys de PaAhpC por CN-ABP1 uma amostra da enzima pré-tratada com o composto e posteriormente oxidada com H₂O₂, não é capaz de formar o dissulfeto intermolecular e as proteínas são detectadas como monômeros como demonstramos previamente (Santos *et al.*, 2022). Caso contrário, se não houver a alquilação das Cys de PaAhpC por CN-ABP1, ela é detectada como dímero em SDS PAGE não redutor devido ao tratamento com peróxido. Sendo assim, amostras da enzima reduzida com DTT (com e sem o redutor na amostra), da enzima oxidada com H₂O₂ e da enzima pré-tratada com o composto CN-ABP1 e posteriormente oxidada com H₂O₂ foram submetidas a SDS PAGE não redutor. Como controle da alquilação das Cys, foi utilizada a PaAhpC tratada com NEM e posteriormente oxidada com H₂O₂ (Figura 21).

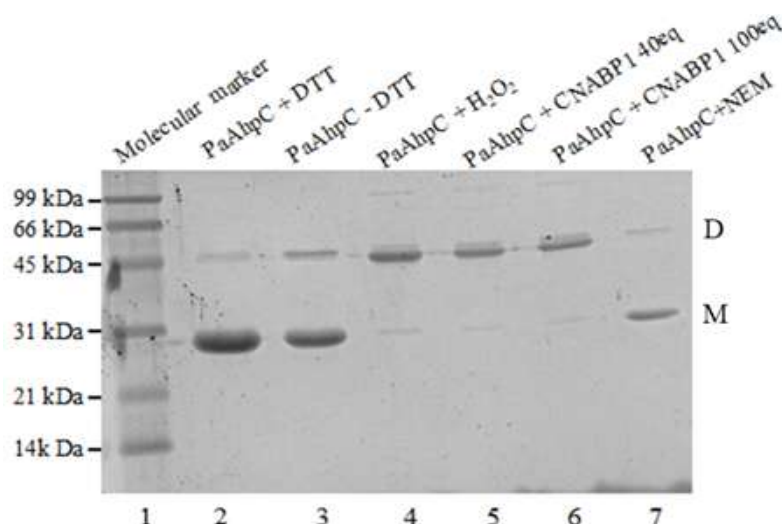


Figura 21. Avaliação da formação de dissulfetos PaAhpC após tratamento com CN-ABP1. Avaliação da formação de dissulfeto intermolecular de PaAhpC por SDS-PAGE não redutor. O marcador de peso molecular Sigma Marker foi aplicado na *lane* 1. 10 μ M de PaAhpC foram reduzidas com DTT 5 mM por 1 h (*lane* 2), o excesso foi removido (*lane* 3) e as proteínas foram tratadas com 2 equivalentes molares de H_2O_2 por 30 min (*lane* 4). As amostras reduzidas e dessalinizadas de DTT foram tratadas com 40 ou 100 equivalentes molares de CN-ABP1 e oxidadas com peróxido de hidrogênio (*lane* 5 e 6). Como controle positivo da inibição da formação de dissulfeto, utilizamos amostras de PaAhpC tratadas com NEM (40 equivalentes molares) (*lane* 7). O gel apresentado é representativo de três experimentos executados de forma independente e com resultados similares.

Como esperado, PaAhpC reduzida foi detectada como monômero (Figura 21, *lanes* 2 e 3), enquanto a amostra oxidada com H_2O_2 foi detectada como dímero (Figura 21, *lane* 4). Para o controle interno de alquilação o resultado também foi o esperado, pois amostras pré-tratadas com NEM e posteriormente oxidadas não foram capazes de formar dímeros (Figura 21, *lane* 7). Já para as amostras pré-tratadas com CN-ABP1 e posteriormente oxidadas com H_2O_2 houve a formação de dímeros (Figura 21, *lanes* 5 e 6). Em conjunto estes resultados revelam que a o tratamento com CN-ABP1 não impede a formação do dissulfeto intermolecular e, portanto, que o modo de inibição não ocorre pelo mecanismo de adição de tiól- Michael sobre os tióis de PaAhpC.

Para melhor compreensão do modo de inibição, utilizamos PaAhpC pré-tratada com CN-ABP1 (50 μ M) e efetuamos o ensaio de oxidação de NADPH com concentrações crescentes de hidroperóxido (de 50 a 750 μ M). Embora a concentração de CN-ABP1 de 50 μ M seja capaz de inibir fortemente a atividade peroxidase de PaAhpC, se a inibição apresenta comportamento competitivo, seria esperado que as concentrações crescentes de peróxidos deslocassem progressivamente o composto CN-ABP1 do sítio ativo restaurando a atividade peroxidásica de PaAhpC. Os nossos resultados revelaram que mesmo em altas concentrações de peróxido foi observada apenas uma atividade residual das amostras de PaAhpC pré-tratadas com CN-ABP1 (Figura 22). A determinação dos parâmetros cinéticos revelou que não foi observado qualquer efeito significativo no K_m . Por outro lado, parâmetros como o K_{cat} e o V_{max} foram expressivamente afetados (Figura 22B; Tabela 6). Este comportamento é compatível com um inibidor irreversível, como esperado por uma química de adição de Michael (Garuti *et al.*, 2011; Leung-Toung *et al.*, 2006; Liu *et al.*, 1992; Liu *et al.*, 2012).

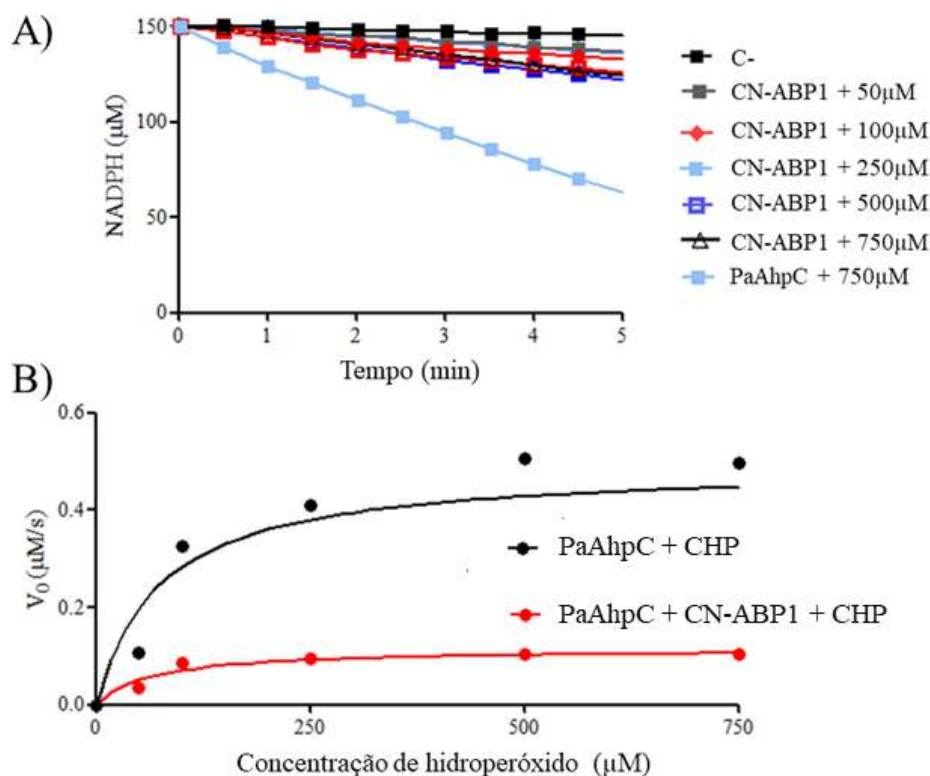


Figura 22. Características de inibição do composto CN-ABP1 sobre PaAhpC com concentrações crescentes de hidropéroxido orgânico. A) Ensaio de oxidação NADPH de amostras de PaAhpC previamente reduzidas com DTT e dessalinizadas e tratadas com 20 μM de CN-ABP1 1h/ RT. As reações continham 3 μM de PaAhpC, 6 μM de EcTrx, 0,9 μM de EcTrxR, 150 μM de NADPH, 50 mM de HEPES (pH = 7,0), 100 μM de DTPA e 1 mM de azida de sódica. As reações foram incubadas a 37°C/3 min e foram iniciadas pela adição de concentrações crescentes de CHP (50 - 750 μM) e monitoradas espectrofotometricamente a 340 nm, 37°C/5 min. Como controle negativo foram utilizadas reações sem a enzima (■) e como controle positivo foi PaAhpC sem tratamento prévio com o composto (■). Todos os experimentos foram realizados três vezes em triplicata. (B) Plot das taxas de oxidação de NADPH de amostras de pré-tratadas com o composto CN-ABP1 (PaAhpC/ CN-ABP1; pontos e linha vermelhos) ou sem tratamento com inibidor (PaAhpC; pontos e linha pretos).

Tabela 6. Parâmetros enzimáticos determinados para o hidropéroxido de (CHP) e PaAhpC pré-tratada ou não com CN-ABP1.

Amostra	K_m (M ⁻¹)	K_{cat} (s ⁻¹)	V_{max}
PaAhpC + CHP	65.93	0.2243	0.6729
PaAhpC/CN-ABP1 + CHP	61.58	0.0765	0.1148

Apesar dos nossos dados sugerirem que a cisteína de PaAhpC não está alquilada pelo composto CN-ABP1 através de uma reação de tiól-Michael, outros grupos reativos como aminas (-NH₂) e o álcool (-OH) podem realizar a química de adição de Michael (por exemplo, adição de aza e oxa-Michael, respectivamente) (Chu *et al.*, 2021; Nising e Brase, 2008), que estão presentes nas cadeias laterais dos resíduos de Arg e Thr de tríade catalítica de PaAhpC, que estão envolvidos na orientação e estabilização do substrato. Neste contexto, é importante salientar que esta modificação é de difícil detecção por espectrometria de massas uma vez que o peptídeo contendo os resíduos da tríade catalítica apresentam baixa ionização (dados não

apresentados). Uma alternativa seria a determinação da estrutura do complexo CN-ABP1/PaAhpC. Aspecto que será discutido posteriormente. É importante salientar que os resultados apresentados nos parecem de grande importância pois foi possível identificar o primeiro composto oriundo da biota costeira do Brasil, pertencente a uma classe química distinta dos demais inibidores identificados para as isoformas humanas, que possui significativa atividade inibitória específica sobre AhpC de uma bactéria patogênica Gram negativa e ainda opera a inibição irreversível por um mecanismo ainda não descrito para as Prx até o presente momento. Pela importância e originalidade os dados apresentados nesta seção compõem um manuscrito recentemente aceito para publicação no periódico PLoS One (Apêndice 1).

4.9 Avaliação do efeito de compostos semi-sintéticos similares a CN-ABP1 sobre a atividade enzimática de PaAhpC

Como CN-ABP1, se mostrou um inibidor para PaAhpC, os seguintes aspectos estruturais/funcionais são passíveis de investigação para um melhor entendimento do modo de inibição: anel benzênico, um grupamento ácido carboxílico, o sistema carbonílico α , β -insaturado e a unidade oxo-geranil (cauda flexível). Sendo assim, se torna interessante a avaliação da interação da enzima com compostos que apresente modificações dos grupos funcionais, acima citados, para um maior entendimento da interação da PaAhpC com CN-ABP1. Curiosamente, moléculas de benzoato são frequentemente encontradas no microambiente de estruturas cristalográficas de Prx, sempre muito próximas de resíduos envolvidos na catálise como no caso da Prx5 humana e Prx6 de *Arenicola marina* (Declercq *et al.*, 2001; Evrard *et al.*, 2004; Smeets *et al.*, 2008). Além disso, o CHP, um peróxido sintético, que apresenta uma função benzóica, é comumente utilizado como substrato para avaliar a atividade peroxidásica das Prx (Baker *et al.*, 2001; Oliveira *et al.*, 2021; Tairum *et al.*, 2016). No caso da função ácido carboxílico e a unidade oxo-geranil (cauda flexível), estruturas similares são encontradas em supostos substratos biológicos de Prx como os hidroperóxidos derivados de lipídios de cadeia longa. Por fim, como mencionado anteriormente, apesar do sistema carbonílico α , β -insaturado não ser capaz de estabelecer uma adição de tiól-Michael com os resíduos de Cys de PaAhpC existem compostos que são capazes de efetuar esta ligação e, portanto, este grupo também merece investigação.

Para tanto, foram fornecidos pelo Prof. Dr. João H. G. Lago vinte e um compostos semi-sintéticos similares ao composto natural CN-ABP1, os quais apresentam modificações combinatórias dos principais grupos funcionais do composto CN-ABP1 (Figura 23).

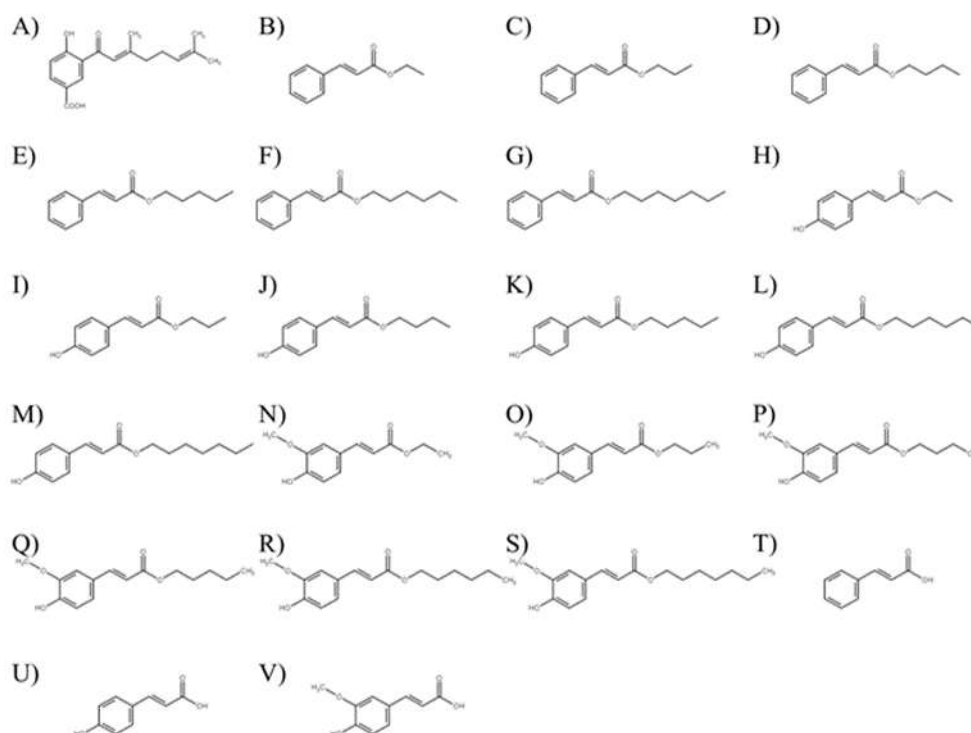


Figura 23. Estrutura química dos compostos semi-sintéticos contendo características funcionais/estruturais similares a CN-ABP1. As abreviações significam, respectivamente: EACN (éster derivado do ácido cinâmico), EAFR (éster derivado do ácido ferúlico), EAPC (éster derivado do ácido-cumárico). **A)** CN-ABP1 (PM = 288 g/mol), **B)** EACN-C2* (PM = 176.21 g/mol), **C)** EACN-C3 (PM = 190.24 g/mol), **D)** EACN-C4 (PM = 204.26 g/mol), **E)** EACN-C5 (PM = 218.29 g/mol), **F)** EACN-C6 (PM = 232.32 g/mol), **G)** EACN-C7 (PM = 246.34 g/mol), **H)** EAPC-C2* (PM = 192.21 g/mol), **I)** EAPC-C3 (PM = 206.24 g/mol), **J)** EAPC-C4 (PM = 220.26 g/mol), **K)** EAPC-C5 (PM = 234.29 g/mol), **L)** EAPC-C6 (PM = 248.36 g/mol), **M)** EAPC-C7 (PM = 262.34 g/mol), **N)** EAFR-C2* (PM = 222.24 g/mol), **O)** EAFR-C3 (PM = 236.26 g/mol), **P)** EAFR-C4 (PM = 250.29 g/mol), **Q)** EAFR-C5 (PM = 264.32 g/mol), **R)** EAFR-C6 (PM = 278.34 g/mol), **S)** EAFR-C7 (PM = 278.34 g/mol), **T)** ácido cinâmico (PM = 148.15 g/mol), **U)** ácido cumárico (PM = 164.04 g/mol) e **V)** ácido ferúlico (PM = 194.18 g/mol).

Os 21 compostos foram classificados em três grupos, sendo eles: éster derivado de ácido cinâmico (EACN), éster derivado de ácido ferúlico (EAFR) e éster derivado de ácido cúmarico (EAPC). Cada grupo contém sete compostos modificados. Inicialmente testamos cinco compostos semi-sintéticos, a fim de avaliar a capacidade de inibição da enzima PaAhpC (Figura 24). Os compostos testados foram: ácido cumárico, EAFR-C2, ácido cinâmico, EACN-C2 e EAPC-C2. O tratamento com estes compostos foi realizado da mesma maneira que o tratamento com os compostos naturais aqui testado tanto para PaAhpC e SeAhpC, utilizando 40 equivalentes molares de composto e o NEM como controle de inibição.

O EAPC-C2 foi o que levou a maior diminuição de v_0 dentre os cinco compostos testados (Tabela 7) e apresentou uma pequena diferença no NADPH residual ao final da reação (Figura 25) quando comparado com a enzima sem tratamento, seguido pelos compostos EAFR-C2 e EACN-C2 ($v_{0\text{PaAhpC} + \text{EAPC-C2}} = 0.38 \pm 0.01 \mu\text{s}^{-1}$, $v_{0\text{PaAhpC} + \text{EAFR-C2}} = 0.44 \pm 0.01 \mu\text{s}^{-1}$, $v_{0\text{PaAhpC} + \text{EAPC-C2}} = 0.44 \pm 0.02 \mu\text{s}^{-1}$). Os compostos ácido cumárico e ácido cinâmico não apresentaram variação significativa de v_0 nem do NADPH residual (Figura 24A, C, Figura 25, Tabela 7). De forma geral os compostos que apresentaram os melhores resultados possuem a cauda mais

longa. No entanto é necessário a continuidade e aprofundamento das análises para se chegar a qualquer conclusão pertinente. Estas investigações deverão ser desenvolvidas em projetos futuros.

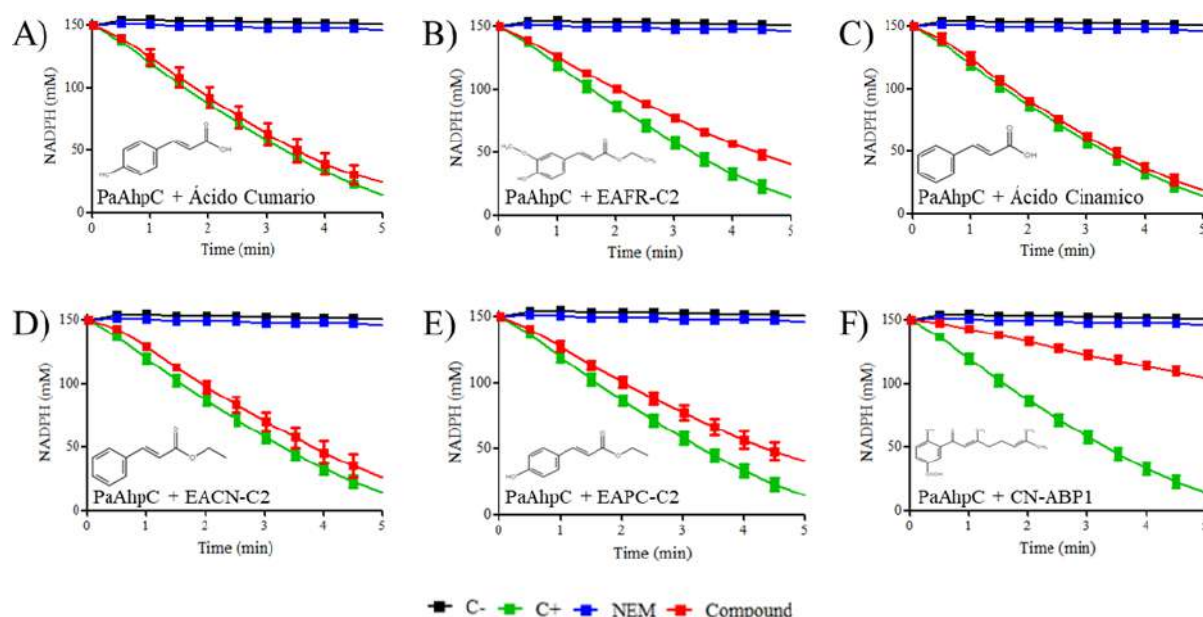


Figura 24. Avaliação da atividade peroxidásica pelo ensaio de oxidação do NADPH de PaAhpC previamente tratadas com os compostos semi-sintéticos. Amostras de PaAhpC foram reduzidas, dessalinizadas e tratadas com 40 equivalentes dos compostos naturais (ácido cumárico (A), EAFC-C2 (B), ácido cinâmico (C), EACN-C2 (D), EAPC-C2 (E) e CN-ABP1 (F)) por 1 hora em temperatura ambiente. Os ensaios foram realizados com 3 μ M de PaAhpC, 6 μ M de EcTrx1, 0.9 μ M de EcTrxR, 150 μ M de NADPH, 50mM de HEPES (pH 7.4), 100 μ M de DTPA e 1mM de azida sódica. As reações foram iniciadas pela adição de 500 μ M de H_2O_2 e monitoradas espectrofotometricamente a 340nm, 37°C, por 5 minutos. Nos gráficos os símbolos representam as seguintes condições: ■ = PaAhpC (controle positivo de atividade), ■ = NEM (controle positivo de inibição), ■ = PaAhpC + composto e ■ = Controle negativo (sem enzima). Os ensaios foram feitos em triplicata.

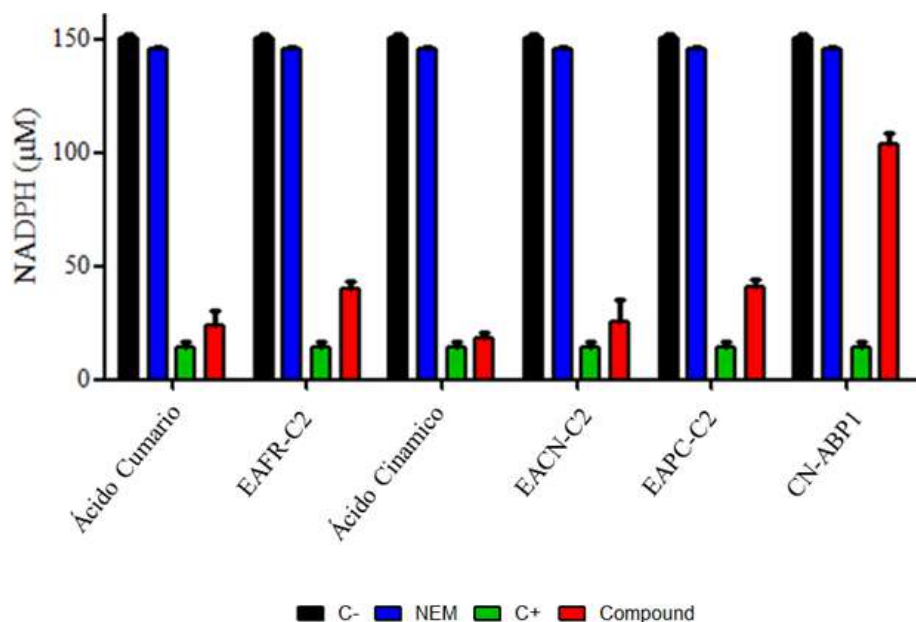


Figura 25. Avaliação da inibição de PaAhpC pelos compostos semi-sintéticos comparado com CN-ABP1. A quantidade de NADPH consumida ao final da reação foi obtida nos ensaios apresentados nas Figuras 6. No gráfico é mostrado a quantidade de NADPH residual no final do ensaio, para os controles (controle negativo e controle positivo de inibição), para enzima sem tratamento e a enzima tratada com cada composto. As barras estão coloridas da seguinte maneira: ■ = PaAhpC (controle positivo de atividade), ■ = NEM (controle positivo de inibição), ■ = PaAhpC + composto e ■ = controle negativo (sem enzima). Os ensaios foram feitos em triplicata.

Tabela 7. Valores de v_0 obtidos para a enzima PaAhpC tratada com os compostos semi-sintéticos.

Amostras	v_0 ($\mu\text{M/s}$)	Decréscimo v_0 (%)
Controle (sem PaAhpC)	0.01 (± 0.01)	-
PaAhpC	0.62 (± 0.01)	-
PaAhpC + NEM	0.02 (± 0.03)	96.78 (± 0.42)
PaAhpC + Ácido cumárico	0.53 (± 0.02)	14.52 (± 4.16)
PaAhpC + EAFR-C2	0.44 (± 0.01)	29.04 (± 1.51)
PaAhpC + Ácido cinâmico	0.52 (± 0.02)	16.13 (± 3.82)
PaAhpC + EACN-C2	0.44 (± 0.02)	29.04 (± 3.74)
PaAhpC + EAPC-C2	0.38 (± 0.03)	38.71 (± 5.54)
PaAhpC + CN-ABP1	0.17 (± 0.03)	72.59 (± 3.90)

4.10 Avaliação da afinidade de PaAhpC para peróxidos derivados de lipídeos de cadeia longa por meio de ensaio do NADPH e fluorescência intrínseca do Trp

Os resultados obtidos neste projeto também nos levaram a uma nova linha de investigação. Como mencionado anteriormente, CN-ABP1 possui um grupo ácido carboxílico e apresenta uma cauda hidrofóbica flexível que aparentemente são importantes para sua atividade inibitória. Vale salientar que ambas as características são compartilhadas com um grupo de biomoléculas que são passíveis de peroxidação, os ácidos graxos de cadeia longa (Bae *et al.*, 2011; Smeets *et al.*, 2008). De fato, a triagem de mutantes sensíveis a hidroperóxidos orgânicos, em especial o peróxido de ácido linoleico, levou a identificação das primeiras AhpCs em *E. coli* e *S. typhimurium* (Jacobson *et al.*, 1989), o que indica que peróxidos lipídicos podem ser substratos biológicos de AhpC. Ainda, trabalhos indicam que AhpCs e seus homólogos em eucariotos também são encontrados associados as membranas celulares, estruturas altamente suscetíveis a peroxidação lipídica (Hunag *et al.*, 2011; Wood *et al.*, 2003).

Estudos apontam que moléculas de ácidos graxos monoinsaturados (MUFA - *monounsaturated fatty acids*) ou poli-insaturados (PUFA - *polyunsaturated fatty acids*) exercem forte atividade bactericida ou bacteriostática, o que pode ser consequência da peroxidação destes compostos (Beavers *et al.*, 2019). Já foi demonstrado que o peróxido derivado de ácido linoleico é bastante tóxico para o fitopatógeno *Xylella fastidiosa* (Alegria *et al.*, 2017). Também foi descrito que em *Caenorhabditis elegans* um dos fatores ligados a suscetibilidade do hospedeiro a infecção por bactérias patogênicas é a abundância de peróxidos de ácido oleico (Anderson *et al.*, 2019). Adicionalmente, estudos mostram que a abundância de MUFAs na membrana bacteriana está relacionada a geração de produtos de peroxidação lipídica incluindo os hidroperóxidos (Libardo *et al.*, 2014; Pradenas *et al.*, 2012; Reis *et al.*, 2005; Wang *et al.*, 2017). Já os PUFAs são extremamente abundantes nas células dos hospedeiros e diversos estudos indicam que são altamente tóxicos para muitas espécies de bactérias Gram-positivas ou negativas (Desbois e Lawlor, 2013; Eijkelkamp *et al.*, 2018; Knapp e Melly, 1986). Temos

como exemplo o ácido araquidônico (ARA), o qual, apesar de ser abundante em células de defesa e participar de cascatas de sinalização imune, possui parcela significativa convertida em hidroperóxidos que são altamente tóxicos para *S. aureus* (Beavers *et al.*, 2019). Existem estudos que avaliaram a afinidade de tiol peroxidases como AhpE e Ohr, que revelaram que peróxidos de lipídios de cadeia longa, como hidroperóxidos de oleico (Ol-OOH), linolênico (Ln-OOH) e de ácido araquidônico (Ar-OOH) têm afinidade muito alta para essas enzimas (Alegria *et al.*, 2017; Longo *et al.*, 2020).

Apesar dos trabalhos mencionados acima, nenhum estudo tentou avaliar a afinidade desse tipo de hidroperóxido para 2-Cys Prx típicas, especialmente AhpCs bacterianas. Sendo assim, avaliamos a afinidade de AhpC para os hidroperóxidos de lipídeos derivados de ácido oleico (Ol-OOH), linoleico (Ln-OOH), linoleico contendo uma ou duas funções peróxido (1-Li-OOH e 2-Li-OOH, respectivamente) para PaAhpC. Inicialmente realizamos simulações computacionais envolvendo *docking* (acoplagem) molecular. Os resultados das simulações computacionais revelaram que os hidroperóxidos derivados de MUFAs e PUFA, possuem um *fitting* significativo no sítio ativo de AhpC, sendo estabilizados por diversas interações hidrofóbicas de três subunidades do decâmero envolvendo os resíduos C_P^{47'}, L^{117'}, E^{115'}, F^{45'}, P^{100''}, P^{179''}, F^{180''}, F^{20*}, F^{77*} e P^{100*} e também interações polares envolvendo os resíduos T^{44'}, R^{120'} (ambos da tríade catalítica), E^{139'} e L^{181''}, o ultimo detectado somente para Ol-OOH (os símbolos ' e '' denotam aminoácidos do homodímero obrigatório e o * da subunidade adjacente do decâmero). Neste ponto vale ressaltar que diversos resíduos envolvidos na estabilização dos peróxidos de lipídeos também respondem pela estabilização de CN-ABP1, o que, a priori, reforça a hipótese de CN-ABP1 mimetizar parcialmente um substrato biológico.

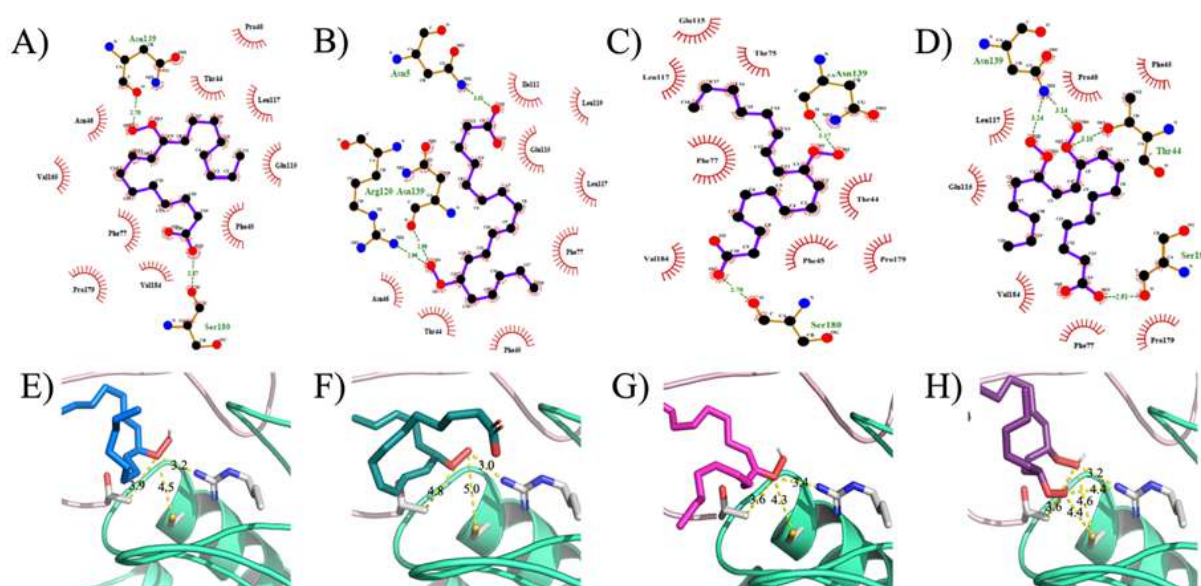


Figura 26. Interações moleculares entre resíduos do microambiente do sítio ativo de AhpC e hidroperóxidos de lipídeos. Os diagramas em 2D mostram as interações polares (tracejados em verdes) e interações apolares

(semi-arcos vermelhos) entre os resíduos de aminoácidos e os compostos: (A) Ol-OOH ($\Delta G = -5.6$ kcal/mol), (B) Ln-OOH ($\Delta G = -5.8$ kcal/mol), (D) 1-Li-OOH ($\Delta G = -5.7$ kcal/mol) e (E) 2-Li-OOH ($\Delta G = -5.7$ kcal/mol). Os diagramas foram gerados utilizando a ferramenta LigPlot⁺. Distâncias para resíduos de importância na catálise, em especial C_P da função peróxido de Ol-OOH (C₁₀) (D), Ln-OOH (C₁₃) (E), Li-OOH (C_{10,13}) (F), e 1-Li-OOH (C_{10,13}) (F). As distâncias estão representadas por linhas tracejadas amarelas e denotam Angstroms. As figuras foram geradas com o programa PyMol e os peróxidos e resíduos de AhpC estão representados em *sticks*.

A energia livre de Gibbs foi determinada como -5.6, -5.8, -5.7 e -5.7 kcal/mol para Ol-OOH, Ln-OOH, 1-Li-OOH e 2-Li-OOH, respectivamente. Outro ponto importante reside na distância da função peróxido das moléculas para C_P que variaram de 3.4 Å a 3.7 Å, o que permitiria o pronto ataque nucleofílico de C_P sobre o hidroperóxido (Figura 26). Também cabe mencionar a grande proximidade com resíduos da tríade catalítica (Thr/Ser e Arg) envolvidos no direcionamento, estabilização e ativação do hidroperóxido para sua decomposição (Figura 26).

Como mencionado na seção de Introdução deste trabalho, quando ocorre a hiperoxidação de C_P, a enzima Srx será responsável por reduzir o C_P-SO₂H em C_P-SOH as custas de ATP (Forshaw *et al.*, 2021; Jang *et al.*, 2004). A Srx é uma enzima encontrada majoritariamente em eucariotos (com raras exceções em cianobactérias) e, portanto, em bactérias a hiperoxidação da enzima resulta na aniquilação da sua atividade peroxidásica sendo necessária que a enzima seja novamente sintetizada (Oliveira *et al.*, 2021). Neste contexto uma alta afinidade para hidroperóxidos de lipídeos poderia levar a uma inativação mais acentuada da Prx o que posicionaria estas moléculas como inibidores biológicos de Prx. Ainda, nossos resultados sugerem que peróxidos derivados de PUFA's contendo mais de uma função peróxido poderiam agir como inibidores ainda mais eficientes da atividade peroxidásica de AhpCs bacterianas (e também de isoformas de eucariotos) mesmo em baixas quantidades, pois uma única molécula de hidroperóxido poderia inativar de forma irreversível a enzima. De fato, os resultados de *docking* revelam grande proximidade de ambas as peroxidações em relação a C_P (Figura 26H). A figura 27, ilustra as diferenças que podem levar a inativação de uma Prx tomando como exemplo o peróxido de hidrogênio e um di-peróxido de lipídeo.

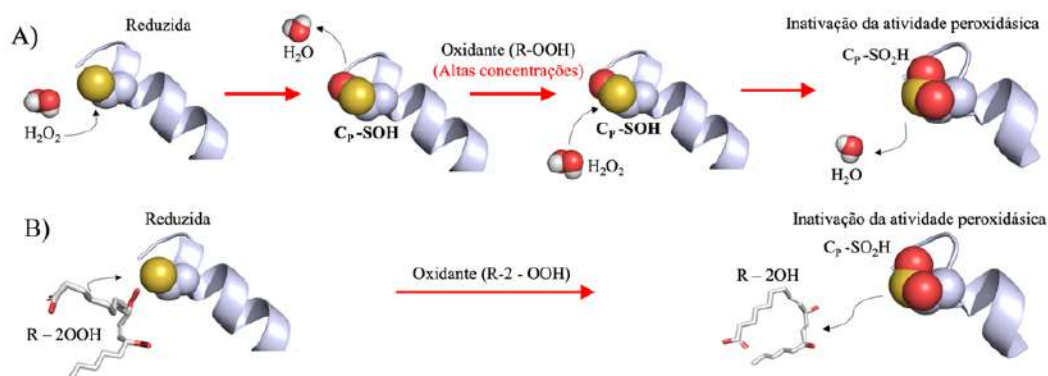


Figura 27. Hiperoxidação de C_P de peroxirredoxinas por monoperóxidos e di-peróxidos. (A) Sob altas concentrações de oxidante, no caso o peróxido de hidrogênio, a C_P é inicialmente oxidada a cisteína ácido sulfênico

(C_P-SOH) e antes da formação do dissulfeto intermolecular ocorre um segundo ataque por uma outra molécula de H₂O₂ levando a inativação da enzima em razão da formação de cisteína ácido sulfinico (C_P-SO₂H). **(B)** No caso de um di-peróxido como um derivado do peróxido de lipídeo poli-insaturado, a hiperoxidação pode ocorrer de forma quase instantânea em somente um passo.

Para averiguar bioquimicamente se peróxidos de lipídeos são bons substratos para PaAhpC estabelecemos colaboração com a Profa. Sayuri Miyamoto (IQ-USP-SP) cujo grupo de pesquisa sintetizou os peróxidos de lipídeos. Em seguida, efetuamos ensaios de oxidação do NADPH com quantidades crescentes dos mesmos peróxidos de lipídeos avaliados por meios de simulações computacionais e calculamos os parâmetros cinéticos para cada um dos peróxidos. Para efeito de comparação também determinamos as constantes para o H₂O₂ e o CHP (Figura 28).

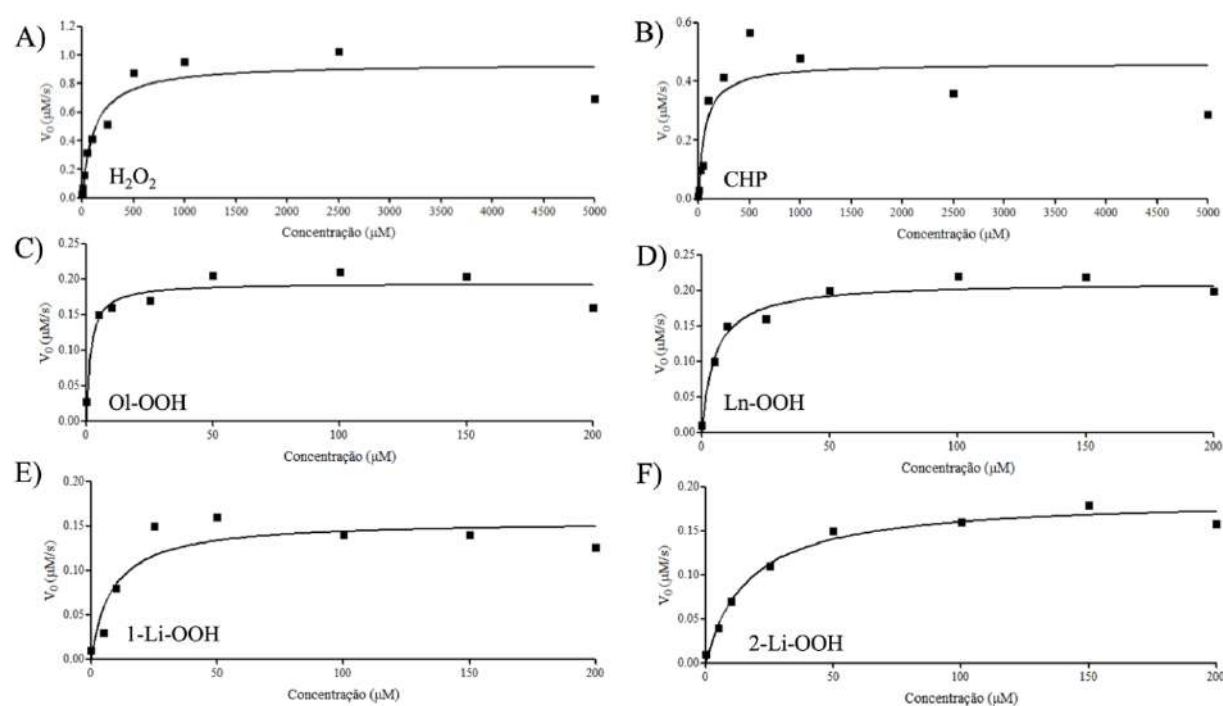


Figura 28. Ensaio de oxidação de NADPH para avaliação da hiperoxidação de PaAhpC. Plotagem dos valores de v_0 para cada concentração dos peróxidos avaliados: H₂O₂ (A), CHP (B), Ol-OOH (C), Ln-OOH (D), 1-Li-OOH (E) e 2-Li-OOH (F). Para obtenção dos valores de v_0 , realizamos o ensaio de oxidação do NADPH com diferentes concentrações dos peróxidos. Os ensaios foram realizados com PaAhpC (3 µM), Trx (6 µM), TrxR (0.9 µM), NADPH (150µM), HEPES (50mM, pH 7.0), DTPA (100µM) e azida sódica (1mM). Os experimentos foram iniciados adicionando concentrações crescentes de H₂O₂, CHP, Ol-OOH, Ln-OOH, 1-Li-OOH e 2-Li-OOH. As concentrações utilizadas foram 5, 10 15, 25, 50 100 150 e 200 µM para os peróxidos de lipídeo. Para o H₂O₂ e CHP também foram utilizadas as concentrações de 500, 1000, 2500 e 5.000 µM. As reações foram incubadas durante 5 min/37°C e iniciadas pela adição de hidroperóxido. A oxidação do NADPH foi monitorada espectrofotometricamente a 340 nm. Todos os experimentos foram realizados em triplicata e repetidos pelo menos três vezes, produzindo resultados consistentes.

Foi possível constatar que nas concentrações de 150 ou 200 µM ocorre a queda das taxas de decomposição para os peróxidos de lipídeos em razão da inibição da reação (Figuras 28C-F), o que pode ser atribuído a hiperoxidação de C_P (Truzzi *et al.*, 2017). É importante salientar que para o H₂O₂ e o CHP, a atividade peroxidásica se mantém elevada em concentrações muito

superiores aos de hidroperóxidos de lipídeos (quase $20 \times$ superiores para H_2O_2 [$2.500 \mu\text{M}$] e $2-4 \times$ mais elevadas para o CHP [$500 \mu\text{M}$]) (Figuras 28A e 28B, respectivamente), indicando uma sensibilidade significativamente maior para os peróxidos de lipídeos.

Tabela 8. Parâmetros cinéticos determinados para os peróxidos com a enzima PaAhpC.

Peróxido	K_m (M^{-1})	K_{cat} (s^{-1})	V_{max}	K_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)
H_2O_2	116.45	0.3131	0.6674	2.7×10^3
CHP	65.93	0.2243	0.6729	2.3×10^3
Ol-OOH	28.30	0.0723	0.2170	2.5×10^3
Ln-OOH	52.32	0.0712	0.2240	1.3×10^3
1-Li-OOH	20.30	0.0557	0.1672	2.7×10^3
2-Li-OOH	17.95	0.0645	0.1938	3.5×10^3

Também iniciamos a avaliação comparativa para SeAhpC, uma vez que a substituição de Thr por Ser na tríade catalítica pode levar a diferenças na afinidade por substratos (Tairum *et al.*, 2021). Como pode ser observado na figura 29, o comportamento na decomposição de hidroperóxidos é distinto, pois alcança o platô muito mais rapidamente quando o substrato é Ol-OOH (Figura 29C), quando comparado com H_2O_2 ou CHP (Figuras 29A e 29B), sugerindo afinidade acentuada pelo peróxido de lipídeo, o que está de acordo com o K_{cat}/K_m (Tabela 9).

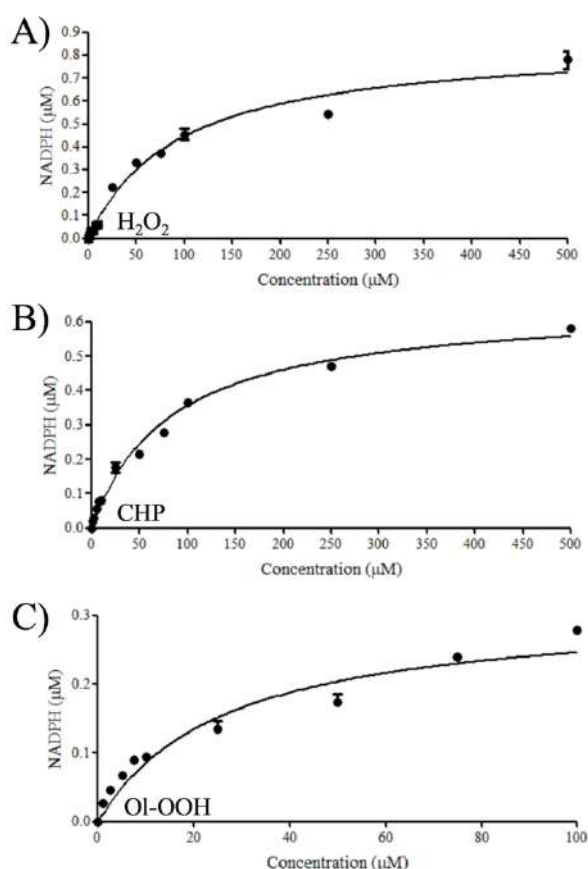


Figura 29. Ensaio de oxidação de NADPH para avaliação da hiperoxidação de SeAhpC. Plotagem dos valores de v_0 para cada concentração dos peróxidos avaliados: H_2O_2 (A), CHP (B) e Ol-OOH (C). Para obtenção dos valores de v_0 , realizamos o ensaio de oxidação do NADPH com diferentes concentrações dos peróxidos. Os ensaios foram realizados com $3 \mu\text{M}$ SeAhpC, $6 \mu\text{M}$ Trx, $0.9 \mu\text{M}$ TrxR, $150 \mu\text{M}$ NADPH, 50mM HEPES (pH 7.0), $100 \mu\text{M}$

DTPA e 1mM azida sódica. Os experimentos foram iniciados adicionando concentrações crescentes de H₂O₂, CHP ou Ol-OOH. As concentrações utilizadas foram 5, 10, 15, 25, 50 e 100 µM para Ol-OOH. Para o H₂O₂ e CHP também foram utilizadas as concentrações de 500µM. As reações foram incubadas durante 5 min/37°C e iniciadas pela adição de hidróperóxido. A oxidação do NADPH foi monitorada espectrofotometricamente a 340 nm. Todos os experimentos foram realizados em triplicata e repetidos pelo menos três vezes, produzindo resultados consistentes.

Tabela 9. Parâmetros cinéticos determinados para os peróxidos com a enzima SeAhpC.

Peróxido	K _m (M ⁻¹)	K _{cat} (s ⁻¹)	V _{max}	K _{cat} /K _m (M ⁻¹ s ⁻¹)
H ₂ O ₂	91.66	0.2858	0.8573	3.1×10 ³
CHP	81.53	0.2160	0.6480	2.6×10 ³
Ol-OOH	5.66	0.0459	0.1488	8.1×10 ³

No entanto, como ainda estamos em fase de padronização acreditamos que as concentrações utilizadas nos ensaios foram altas e, neste contexto, podendo levar a inativação parcial da população da enzima o que mascararia os resultados. Para tanto é importante utilizar doses menores, partindo de doses subestequiométricas, para evitar a superoxidação parcial. Neste contexto, refizemos os ensaios com menores doses para o Ol-OOH, não foi possível fazer para os demais peróxidos de lipídeos (1-Li-OOH e 2-Li-OOH) em razão das quantidades sintetizadas serem menores e terem se esgotado.

Quando são analisados somente os dados de baixas concentrações os resultados indicam fortemente que o Ol-OOH apresenta taxas iniciais de decomposição por PaAhpC significativamente superiores quando comparado com H₂O₂ ou CHP (Figura 30A), portanto é bastante plausível que uma maior afinidade possa resultar na hiperoxidação de C_P e perda da atividade peroxidásica de PaAhpC. Por outro lado, quando se analisa as mesmas condições para SeAhpC é possível constatar que a velocidade inicial de PaAhpC com Ol-OOH (Figura 30A) é significativamente superior as detectadas para SeAhpC (Figura 30B). Ainda, as taxas de decomposição para os três peróxidos são muito similares (Figura 30B). Portanto, os dados reforçam a hipótese que a interação com ligantes entre as enzimas é distinta. O que está em acordo com trabalhos anteriores de nosso grupo de pesquisa (Tairum *et al.*, 2016; Tairum *et al.*, 2021) e também os resultados obtidos, ao menos em parte para CN-ABP1.

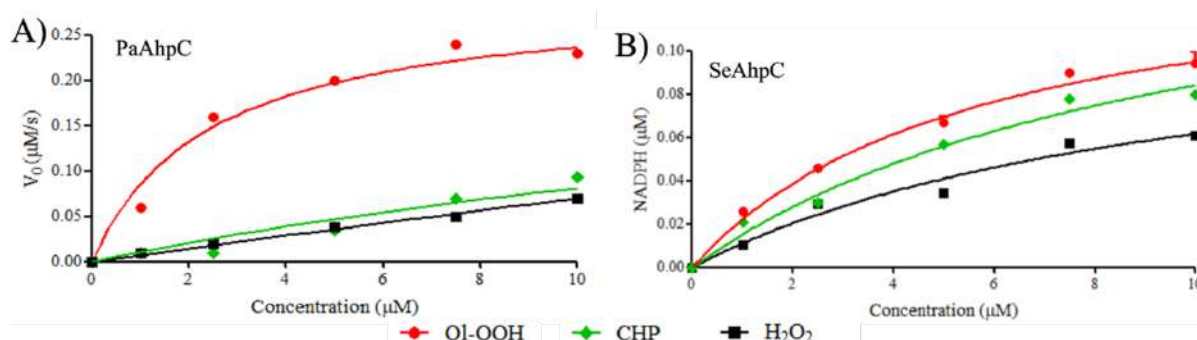


Figura 30. Plotagem comparativa das velocidades iniciais × baixas concentrações de peróxido de hidrogênio, e os hidróperóxidos orgânicos de cumeno e oleico para PaAhpC e SeAhpC. Plotagem dos valores de v₀ para

baixas concentrações (1.0, 2.5, 5.0, 7.5 e 10 μM) dos peróxidos de H_2O_2 (preto), CHP (verde) e Ol-OOH (vermelho) para as enzimas PaAhpC (A) e SeAhpC (B). Os ensaios foram realizados com 3 μM AhpC, 6 μM Trx, 0.9 μM TrxR, 150 μM NADPH, 50mM HEPES (pH 7.0), 100 μM DTPA e 1mM azida sódica. Os experimentos foram iniciados adicionando concentrações crescentes hidroperóxidos. Todos os experimentos foram realizados a 37° C, em triplicata e repetidos pelo menos três vezes produzindo resultados consistentes.

Uma vez que as taxas de hiperoxidação não podem ser determinadas pelo ensaio de NADPH, estabelecemos colaboração com o grupo de pesquisa da Profa. Daniela Truzzi (IQ-USP-SP) para a determinação das taxas de hiperoxidação por fluorescência em espectrofluorímetro com *stopped flow* (Figura 31).

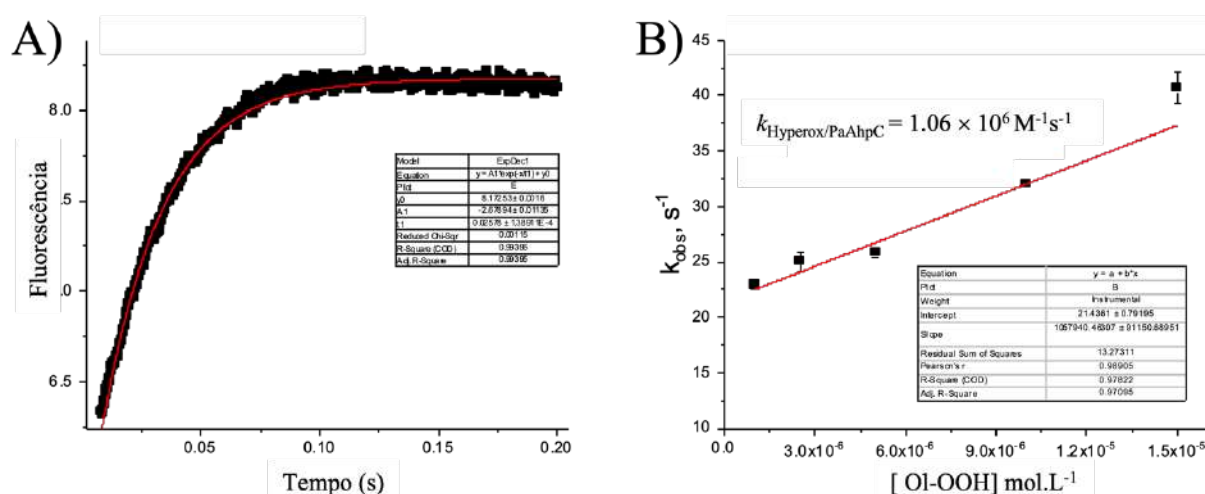


Figura 31. Cinética de hiperoxidação de PaAhpC por hidroperóxido de ácido oleico. (A) Aumento representativo da fluorescência intrínseca da PaAhpC oxidada) ao longo do tempo após a adição de 15 μM de Ol-OOH. (B) Determinação das constantes de hiperoxidação de PaAhpC por Ol-OOH utilizando concentrações crescentes de Ol-OOH. Todos os experimentos foram efetuados a 37° C em triplicada.

Os resultados, apesar de iniciais, foram bastante significativos e revelam que fase de hiperoxidação é dependente do tempo (Figura 31A) e indicam fortemente que Ol-OOH é capaz de hiperoxidar PaAhpC com taxas de $1.06 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, ou seja, muito elevadas (Figura 31A). De fato, as taxas de hiperoxidação já determinadas até o presente momento, no caso para AhpE, uma 1-Cys Prx de *M. tuberculosis* e, portanto, muito distinta de PaAhpC, são $10 \times$ menores que a determinada para PaAhpC ($10^5 \text{ M}^{-1}\text{s}^{-1}$) o que indica que o peróxido de lipídeo é um substrato melhor para enzima de *P. aeruginosa* (Reyes *et al.*, 2011). Apesar de ser necessário a determinação das constantes de oxidação e hiperoxidação para os outros peróxidos de lipídeo, incluindo o contendo duas peroxidações (2-Li-OOH) e para SeAhpC, os resultados indicam que os peróxidos são capazes de hiperoxidar de forma acentuada AhpC e, portanto, inativar a enzima e podem representar um inibidor biológico para este grupo de enzimas. Algo inédito na literatura e que pode ter impacto significativo nesta área.

4.11 Cristalização de PaAhpC em diferentes estados oxidativos e contendo ligante

Como discutido em seções anteriores, um dos desafios para o entendimento das relações entre PaAhpC/SeAhpC e ligantes, sejam eles substratos ou inibidores, é a ausência de estruturas tridimensionais determinadas para estas enzimas. De fato, neste estudo construímos modelos teóricos para realizar as análises de *docking* molecular. No entanto, é importante ressaltar que este tipo de análise é uma aproximação. O ideal seria a determinação das estruturas destas proteínas para se ter dados estruturais mais confiáveis.

Neste contexto, estabelecemos colaboração com o grupo de pesquisa do Prof. Otávio Henrique Thieman do Instituto de Física da USP de São Carlos para determinar a estrutura das proteínas. Cabe ressaltar que esta é uma tarefa complexa uma vez que a estrutura quaternária de AhpC varia com o estado oxidativo (decâmeros - reduzidas; dímeros - oxidada ou complexos de alto peso molecular -hiperoxidada). Para tanto, realizamos experimentos de cristalização de proteínas utilizando inicialmente PaAhpC em dois estados oxidativos distintos (reduzida com DTT e oxidada com baixas concentrações de peróxido de hidrogênio) e da proteína previamente tratada com NEM (ligado covalentemente). Neste contexto foi nosso objetivo não só obter cristais de PaAhpC em diferentes estados oligoméricos, mas também da enzima contendo um ligante no sítio ativo da proteína, o que pode auxiliar em um maior entendimento de complexos ligante-enzima. Para tanto foram testadas ~1.800 condições de cristalização e obtivemos sucesso na formação de cristais para PaAhpC para os três tratamentos (Figura 32).

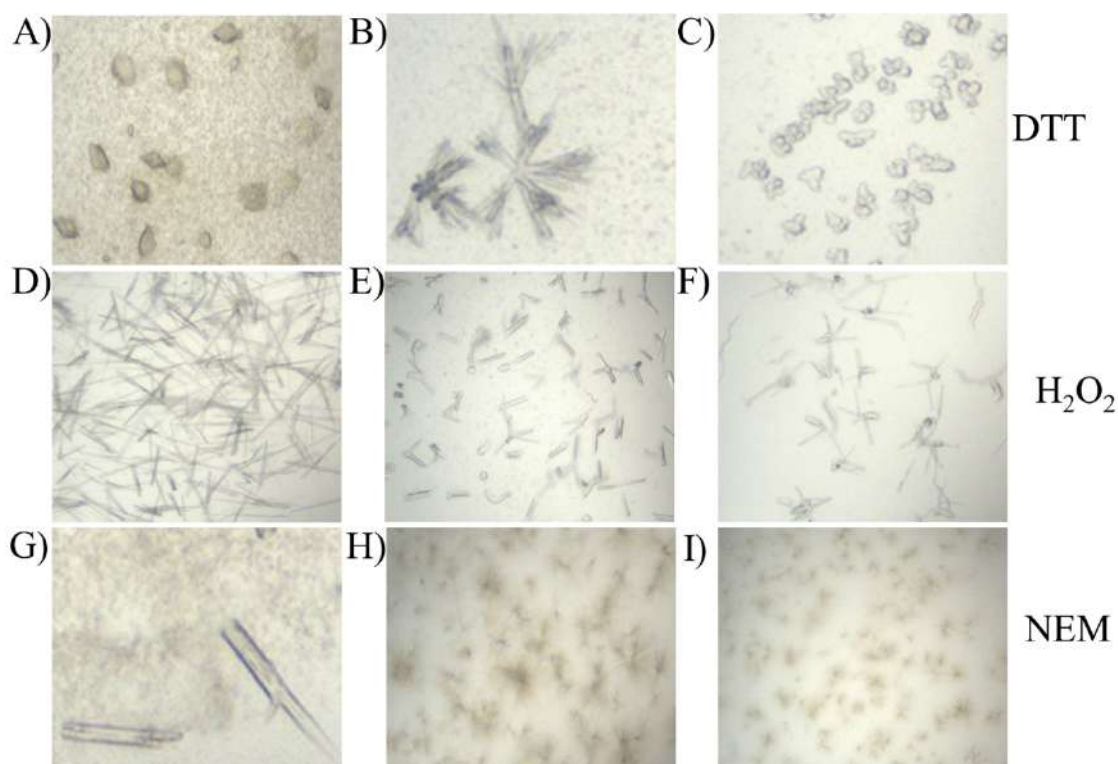


Figura 32. Resultados de experimentos de cristalização de PaAhpC em diferentes estados oxidativos e contendo ligante. Cristais de PaAhpC da enzima tratada com DTT 50mM (A - C), H₂O₂ (D - F), ou NEM (G -

I). As soluções de cristalização utilizadas foram: **(A)** PEG 3350 25% w/v (precipitante) e HEPES 0.1 M pH 7.5 (tampão); **(B)** M1K335 37.5% w/v (precipitante), MB3 0.1 M pH 8.5 (tampão) e halogênio 0.09 M (ingrediente complexo); **(C)** NaBr 0.2 M (sal), PEG 3350 20% w/v (precipitante) e Bis-Tris 0.1 M pH 7.5 (tampão); **(D)** 20% w/v PEG 3350 (precipitante) citrato 0.2M (sal); **(E)** 0.35M NaH₂PO₄ 0.35M (sal) e K₂HPO₄ 0.65M (sal); **(F)** 0.2 M MgCl₂ (sal), EG 10% v/v (precipitante), propanol 5% v/v (precipitante), 0.1 M HEPES pH 7.5 (tampão), PSM 15% v/v (precipitante); **(G)** condições utilizadas foram: PEG 3350 20% w/v (precipitante) e MgCl₂ 0.2 M (precipitante); **(H)** M1K3350 37.5% w/v (precipitante), MB2 0.1 M pH 7.5 (tampão) e MCA 0.1 M (ingrediente complexo) e **(I)** 550M_20K 30% w/v (precipitante), MB2 0.1 M pH 7.5 (tampão) e MCA 0.1 M (ingrediente complexo).

É importante mencionar que as condições de cristalização identificadas para os tratamentos com DTT e H₂O₂ (Figura 32 A-F) possuem dimensões passíveis de coleta de dados de difração de raios-X, assim como uma das condições de PaAhpC tratada com NEM (Figura 32G). Ainda, todas são passíveis de refinamento o que abre perspectivas para a determinação das estruturas nas diferentes condições.

5 CONCLUSÕES E CONSIDERAÇÕES FINAIS

Este projeto de pesquisa teve por objetivo a busca de inibidores oriundos da biota costeira do Estado de São Paulo para AhpCs bacterianas de *P. aeruginosa* e *S. epidermidis* através de ensaios bioquímicos e análises estruturais das enzimas. Neste contexto, foi possível estabelecer as condições de expressão e purificação de PaAhpC e SeAhpC com elevado rendimento e pureza (> 95%) e padronizado os testes de inibição envolvendo o tratamento da enzima com os compostos seguido pelo ensaio acoplado de oxidação de NADPH. O isolamento, caracterização e seleção dos compostos oriundos da biota costeira do estado de São Paulo foi efetuada em parceria com o grupo de pesquisa do Prof. Dr. João H. G. Lago da Universidade Federal do ABC (UFABC). Foram testados os efeitos de quatro compostos sobre a enzima PaAhpC de classes químicas distintas: CN-LS5 (lactona sesquiterpênica) de *Pentacalia desiderabilis*, CN-HP1 (hidroquinona prenilada) e CN-ABP1 (ácido benzóico prenilado), ambos de *Piper crassinervium*, e CN-LG1 (lignana) de *Nectandra cupidata*. Demonstramos que somente o composto CN-ABP1 é capaz de inibir de forma significativa PaAhpC com $IC_{50} = 20.3 (\pm 0.4) \mu M$, e de forma bastante seletiva uma vez que não é capaz de inibir as isoformas de *S. epidermidis* (SeAhpC) e de humanos (HsPrx2) ou mesmo outras tiól proteínas testadas (EcTrx e EcTrxR). Também investigamos a ligação dos compostos por meio de simulações computacionais e identificamos resíduos que provavelmente são responsáveis pela estabilização de CN-ABP1 no microambiente do sítio ativo de PaAhpC, através de uma série de interações polares e apolares. Ainda, o composto identificado além de ser oriundo da biota brasileira e pertencer a uma classe química ainda não descrita como inibidor de 2-Cys Prx, também efetua a inibição por meio de um mecanismo distinto da adição tiól-Michael. Conjuntamente consideramos os resultados bastante originais e relevantes, incluindo pelo fato de valorizar compostos bioativos oriundos do Brasil que podem ser obtidos de forma sustentável, e os dados foram compilados em um manuscrito que foi aceito para publicação em periódico de circulação internacional (Apêndice 1).

Também é importante mencionar que os dados obtidos abriram três novas linhas de investigação sobre inibidores de AhpC. Na primeira, através da colaboração com o grupo do Prof. Dr. João H. G. Lago foram iniciadas a avaliação de propriedades inibitórias de uma série de compostos que guardam semelhanças funcionais e estruturais com CN-ABP1 e que devem levar a um maior entendimento das interações entre as 2-Cys Prx e inibidores. A segunda, também foi baseada na estrutura alongada e grupo funcional (ácido carboxílico) que sugeriu que peróxidos derivados de lipídeos de cadeia longa possam ser substratos biológicos de AhpC. Para desenvolver esta linha estabelecemos colaboração com os grupos de pesquisa da Prof^a.

Sayuri Miyamoto e Prof^ª. Daniela Truzzi, ambas do Instituto de Química da USP (São Paulo) e os dados obtidos indicam que peróxidos derivados de lipídeos são capazes de hiperoxidar e inativar AhpC em doses muito menores que H₂O₂ ou CHP, sugerindo que estes substratos possam ser inibidores biológicos de AhpC, e que di-peróxidos possam desempenhar um papel importante neste sentido. Como terceira linha de investigação iniciamos procedimentos de cristalização visando a determinação das estruturas de PaAhpC e SeAhpC em colaboração com o grupo de pesquisa do Prof. Otávio Henrique Thieman do Instituto de Física da USP (São Carlos) em diferentes estados oxidativos e oligoméricos e contendo ligante (NEM). Foi possível obter cristais passíveis de utilização em experimentos de difração. Esta linha de investigação abre a perspectiva de um maior entendimento da biologia básica destas proteínas bem como a possibilidade de co-cristalização de compostos que possam atuar como inibidores de AhpC, em especial CN-ABP1 ou mesmo os compostos similares que estão sendo avaliados.

Cabe ainda ressaltar que durante o período desta dissertação, em especial durante os períodos em que a pandemia de COVID-19 reduziu substancialmente a acessibilidade aos laboratórios de pesquisa, foi possível elaborar e publicar dois artigos em periódicos de circulação internacional com dados inéditos (*Antioxidants*. 2021 10(7):1032. doi: 10.3390/antiox10071032. PMID: 34202406 – Apêndice 2; *Chem. Res. Toxicol.* 2022. doi: 10.1021/acs.chemrestox.2c00049 – Apêndice 3) e de uma revisão (*Appl Microbiol Biotechnol.* 2021 105(14-15):5701-5717. doi: 10.1007/s00253-021-11360-5 – Apêndice 4), todos com temática intrinsicamente ligados a este projeto de pesquisa, além de um capítulo de livro voltado para o ensino de Biologia Molecular para a graduação em Ciências Biológicas. Portanto, apesar de boa parte do projeto ter sido desenvolvido sob a pandemia de COVID-19 (primeiro ano e parte do segundo) acreditamos que os objetivos foram cumpridos de forma bastante significativa superando as expectativas iniciais.

6 PERSPECTIVAS

Abaixo são enumeradas de forma sucinta algumas perspectivas abertas no âmbito dos estudos desenvolvidos que poderão ser investigados em projeto(s) futuro(s):

- ◆ Investigação da forma de ligação de CN-ABP1 / PaAhpC por meio de estudos envolvendo espectrometria de massas.
- ◆ Avaliação do crescimento de células de *P. aeruginosa* e neutrófilos humanos submetidas a doses crescentes de CN-ABP1, em conjunto ou não com antibióticos.
- ◆ Investigação do potencial inibitório de compostos similares a CN-ABP1 sobre PaAhpC e SeAhpC. Havendo inibição, também será efetuada a determinação de IC₅₀ e especificidade para a 2-Cys Prx.
- ◆ Determinação das taxas de oxidação e hiperoxidação de PaAhpC e SeAhpC por ensaios fluorométricos e *western blot*, por diferentes peróxidos derivados de lipídeos (ácidos araquidônico e oleico, linoleico) e contendo uma ou duas peroxidações (linoleico).
- ◆ Avaliação do crescimento de células de *P. aeruginosa* e *S. epidermidis* submetidas a doses crescentes de peróxidos de lipídeos e/ou de drogas promotoras de peroxidação lipídica.
- ◆ Determinação de estruturas cristalográficas de PaAhpC e SeAhpC em diferentes estados oxidativos/oligoméricos visando investigar aspectos fundamentais de diferenças de estrutura e função de AhpC contendo Ser ou Thr como parte da tríade catalítica.
- ◆ Determinação de estruturas cristalográficas de PaAhpC contendo ligante (s), incluindo CN-ABP1 e compostos da série, para investigação detalhada de interações enzima-ligante.

REFERÊNCIAS

- Alegria TG, Meireles DA, Cussiol JR, Hugo M, Trujillo M, de Oliveira MA (2017). Ohr plays a central role in bacterial responses against fatty acid hydroperoxides and peroxynitrite. *Proc Natl Acad Sci U S A*. 114(2):E132-E41.
- Alharbi, A., Rabadi, S. M., Alqahtani, M., Marghani, D., Worden, M., Ma, Z., Malik, M., & Bakshi, C. S. (2019). Role of peroxiredoxin of the AhpC/TSA family in antioxidant defense mechanisms of *Francisella tularensis*. *PloS one*, 14(3), e0213699.
- Aguirre, C., ten Brink, T., Guichou, J. F., Cala, O., & Krimm, I. (2014). Comparing binding modes of analogous fragments using NMR in fragment-based drug design: application to PRDX5. *PloS one*, 9 (7), e102300.
- Anderson SM, Cheesman HK, Peterson ND, Salisbury JE, Soukas AA, Pukkila-Worley R. (2019). The fatty acid oleate is required for innate immune activation and pathogen defense in *Caenorhabditis elegans*. *PLoS Pathog*. 15(6):e1007893.
- Antimicrobial Resistance Collaborators (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet* (London, England). 399(10325), 629–655.
- Aslam, B., Wang, W., Arshad, M. I., Khurshid, M., Muzammil, S., Rasool, M. H., Nisar, M. A., Alvi, R. F., Aslam, M. A., Qamar, M. U., Salamat, M., & Baloch, Z. (2018). Antibiotic resistance: a rundown of a global crisis. *Infection and drug resistance*, 11, 1645–1658.
- Arai, T., Kimata, S., Mochizuki, D., Hara, K., Zako, T., Odaka, M., Yohda, M., Arisaka, F., Kanamaru, S., Matsumoto, T., Yajima, S., Sato, J., Kawasaki, S., & Niimura, Y. (2015). NADH oxidase and alkyl hydroperoxide reductase subunit C (peroxiredoxin) from *Amphibacillus xylanus* form an oligomeric assembly. *FEBS open bio*, 5, 124–131.
- Bae YA, Kim SH, Lee EG, Sohn WM, Kong Y. (2011). Identification and biochemical characterization of two novel peroxiredoxins in a liver fluke, *Clonorchis sinensis*. *Parasitology*. 138(9):1143-53
- Bajor, M., Zych, A. O., Graczyk-Jarzynka, A., Muchowicz, A., Firczuk, M., Trzeciak, L., Gaj, P., Domagala, A., Siernicka, M., Zagodzón, A., Siedlecki, P., Kniotek, M., O'Leary, P. C., Golab, J., & Zagodzón, R. (2018). Targeting peroxiredoxin 1 impairs growth of breast cancer cells and potentially sensitises these cells to prooxidant agents. *British journal of cancer*, 119(7), 873–884.
- Baker LM, Raudonikienė A, Hoffman PS, Poole LB. (2001). Essential thioredoxin-dependent peroxiredoxin system from *Helicobacter pylori*: genetic and kinetic characterization. *J Bacteriol*. 183(6):1961-73.
- Blázquez, J., Rodríguez-Beltrán, J., & Matic, I. (2018). Antibiotic-Induced Genetic Variation: How It Arises and How It Can Be Prevented. *Annual review of microbiology*, 72, 209–230.
- Brennan, R. E., Kiss, K., Baalman, R., & Samuel, J. E. (2015). Cloning, expression, and characterization of a *Coxiella burnetii* Cu/Zn Superoxide dismutase. *BMC microbiology*, 15, 99.
- Brindisi, M., Brogi, S., Relitti, N., Vallone, A., Butini, S., Gemma, S., Novellino, E., Colotti, G., Angiulli, G., Di Chiaro, F., Fiorillo, A., Ilari, A., & Campiani, G. (2015). Structure-based discovery of the first non-covalent inhibitors of *Leishmania major* trypanothione peroxidase by high throughput docking. *Scientific reports*, 5, 9705.
- Brito IA, Thevenard F, Costa-Silva TA, Oliveira SS, Cunha RLOR, de Oliveira EA, Sartorelli P, Guadagnin RC, Romanelli MM, Tempone AG, Lago JHG. (2022). Antileishmanial Effects of Acetylene Acetogenins from Seeds of *Porcelia macrocarpa* (Warm.) R.E. Fries (Annonaceae) and Semisynthetic Derivatives. *Molecules*. (3):893.

Calcerrada, P., Peluffo, G., & Radi, R. (2011). Nitric oxide-derived oxidants with a focus on peroxynitrite: molecular targets, cellular responses and therapeutic implications. *Current pharmaceutical design*, 17(35), 3905–3932.

Carvalho, L., Truzzi, D. R., Fallani, T. S., Alves, S. V., Toledo, J. C., Jr, Augusto, O., Netto, L., & Meotti, F. C. (2017). Urate hydroperoxide oxidizes human peroxiredoxin 1 and peroxiredoxin 2. *The Journal of biological chemistry*, 292(21), 8705–8715.

Chang HH, Cohen T, Grad YH, Hanage WP, O'Brien TF, Lipsitch M. (2015). Origin and proliferation of multiple-drug resistance in bacterial pathogens. *Microbiol Mol Biol Rev*. 79(1):101-16.

Chaves-Moreno, D., Wos-Oxley, M. L., Jáuregui, R., Medina, E., Oxley, A. P., & Pieper, D. H. (2016). Exploring the transcriptome of *Staphylococcus aureus* in its natural niche. *Scientific reports*, 6, 33174.

Chen, H., & Zhong, Q. (2018). Antibacterial activity of acidified sodium benzoate against *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* in tryptic soy broth and on cherry tomatoes. *International journal of food microbiology*, 274, 38–44.

Choi, J., Choi, S., Choi, J., Cha, M. K., Kim, I. H., & Shin, W. (2003). Crystal structure of *Escherichia coli* thiol peroxidase in the oxidized state: insights into intramolecular disulfide formation and substrate binding in atypical 2-Cys peroxiredoxins. *The Journal of biological chemistry*, 278(49), 49478–49486.

Chopra I, Hesse L, O'Neill AJ. (2002). Exploiting current understanding of antibiotic action for discovery of new drugs. *J Appl Microbiol*. 2002;92 Suppl:4S-15S.

Chu HW, Sethy B, Hsieh PW, Horng JT. (2021). Identification of Potential Drug Targets of Broad-Spectrum Inhibitors with a Michael Acceptor Moiety Using Shotgun Proteomics. *Viruses*. 13(9).

Cox AG, Winterbourn CC, Hampton MB. (2009). Mitochondrial peroxiredoxin involvement in antioxidant defence and redox signalling. *Biochem J*. 425(2):313-25.

de Souza, L. F., Pearson, A. G., Pace, P. E., Dafre, A. L., Hampton, M. B., Meotti, F. C., & Winterbourn, C. C. (2019). Peroxiredoxin expression and redox status in neutrophils and HL-60 cells. *Free radical biology & medicine*, 135, 227–234.

Desbois AP, Lawlor KC. Antibacterial activity of long-chain polyunsaturated fatty acids against *Propionibacterium acnes* and *Staphylococcus aureus*. *Mar Drugs*. 2013 Nov 13;11(11):4544-57.

Declercq, J. P., Evrard, C., Clippe, A., Stricht, D. V., Bernard, A., & Knoop, B. (2001). Crystal structure of human peroxiredoxin 5, a novel type of mammalian peroxiredoxin at 1.5 Å resolution. *Journal of molecular biology*, 311(4), 751–759.

Diniz do Nascimento L, Moraes AAB, Costa KSD, Pereira Galúcio JM, Taube PS, Costa CML, Neves Cruz J, de Aguiar Andrade EH, Faria LJG. (2020) Bioactive Natural Compounds and Antioxidant Activity of Essential Oils from Spice Plants: New Findings and Potential Applications. *Biomolecules*.10(7):988.

Docquier JD, Mangani S. (2018). An update on β -lactamase inhibitor discovery and development. *Drug Resist Updat*. 36:13-29.

Dong, Y., Speer, C. P., & Glaser, K. (2018). Beyond sepsis: *Staphylococcus epidermidis* is an underestimated but significant contributor to neonatal morbidity. *Virulence*, 9(1), 621–633.

Drogosz, J., & Janecka, A. (2019). Helenalin - A Sesquiterpene Lactone with Multidirectional Activity. *Current drug targets*, 20(4), 444–452.

Dutra, L. M., Bomfim, L. M., Rocha, S. L., Nepel, A., Soares, M. B., Barison, A., Costa, E. V., & Bezerra, D. P. (2014). ent-Kaurane diterpenes from the stem bark of *Annona vepretorum* (Annonaceae) and cytotoxic evaluation. *Bioorganic & medicinal chemistry letters*, 24(15), 3315–3320.

Edwards, S. E., & Morel, C. M. (2019). Learning from our mistakes: using key opportunities to remove the perverse incentives that help drive antibiotic resistance. *Expert review of pharmacoeconomics & outcomes research*, 19(6), 685–692.

Eijkelpamp BA, Begg SL, Pederick VG, Trapetti C, Gregory MK, Whittall JJ, Paton JC, McDevitt CA. (2018). Arachidonic Acid Stress Impacts Pneumococcal Fatty Acid Homeostasis. *Front Microbiol*. 9:813.

Enany, S., Yoshida, Y., & Yamamoto, T. (2014). Exploring extra-cellular proteins in methicillin susceptible and methicillin resistant *Staphylococcus aureus* by liquid chromatography-tandem mass spectrometry. *World journal of microbiology & biotechnology*, 30(4), 1269–1283.

Evrard, C., Capron, A., Marchand, C., Clippe, A., Wattiez, R., Soumillon, P., Knoops, B., & Declercq, J. P. (2004). Crystal structure of a dimeric oxidized form of human peroxiredoxin 5. *Journal of molecular biology*, 337(5), 1079–1090.

Eswar, N., Webb, B., Marti-Renom, M. A., Madhusudhan, M. S., Eramian, D., Shen, M. Y., Pieper, U., & Sali, A. (2006). Comparative protein structure modeling using Modeller. *Current protocols in bioinformatics*, Chapter 5, Unit–5.6.

Ferrer, N., Folch, A., Masó, G., Sanchez, S., & Sanchez-Vila, X. (2020). What are the main factors influencing the presence of faecal bacteria pollution in groundwater systems in developing countries?. *Journal of contaminant hydrology*, 228, 103556.

Ferrer-Sueta, G., Manta, B., Botti, H., Radi, R., Trujillo, M., & Denicola, A. (2011). Factors affecting protein thiol reactivity and specificity in peroxide reduction. *Chemical research in toxicology*, 24(4), 434–450.

Fidyt, K., Pastorczak, A., Goral, A., Szczygiel, K., Fendler, W., Muchowicz, A., Bartłomiejczyk, M. A., Madzio, J., Cyran, J., Graczyk-Jarzynka, A., Jansen, E., Patkowska, E., Lech-Maranda, E., Pal, D., Blair, H., Burdzinska, A., Pedzisz, P., Głodkowska-Mrowka, E., Demkow, U., Gawle-Krawczyk, K., ... Firczuk, M. (2019). Targeting the thioredoxin system as a novel strategy against B-cell acute lymphoblastic leukemia. *Molecular oncology*, 13(5), 1180–1195.

Flohé, L., Toppo, S., Cozza, G., & Ursini, F. (2011). A comparison of thiol peroxidase mechanisms. *Antioxidants & redox signaling*, 15(3), 763–780.

Forshaw TE, Reisz JA, Nelson KJ, Gumpena R, Lawson JR, Jönsson TJ, Wu H, Clodfelter JE, Johnson LC, Furdui CM, Lowther WT. (2021). Specificity of Human Sulfiredoxin for Reductant and Peroxiredoxin Oligomeric State. *Antioxidants (Basel)*.10(6):946.

Foti, J. J., Devadoss, B., Winkler, J. A., Collins, J. J., & Walker, G. C. (2012). Oxidation of the guanine nucleotide pool underlies cell death by bactericidal antibiotics. *Science (New York, N.Y.)*, 336(6079), 315–319.

Garuti L, Roberti M, Bottegoni G. (2011). Irreversible protein kinase inhibitors. *Curr Med Chem*. 18(20):2981-94.

Golkar Z, Bagasra O, Pace DG. (2014). Bacteriophage therapy: a potential solution for the antibiotic resistance crisis. *J Infect Dev Ctries*. 8(2):129-36.

Grant, S. S., Kaufmann, B. B., Chand, N. S., Haseley, N., & Hung, D. T. (2012). Eradication of bacterial persisters with antibiotic-generated hydroxyl radicals. *Proceedings of the National Academy of Sciences of the United States of America*, 109(30), 12147–12152.

Hall, A., Parsonage, D., Poole, L. B., & Karplus, P. A. (2010). Structural evidence that peroxiredoxin catalytic power is based on transition-state stabilization. *Journal of molecular biology*, 402(1), 194–209.

Hall, A., Nelson, K., Poole, L. B., & Karplus, P. A. (2011). Structure-based insights into the catalytic power and conformational dexterity of peroxiredoxins. *Antioxidants & redox signaling*, 15(3), 795–815.

- Halliwell, B. & Gutteridge, J.M. 2015. *Free Radicals in Biology and Medicine*. USA: Oxford University Press. 5^a edição, 823.
- Hanna, S. L., Sherman, N. E., Kinter, M. T., & Goldberg, J. B. (2000). Comparison of proteins expressed by *Pseudomonas aeruginosa* strains representing initial and chronic isolates from a cystic fibrosis patient: an analysis by 2-D gel electrophoresis and capillary column liquid chromatography-tandem mass spectrometry. *Microbiology (Reading, England)*, 146 (Pt 10), 2495–2508.
- Hu, A.; Li, J.; Fang, Y. & Li, W. 2019. Adenanthin induces G2/M arrest and apoptosis in human nonsmall-cell lung cancer cells. *International Journal of Clinical and Experimental Medicine*., 12(3): 2354-2363.
- Hou, J.K.; Huang, Y.; He, W.; Yan, Z.W.; Fan, L.; Liu, M.H. & Chen, G.Q. 2014. Adenanthin targets peroxiredoxin I/II to kill hepatocellular carcinoma cells. *Cell Death & Disease*., 5(9): e1400-e1400.
- Ibacache-Quiroga, C., Oliveros, J. C., Couce, A., & Blázquez, J. (2018). Parallel Evolution of High-Level Aminoglycoside Resistance in *Escherichia coli* Under Low and High Mutation Supply Rates. *Frontiers in microbiology*, 9, 427.
- Irvine, S.; Bunk, B.; Bayes, H. K.; Spröer, C.; Connolly, J. P. R.; Six A.; Evans T. J.; Roe, A. J.; Overmann, J.; Walker, D. (2018). Genomic inversion drives small colony variant formation and increased virulence in *P. aeruginosa*. *bioRxiv* 356386
- Jacobson FS, Morgan RW, Christman MF, Ames BN. (1989). An alkyl hydroperoxide reductase from *Salmonella typhimurium* involved in the defense of DNA against oxidative damage. Purification and properties. *J Biol Chem*. 264(3):1488-96.
- Jang, H. H., Lee, K. O., Chi, Y. H., Jung, B. G., Park, S. K., Park, J. H., Lee, J. R., Lee, S. S., Moon, J. C., Yun, J. W., Choi, Y. O., Kim, W. Y., Kang, J. S., Cheong, G. W., Yun, D. J., Rhee, S. G., Cho, M. J., & Lee, S. Y. (2004). Two enzymes in one; two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function. *Cell*, 117(5), 625–635.
- Jang, S., & Imlay, J. A. (2010). Hydrogen peroxide inactivates the *Escherichia coli* Isc iron-sulphur assembly system, and OxyR induces the Suf system to compensate. *Molecular microbiology*, 78(6), 1448–1467.
- Kalghatgi, S., Spina, C. S., Costello, J. C., Liesa, M., Morones-Ramirez, J. R., Slomovic, S., Molina, A., Shiriha, O. S., & Collins, J. J. (2013). Bactericidal antibiotics induce mitochondrial dysfunction and oxidative damage in Mammalian cells. *Science translational medicine*, 5(192), 192ra85.
- Kamariah, N.; Eisenhaber, B.; Eisenhaber, F. & Grüber, G. 2018. Molecular mechanism of the *Escherichia coli* AhpC in the function of a chaperone under heat-shock conditions. *Scientific Reports*., 8(1): 1-10.
- Kampf G. (2019). Antibiotic Resistance Can Be Enhanced in Gram-Positive Species by Some Biocidal Agents Used for Disinfection. *Antibiotics (Basel, Switzerland)*, 8(1), 13.
- Kim, S. Y., Park, C., Jang, H. J., Kim, B. O., Bae, H. W., Chung, I. Y., Kim, E. S., & Cho, Y. H. (2019). Antibacterial strategies inspired by the oxidative stress and response networks. *Journal of microbiology (Seoul, Korea)*, 57(3), 203–212.
- Khan, A., Sharma, D., Faheem, M., Bisht, D., & Khan, A. U. (2017). Proteomic analysis of a carbapenem-resistant *Klebsiella pneumoniae* strain in response to meropenem stress. *Journal of global antimicrobial resistance*, 8, 172–178.
- Kohanski, M. A., Dwyer, D. J., & Collins, J. J. (2010). How antibiotics kill bacteria: from targets to networks. *Nature reviews. Microbiology*, 8(6), 423–435.
- Ter Kuile, B. H., Kraupner, N., & Brul, S. (2016). The risk of low concentrations of antibiotics in agriculture for resistance in human health care. *FEMS microbiology letters*, 363(19), fnw210.

Lee, J., Monk, I. R., Gonçalves da Silva, A., Seemann, T., Chua, K., Kearns, A., Hill, R., Woodford, N., Bartels, M. D., Strommenger, B., Laurent, F., Dodémont, M., Deplano, A., Patel, R., Larsen, A. R., Korman, T. M., Stinear, T. P., & Howden, B. P. (2018). Global spread of three multidrug-resistant lineages of *Staphylococcus epidermidis*. *Nature microbiology*, 3(10), 1175–1185.

Leung-Toung R, Zhao Y, Li W, Tam TF, Karimian K, Spino M. (2006). Thiol proteases: inhibitors and potential therapeutic targets. *Curr Med Chem*. 13(5):547-81.

Li H, Zhou X, Huang Y, Liao B, Cheng L, Ren B. (2021). Reactive Oxygen Species in Pathogen Clearance: The Killing Mechanisms, the Adaption Response, and the Side Effects. *Front Microbiol*. 11:622534.

Libardo MD, Cervantes JL, Salazar JC, Angeles-Boza AM. (2014). Improved bioactivity of antimicrobial peptides by addition of amino-terminal copper and nickel (ATCUN) binding motifs. *Chem Med Chem*. 9(8):1892-901.

Lin, Y., Wang, J., Zheng, W., Luo, Q., Wu, K., Du, J., Zhao, Y., & Wang, F. (2019). Organometallic ruthenium anticancer complexes inhibit human peroxiredoxin I activity by binding to and inducing oxidation of its catalytic cysteine residue. *Metallomics : integrated biometal science*, 11(3), 546–555.

Link, A. J., Robison, K., & Church, G. M. (1997). Comparing the predicted and observed properties of proteins encoded in the genome of *Escherichia coli* K-12. *Electrophoresis*, 18(8), 1259–1313.

Liu S, Hanzlik RP. (1992). Structure-activity relationships for inhibition of papain by peptide Michael acceptors. *J Med Chem*. 35(6):1067-75.

Liu, C. X., Yin, Q. Q., Zhou, H. C., Wu, Y. L., Pu, J. X., Xia, L., Liu, W., Huang, X., Jiang, T., Wu, M. X., He, L. C., Zhao, Y. X., Wang, X. L., Xiao, W. L., Chen, H. Z., Zhao, Q., Zhou, A. W., Wang, L. S., Sun, H. D., & Chen, G. Q. (2012). Adenanthin targets peroxiredoxin I and II to induce differentiation of leukemic cells. *Nature chemical biology*, 8(5), 486–493.

Longo LVG, Breyer CA, Novaes GM, Gegembauer G, Leitao NP, Jr., Octaviano CE. (2020). The Human Pathogen *Paracoccidioides brasiliensis* Has a Unique 1-Cys Peroxiredoxin That Localizes Both Intracellularly and at the Cell Surface. *Front Cell Infect Microbiol*. 10:394.

Machowska, A., & Stålsby Lundborg, C. (2018). Drivers of Irrational Use of Antibiotics in Europe. *International journal of environmental research and public health*, 16(1), 27.

Mattos, K., Visacri, M. B., Quintanilha, J., Lloret, G. R., Cursino, M. A., Levin, A. S., Levy, C. E., & Moriel, P. (2017). Brazil's resolutions to regulate the sale of antibiotics: Impact on consumption and *Escherichia coli* resistance rates. *Journal of global antimicrobial resistance*, 10, 195–199.

Maura, D., Hazan, R., Kitao, T., Ballok, A. E., & Rahme, L. G. (2016). Evidence for Direct Control of Virulence and Defense Gene Circuits by the *Pseudomonas aeruginosa* Quorum Sensing Regulator, MvfR. *Scientific reports*, 6, 34083.

Mishra, S., & Imlay, J. (2012). Why do bacteria use so many enzymes to scavenge hydrogen peroxide?. *Archives of biochemistry and biophysics*, 525(2), 145–160.

Muchowicz, A., Firczuk, M., Chlebowska, J., Nowis, D., Stachura, J., Barankiewicz, J., Trzeciecka, A., Kłossowski, S., Ostaszewski, R., Zagożdżon, R., Pu, J. X., Sun, H. D., & Golab, J. (2014). Adenanthin targets proteins involved in the regulation of disulphide bonds. *Biochemical pharmacology*, 89(2), 210–216.

Muntel, J., Fromion, V., Goelzer, A., Maaß, S., Mäder, U., Büttner, K., Hecker, M., & Becher, D. (2014). Comprehensive absolute quantification of the cytosolic proteome of *Bacillus subtilis* by data independent, parallel fragmentation in liquid chromatography/mass spectrometry (LC/MS(E)). *Molecular & cellular proteomics*, 13(4), 1008–1019.

- Nalca, Y., Jänsch, L., Bredenbruch, F., Geffers, R., Buer, J., & Häussler, S. (2006). Quorum-sensing antagonistic activities of azithromycin in *Pseudomonas aeruginosa* PAO1: a global approach. *Antimicrobial agents and chemotherapy*, 50(5), 1680–1688.
- Nakamura, T., Kado, Y., Yamaguchi, T., Matsumura, H., Ishikawa, K., & Inoue, T. (2010). Crystal structure of peroxiredoxin from *Aeropyrum pernix* K1 complexed with its substrate, hydrogen peroxide. *Journal of biochemistry*, 147(1), 109–115.
- Nelson, K. J., Perkins, A., Van Swearingen, A., Hartman, S., Brereton, A. E., Parsonage, D., Salsbury, F. R., Jr, Karplus, P. A., & Poole, L. B. (2018). Experimentally Dissecting the Origins of Peroxiredoxin Catalysis. *Antioxidants & redox signaling*, 28(7), 521–536.
- Netto, L. E., & Antunes, F. (2016). The Roles of Peroxiredoxin and Thioredoxin in Hydrogen Peroxide Sensing and in Signal Transduction. *Molecules and cells*, 39(1), 65–71.
- Nising CF, Brase S. (2008). The oxa-Michael reaction: from recent developments to applications in natural product synthesis. *Chem Soc Rev*. 37(6):1218-28
- Novaes, R. D., Teixeira, A. L., & de Miranda, A. S. (2019). Oxidative Stress in Microbial Diseases: Pathogen, Host, and Therapeutics. *Oxidative medicine and cellular longevity*, 2019, 8159562.
- Oliveira, M. A., Discola, K. F., Alves, S. V., Medrano, F. J., Guimarães, B. G., & Netto, L. E. (2010). Insights into the specificity of thioredoxin reductase-thioredoxin interactions. A structural and functional investigation of the yeast thioredoxin system. *Biochemistry*, 49(15), 3317–3326.
- Otto M. (2009). *Staphylococcus epidermidis*--the 'accidental' pathogen. *Nature reviews. Microbiology*, 7(8), 555–567.
- de Oliveira, M. A., Tairum, C. A., Netto, L., de Oliveira, A., Aleixo-Silva, R. L., Cabrera, V., Breyer, C. A., & Dos Santos, M. C. (2021). Relevance of peroxiredoxins in pathogenic microorganisms. *Applied microbiology and biotechnology*, 105(14-15), 5701–5717.
- Ouidir, T., Jarnier, F., Cosette, P., Jouenne, T., & Hardouin, J. (2014). Extracellular Ser/Thr/Tyr phosphorylated proteins of *Pseudomonas aeruginosa* PA14 strain. *Proteomics*, 14(17-18), 2017–2030.
- Pastor-Flores, D., Talwar, D., Pedre, B., & Dick, T. P. (2020). Real-time monitoring of peroxiredoxin oligomerization dynamics in living cells. *Proceedings of the National Academy of Sciences of the United States of America*, 117(28), 16313–16323.
- Parsonage, D., Youngblood, D. S., Sarma, G. N., Wood, Z. A., Karplus, P. A., & Poole, L. B. (2005). Analysis of the link between enzymatic activity and oligomeric state in AhpC, a bacterial peroxiredoxin. *Biochemistry*, 44(31), 10583–10592.
- Peskin, A. V., Cox, A. G., Nagy, P., Morgan, P. E., Hampton, M. B., Davies, M. J., & Winterbourn, C. C. (2010). Removal of amino acid, peptide and protein hydroperoxides by reaction with peroxiredoxins 2 and 3. *The Biochemical journal*, 432(2), 313–321.
- Perkins, A., Nelson, K. J., Williams, J. R., Parsonage, D., Poole, L. B., & Karplus, P. A. (2013). The sensitive balance between the fully folded and locally unfolded conformations of a model peroxiredoxin. *Biochemistry*, 52(48), 8708–8721.
- Pradenas GA, Díaz-Vásquez WA, Pérez-Donoso JM, Vásquez CC. (2013). Monounsaturated fatty acids are substrates for aldehyde generation in tellurite-exposed *Escherichia coli*. *Biomed Res Int*. 2013:563756.
- Prasad S, V P S, Abbas HS, Kotakonda M. (2022). Mechanisms of Antimicrobial Resistance: Highlights on Current Advance Methods for Detection of Drug Resistance and Current Pipeline Antitubercular Agents. *Curr Pharm Biotechnol*. 23(15):1824-1836.
- Puente, V., Laurella, L. C., Spina, R. M., Lozano, E., Martino, V. S., Sosa, M. A., Sülsen, V. P., & Lombardo, E. (2019). Primary targets of the sesquiterpene lactone deoxymikanolide on *Trypanosoma cruzi*. *Phytomedicine international journal of phytotherapy and phytopharmacology*, 56, 27–34.

- Rathbone, C. R., Cross, J. D., Brown, K. V., Murray, C. K., & Wenke, J. C. (2011). Effect of various concentrations of antibiotics on osteogenic cell viability and activity. *Journal of orthopaedic research official publication of the Orthopaedic Research Society*, 29(7), 1070–1074.
- Reis A, Domingues MR, Amado FM, Ferrer-Correia AJ, Domingues P.(2005). Separation of peroxidation products of diacyl-phosphatidylcholines by reversed-phase liquid chromatography-mass spectrometry. *Biomed Chromatogr.* 19(2):129-37
- Rello, J., Kalwaje Eshwara, V., Lagunes, L., Alves, J., Wunderink, R. G., Conway-Morris, A., Rojas, J. N., Alp, E., & Zhang, Z. (2019). A global priority list of the TOP TEN resistant Microorganisms (TOTEM) study at intensive care: a prioritization exercise based on multi-criteria decision analysis. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology*, 38(2), 319–323.
- Reyes AM, Hugo M, Trostchansky A, Capece L, Radi R, Trujillo M. (2011). Oxidizing substrate specificity of Mycobacterium tuberculosis alkyl hydroperoxide reductase E: kinetics and mechanisms of oxidation and overoxidation. *Free Radic Biol Med.* 51(2):464-73.
- Rhee, S. G., & Kil, I. S. (2017). Multiple Functions and Regulation of Mammalian Peroxiredoxins. *Annual review of biochemistry*, 86, 749–775.
- Rocha, M. C., de Godoy, K. F., Bannitz-Fernandes, R., Fabri, J., Barbosa, M., de Castro, P. A., Almeida, F., Goldman, G. H., da Cunha, A. F., Netto, L., de Oliveira, M. A., & Malavazi, I. (2018). Analyses of the three 1-Cys Peroxiredoxins from *Aspergillus fumigatus* reveal that cytosolic Prx1 is central to H₂O₂ metabolism and virulence. *Scientific reports*, 8(1), 12314.
- Rocha, L. S., Silva, B., Correia, T., Silva, R., Meireles, D. A., Pereira, R., Netto, L., Meotti, F. C., & Queiroz, R. F. (2021). Peroxiredoxin AhpC1 protects *Pseudomonas aeruginosa* against the inflammatory oxidative burst and confers virulence. *Redox biology*, 46, 102075.
- Rosa W, da Silva Domingos O, de Oliveira Salem PP, Caldas IS, Murgu M, Lago JHG, Sartorelli P, Dias DF, Chagas-Paula DA, Soares MG. (2021). In vivo anti-inflammatory activity of Fabaceae species extracts screened by a new ex vivo assay using human whole blood. *Phytochem Anal.* 32(5):859-883.
- Saini, R. & Singh, S. 2019. Inducible nitric oxide synthase: An asset to neutrophils. *Journal of Leukocyte Biology.*, 105(1), 49-61
- Santos M.C.; Oliveira, MA.; Monteiro, G.; Breyer, C.A., Tairum, C.A.; Oliveira, A.J.; Zampieri, N.; Andrade, V.C; Ié, R. S.; Toyama, M.H. Inibição de enzimas peroxirredoxinas de microrganismos: uma nova abordagem para o combate a infecções microbianas. Número do registro: BR 10 2020 016245 4.
- Sarwar MS, Xia YX, Liang ZM, Tsang SW, Zhang HJ (2020). Mechanistic Pathways and Molecular Targets of Plant-Derived Anticancer ent-Kaurane Diterpenes. *Biomolecules.* 10(1):144.
- Shatalin, K., Shatalina, E., Mironov, A., & Nudler, E. (2011). H₂S: a universal defense against antibiotics in bacteria. *Science (New York, N.Y.)*, 334(6058), 986–990.
- Shi, X. J., Ding, L., Zhou, W., Ji, Y., Wang, J., Wang, H., Ma, Y., Jiang, G., Tang, K., Ke, Y., Zhao, W., & Liu, H. M. (2017). Pro-Apoptotic Effects of JDA-202, a Novel Natural Diterpenoid, on Esophageal Cancer Through Targeting Peroxiredoxin I. *Antioxidants & redox signaling*, 27(2), 73–92.
- Smeets, A., Loumaye, E., Clippe, A., Rees, J. F., Knoops, B., & Declercq, J. P. (2008). The crystal structure of the C45S mutant of annelid *Arenicola marina* peroxiredoxin 6 supports its assignment to the mechanistically typical 2-Cys subfamily without any formation of toroid-shaped decamers. *Protein science a publication of the Protein Society*, 17(4), 700–710.
- Smith T. J. (1995). MolView: a program for analyzing and displaying atomic structures on the Macintosh personal computer. *Journal of molecular graphics*, 13(2), 122–115.

Soethoudt, M., Peskin, A. V., Dickerhof, N., Paton, L. N., Pace, P. E., & Winterbourn, C. C. (2014). Interaction of adenanthin with glutathione and thiol enzymes: selectivity for thioredoxin reductase and inhibition of peroxiredoxin recycling. *Free radical biology & medicine*, 77, 331–339.

Tairum, C. A., Jr, de Oliveira, M. A., Horta, B. B., Zara, F. J., & Netto, L. E. (2012). Disulfide biochemistry in 2-cys peroxiredoxin: requirement of Glu50 and Arg146 for the reduction of yeast Tsa1 by thioredoxin. *Journal of molecular biology*, 424(1-2), 28–41.

Tairum, C.A.; Santos, M.C.; Breyer, C.A.; Geyer, R.R.; Nieves, C.J.; Portillo-Ledesma, S. & Netto, L.E. (2016). Catalytic Thr or Ser residue modulates structural switches in 2-Cys peroxiredoxin by distinct mechanisms. *Scientific Reports.*, 6: 33133.

Tairum, C. A., Santos, M. C., Breyer, C. A., de Oliveira, A., Cabrera, V., Toledo-Silva, G., Mori, G. M., Toyama, M. H., Netto, L., & de Oliveira, M. A. (2021). Effects of Serine or Threonine in the Active Site of Typical 2-Cys Prx on Hyperoxidation Susceptibility and on Chaperone Activity. *Antioxidants (Basel, Switzerland)*, 10(7), 1032.

Trott, O., & Olson, A. J. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of computational chemistry*, 31(2), 455–461.

Truzzi, D.R.; Coelho, F.R.; Paviani, V.; Alves, S.V.; Netto, L.E. & Augusto, O. (2019). The bicarbonate/carbon dioxide pair increases hydrogen peroxide-mediated hyperoxidation of human peroxiredoxin 1. *Journal of Biological Chemistry.*, 294(38), 14055-14067.

Ventola C. L. (2015). The antibiotic resistance crisis: part 1: causes and threats. *P & T : a peer-reviewed journal for formulary management*, 40(4), 277–283.

Wang, X., & Zhao, X. (2009). Contribution of oxidative damage to antimicrobial lethality. *Antimicrobial agents and chemotherapy*, 53(4), 1395–1402.

Wang, X., Zhao, X., Malik, M., & Drlica, K. (2010). Contribution of reactive oxygen species to pathways of quinolone-mediated bacterial cell death. *The Journal of antimicrobial chemotherapy*, 65(3), 520–524.

Wang, Y., Hou, Y., Wang, Y., Lu, Z., Song, C., Xu, Y., Wei, N., & Wang, Q. (2019). Cloning, expression and enzymatic characteristics of a 2-Cys peroxiredoxin from Antarctic sea-ice bacterium *Psychrobacter* sp. ANT206. *International journal of biological macromolecules*, 129, 1047–1055.

Wei W, Ma C, Cao Y, Yang L, Huang Z, Qin D, Chen Y, Liu C, Xia L, Wang T, Lei H, Yu Y, Huang M, Tong Y, Xu H, Gao F, Zhang J, Wu YL (2016). Identification of H7 as a novel peroxiredoxin I inhibitor to induce differentiation of leukemia cells. *Oncotarget*. 7(4):3873-83.

Wongsaroj, L., Saninjuk, K., Romsang, A., Duang-Nkern, J., Trinachartvanit, W., Vattanaviboon, P., & Mongkolsuk, S. (2018). *Pseudomonas aeruginosa* glutathione biosynthesis genes play multiple roles in stress protection, bacterial virulence and biofilm formation. *PloS one*, 13(10), e0205815.

Wood, Z. A., Schröder, E., Robin Harris, J., & Poole, L. B. (2003). Structure, mechanism and regulation of peroxiredoxins. *Trends in biochemical sciences*, 28(1), 32–40.

Wood, Z. A., Poole, L. B., & Karplus, P. A. (2003). Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science (New York, N.Y.)*, 300(5619), 650–653.

Wu, Z., Wang, C., Huang, M., Tao, Z., Yan, W., & Du, Y. (2019). Naturally Occurring Sesquiterpene Lactone-Santonin, Exerts Anticancer Effects in Multi-Drug Resistant Breast Cancer Cells by Inducing Mitochondrial Mediated Apoptosis, Caspase Activation, Cell Cycle Arrest, and by Targeting Ras/Raf/MEK/ERK Signaling Pathway. *Medical science monitor : international medical journal of experimental and clinical research*, 25, 3676–3682.

Ye, Q., Zhang, Y., Cao, Y., Wang, X., Guo, Y., Chen, J., Horn, J., Ponomareva, L. V., Chaiswing, L., Shaaban, K. A., Wei, Q., Anderson, B. D., St Clair, D. K., Zhu, H., Leggas, M., Thorson, J. S., & She, Q. B. (2019). Frenolicin B Targets Peroxiredoxin 1 and Glutaredoxin 3

to Trigger ROS/4E-BP1-Mediated Antitumor Effects. *Cell chemical biology*, 26(3), 366–377.e12.

Chang, Y., Goldberg, V. M., & Caplan, A. I. (2006). Toxic effects of gentamicin on marrow-derived human mesenchymal stem cells. *Clinical orthopaedics and related research*, 452, 242–249.

Zhu, H., Wang, B., Kong, L., An, T., Li, G., Zhou, H., Gong, L., Zhao, Z., Gong, Y., Sun, H., Puno, P., & Li, Y. (2019). Parvifoline AA Promotes Susceptibility of Hepatocarcinoma to Natural Killer Cell-Mediated Cytolysis by Targeting Peroxiredoxin. *Cell chemical biology*, 26(8), 1122–1132.e6.

APÊNDICE 1

PONE-D-22-35457: Final Decision Being Processed - [EMID:b7e9ff36a5f9cf20]

4 mensagens

PLOS ONE <em@editorialmanager.com>

20 de janeiro de 2023 às 10:31

Responder a: PLOS ONE <plosone@plos.org>

Para: "Marcos A. de Oliveira" <marcos.a.oliveira@unesp.br>

CC: "Vitoria Isabela Montanhero Cabrera" vitoria.isabela@unesp.br, "Gabrielle Nascimento Sividanes" g.sividanes@unesp.br, "Natalia Fernanda Quintiliano" nataliafq@ufabc.edu.br, "Marcos Hikari Toyama" marcos.toyama@unesp.br, "João Henrique Guilhardi Lago" joao.lago@ufabc.edu.br

Exploring functional and structural features of chemically related natural prenylated hydroquinone and benzoic acid from *Piper crassinervium* (Piperaceae) on bacterial peroxiredoxin inhibition
PONE-D-22-35457

Dear Dr. Oliveira,

We're pleased to inform you that your manuscript has been judged scientifically suitable for publication and will be formally accepted for publication once it meets all outstanding technical requirements.

Within one week, you'll receive an e-mail detailing the required amendments. When these have been addressed, you'll receive a formal acceptance letter and your manuscript will be scheduled for publication.

An invoice for payment will follow shortly after the formal acceptance. To ensure an efficient process, please log into Editorial Manager at <http://www.editorialmanager.com/pone/>, click the 'Update My Information' link at the top of the page, and double check that your user information is up-to-date. If you have any billing related questions, please contact our Author Billing department directly at authorbilling@plos.org.

If your institution or institutions have a press office, please notify them about your upcoming paper to help maximize its impact. If they'll be preparing press materials, please inform our press team as soon as possible -- no later than 48 hours after receiving the formal acceptance. Your manuscript will remain under strict press embargo until 2 pm Eastern Time on the date of publication. For more information, please contact onepress@plos.org.

Kind regards,

A Ganesan
Academic Editor
PLOS ONE

Journal requirements:

When submitting your revision, we need you to address these additional requirements.

1. Please ensure that your manuscript meets PLOS ONE's style requirements, including those for file naming. The PLOS ONE style templates can be found at

https://journals.plos.org/plosone/s/file?id=wjVg/PLOOne_formatting_sample_main_body.pdf and

https://journals.plos.org/plosone/s/file?id=ba62/PLOOne_formatting_sample_title_authors_affiliations.pdf

2. We noticed you have some minor occurrence of overlapping text with the following previous publication(s), which needs to be addressed:

<https://www.sciencedirect.com/science/article/abs/pii/S0891584920314611?via%3Dihub>

In your revision ensure you cite all your sources (including your own works), and quote or rephrase any duplicated text outside the methods section. Further consideration is dependent on these concerns being addressed.

PLOS ONE

Exploring functional and structural features of chemically related natural prenylated hydroquinone and benzoic acid from *Piper crassinervium* (Piperaceae) on bacterial peroxiredoxin inhibition

--Manuscript Draft--

Manuscript Number:	PONE-D-22-35457
Article Type:	Research Article
Full Title:	Exploring functional and structural features of chemically related natural prenylated hydroquinone and benzoic acid from <i>Piper crassinervium</i> (Piperaceae) on bacterial peroxiredoxin inhibition
Short Title:	Bacterial peroxiredoxin inhibitor
Corresponding Author:	Marcos A. de Oliveira, Ph.D UNESP: Universidade Estadual Paulista Julio de Mesquita Filho São Vicente, SP BRAZIL
Keywords:	Prenylated benzoic acid; prenylated hydroquinone; long chain lipids hydroperoxides; alkyl hydroperoxide reductase C (AhpC); peroxidase activity; molecular simulations
Abstract:	The resistance of pathogenic bacteria to multiple antibiotics has increased all over the world in recent years, demanding novel antibacterial compounds. Recently, some studies have shown that distinct antibiotics with different targets have the convergent ability to produce reactive oxygen species (ROS), which contribute to the killing of bacterial pathogens. Bacteria possess several antioxidant enzymes able to decompose ROS. Among them, the typical 2-Cys peroxiredoxins, so-called AhpC in bacteria, are extremely abundant in bacterial cells, and some studies indicate that AhpC have virulent properties. The high reactivity of AhpC is related to a catalytic triad, composed of two polar residues (Thr/Ser and Arg) that maintain CysP sulfur in the thiolate form (S-) and are involved in targeting and stabilizing the substrate to allow bimolecular nucleophilic substitution (SN2) reaction. Despite the importance of AhpC in bacteria, few studies aimed at using natural compounds as inhibitors of this class of enzymes. Some natural products were identified as human isoforms, presenting as common characteristics a bulk hydrophobic moiety and an α, β-unsaturated carbonylic system able to perform a thiol-Michael reaction. In this work, we evaluated two chemically related natural products: 1,4-dihydroxy-2-(3',7'-dimethyl-1'-oxo-2'E,6'-octadienyl) benzene (C1) and 4-hydroxy-2-(3',7'-dimethyl-1'-oxo-2'E,6'-octadienyl) benzoic acid (C2), both were isolated from branches <i>Piper crassinervium</i> (Piperaceae), over the peroxidase activity of AhpC from <i>Pseudomonas aeruginosa</i> (PaAhpC) and <i>Staphylococcus epidermidis</i> (SeAhpC). By biochemical assays we show that although both compounds can perform the Michael addition reaction, only compound C2 was able to inhibit the PaAhpC peroxidase activity but not SeAhpC, presenting IC50 = 20.3 μM. SDS-PAGE analysis revealed that the compound was not able to perform a thiol-Michael addition, suggesting another inhibition behavior. Using computer-assisted simulations, we also show that an acidic group present in the structure of compound C2 may be involved in the stabilization by polar interactions with the Thr and Arg residues from the catalytic triad and several apolar interactions with hydrophobic residues. Finally, C2 was not able to interfere in the peroxidase activity of the isoform Prx2 from humans or even the thiol proteins of the Trx reducing system from <i>Escherichia coli</i> (EcTrx and EcTrxR), indicating specificity for <i>P. aeruginosa</i> AhpC.
Order of Authors:	Vitoria Isabela Montanhero Cabrera, MSc Gabrielle Nascimento Sividanes, BSc Natalia Fernanda Quintiliano, MSc Marcos Hikari Toyama, PhD João Henrique Guilhardi Lago, PhD Marcos A. de Oliveira, PhD
Opposed Reviewers:	

Additional Information:	
Question	Response
Financial Disclosure Enter a financial disclosure statement that describes the sources of funding for the work included in this submission. Review the submission guidelines for detailed requirements. View published research articles from PLOS ONE for specific examples. This statement is required for submission and will appear in the published article if the submission is accepted. Please make sure it is accurate. Unfunded studies Enter: <i>The author(s) received no specific funding for this work.</i> Funded studies Enter a statement with the following details: • Initials of the authors who received each award • Grant numbers awarded to each author • The full name of each funder • URL of each funder website • Did the sponsors or funders play any role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript? • NO - Include this sentence at the end of your statement: <i>The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.</i> • YES - Specify the role(s) played. * typeset	The authors M.A.O., V.I.M.C. and G.N.S. are members of the CEPID REDOXOMA from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (grant number 2013/07937-8). This work was also supported by grants from FAPESP to M.A.O. (2017/19942-7), M.H.T. (2017/20291-0) and J.H.G.L (2021/02789-7). V.I.M.C. is recipient of fellowship from FAPESP (2020/02868-1). We also thank CNPq for the fellowships. NO.
Competing Interests Use the instructions below to enter a competing interest statement for this submission. On behalf of all authors, disclose any competing interests that could be perceived to bias this work—acknowledging all financial support and any other relevant financial or non-financial competing interests.	The authors have declared that no competing interests exist.

This statement is required for submission and will appear in the published article if the submission is accepted. Please make sure it is accurate and that any funding sources listed in your Funding Information later in the submission form are also declared in your Financial Disclosure statement. View published research articles from PLOS ONE for specific examples. NO authors have competing interests Enter: <i>The authors have declared that no competing interests exist.</i> Authors with competing interests Enter competing interest details beginning with this statement: <i>I have read the journal's policy and the authors of this manuscript have the following competing interests: [insert competing interests here]</i> * typeset	
Ethics Statement Enter an ethics statement for this submission. This statement is required if the study involved: • Human participants • Human specimens or tissue • Vertebrate animals or cephalopods • Vertebrate embryos or tissues • Field research Write "N/A" if the submission does not require an ethics statement. General guidance is provided below. Consult the submission guidelines for detailed instructions. Make sure that all information entered here is included in the	N/A

Methods section of the manuscript. Format for specific study types Human Subject Research (involving human participants and/or tissue) • Give the name of the institutional review board or ethics committee that approved the study • Include the approval number and/or a statement indicating approval of this research • Indicate the form of consent obtained (written/oral) or the reason that consent was not obtained (e.g. the data were analyzed anonymously) Animal Research (involving vertebrate animals, embryos or tissues) • Provide the name of the Institutional Animal Care and Use Committee (IACUC) or other relevant ethics board that reviewed the study protocol, and indicate whether they approved this research or granted a formal waiver of ethical approval • Include an approval number if one was obtained • If the study involved <i>non-human primates</i>, add <i>additional details</i> about animal welfare and steps taken to ameliorate suffering • If anesthesia, euthanasia, or any kind of animal sacrifice is part of the study, include briefly which substances and/or methods were applied Field Research Include the following details if this study involves the collection of plant, animal, or other materials from a natural setting: • Field permit number • Name of the institution or relevant body that granted permission	
Data Availability Authors are required to make all data underlying the findings described fully available, without restriction, and from the time of publication. PLOS allows rare exceptions to address legal and ethical concerns. See the PLOS Data Policy and FAQ for detailed information.	Yes - all data are fully available without restriction

A Data Availability Statement describing where the data can be found is required at submission. Your answers to this question constitute the Data Availability Statement and will be published in the article, if accepted. Important: Stating 'data available on request from the author' is not sufficient. If your data are only available upon request, select 'No' for the first question and explain your exceptional situation in the text box. Do the authors confirm that all data underlying the findings described in their manuscript are fully available without restriction?	
Describe where the data may be found in full sentences. If you are copying our sample text, replace any instances of XXX with the appropriate details. • If the data are held or will be held in a public repository, include URLs, accession numbers or DOIs. If this information will only be available after acceptance, indicate this by ticking the box below. For example: <i>All XXX files are available from the XXX database (accession number(s) XXX, XXX).</i> • If the data are all contained within the manuscript and/or Supporting Information files, enter the following: <i>All relevant data are within the manuscript and its Supporting Information files.</i> • If neither of these applies but you are able to provide details of access elsewhere, with or without limitations, please do so. For example: <i>Data cannot be shared publicly because of [XXX]. Data are available from the XXX Institutional Data Access / Ethics Committee (contact via XXX) for researchers who meet the criteria for access to confidential data.</i> <i>The data underlying the results</i>	All relevant data are within the manuscript and its Supporting Information files.

<i>presented in the study are available from (include the name of the third party and contact information or URL).</i> • This text is appropriate if the data are owned by a third party and authors do not have permission to share the data. * typeset	
Additional data availability information:	Tick here if the URLs/accession numbers/DOIs will be available only after acceptance of the manuscript for publication so that we can ensure their inclusion before publication.



SAO PAULO STATE UNIVERSITY
BIOSCIENCES INSTITUTE
COSTAL CAMPUS



São Vicente, December 20nd, 2022

To editorial board of PLoS One

The manuscript entitled “Exploring functional and structural features of chemically related natural prenylated hydroquinone and benzoic acid from *Piper crassinervium* (Piperaceae) on bacterial peroxiredoxin inhibition” is presented here for submission to *the PLoS One Journal*. In our investigation we evaluated two natural products (compound 1 and compound 2) from *Piper crassinervium* over peroxiredoxin AhpC from the human pathogen *Pseudomonas aeruginosa*.

The motivation of our research resides in the fact that the emergence and re-emergence of bacterial strains resistant to multiple drugs is a global health threat and the search for novel biological targets is a worldwide concern. AhpC is ubiquitous bacterial peroxidase, from peroxiredoxin (Prx) family, involved in the redox homeostasis in cells metabolizing the most diverse kinds of hydroperoxides generated throughout several biological processes, using reactive cysteines residues. In pathogenic bacteria these enzymes are related in the infection, establishment, and survival in their hosts, subjugating the oxidative burst generated by the immune system and antibiotics and were characterized as virulence factors in some species. Despite their importance, no inhibitor was systematically evaluated to date. Conversely, in humans, some Prx isoforms are overexpressed in genetic diseases as cancer, and inhibitors were identified, including natural products. Among them some natural compounds which share common characteristics as a bulky hydrophobic carbonylic skeleton and a group able to perform a thiol-Michael addition to the peroxidatic residue of human Prx. Here we show that two natural products chemically related and both can perform a thiol-Michael addition, but only the compound containing an acidic group was able to exert inhibitory effects over the PaAhpC, but not by the thiol-Michael chemistry. We also evaluated the inhibitory over Prx isoforms from *Staphylococcus epidermidis* (ScAhpC), humans (HsPrx2) and other thiols proteins, but no significative inhibition was detected, revealing specificity over PaAhpC. The biochemical results were explored by molecular docking simulations and a rationalization of the results is proposed based in structural studies and biological oxidizing substrates. Since *P. aeruginosa* is an emerging pathogen with several strains resistant to multiple drugs and this characterization may impact in the discovery of novel Prx inhibitors, our findings may be of importance to human health, are in accordance with the scope of the *PLoS One* and will be of interest to its readers.

Thank you in advance

Prof. Marcos A. de Oliveira, PhD
Departamento de Ciências Biológicas
e Ambientais Instituto de Biociências - UNESP
Campus do Litoral Paulista
Praça Infante Dom Henrique S/N
Sao Vicente - SP - Brazil
11380-972
Phone +55 13 3569-7108/Fax+55 13 3569-714

UNESP - Biosciences Institute - Coastal Campus
Praça Infante D. Henrique s/n° - CEP 11330-900 - São Vicente (SP) - Brazil
(55)13 3569-7148 - Fax (55)13 3569-7146 - mao@clp.unesp.br

**Exploring functional and structural features of chemically related
natural prenylated hydroquinone and benzoic acid from *Piper
crassinervium* (Piperaceae) on bacterial peroxiredoxin inhibition**

Vitoria Isabela Montanhero Cabrera¹, Gabrielle do Nascimento Sividanes¹,
Natalia Fernanda Quintiliano², Marcos Hikari Toyama¹, João Henrique Ghilardi Lago^{2*} and
Marcos Antonio de Oliveira^{1*}

¹Instituto de Biociências, Universidade Estadual Paulista, UNESP, São Vicente, SP, Brazil.

²Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, Santo André, SP
09210-580, Brazil.

*Correspondence:

Marcos A. de Oliveira – marcos.a.oliveira@unesp.br; João H. G. Lago –
joao.lago@ufabc.edu.br

Abstract

The resistance of pathogenic bacteria to multiple antibiotics has increased all over the world in recent years, demanding novel antibacterial compounds. Recently, some studies have shown that distinct antibiotics with different targets have the convergent ability to produce reactive oxygen species (ROS), which contribute to the killing of bacterial pathogens. Bacteria possess several antioxidant enzymes able to decompose ROS. Among them, the typical 2-Cys peroxiredoxins, so-called AhpC in bacteria, are extremely abundant in bacterial cells, and some studies indicate that AhpC have virulent properties. The high reactivity of AhpC is related to a catalytic triad, composed of two polar residues (Thr/Ser and Arg) that maintain Cys_P sulfur in the thiolate form (S⁻) and are involved in targeting and stabilizing the substrate to allow bimolecular nucleophilic substitution (S_N2) reaction. Despite the importance of AhpC in bacteria, few studies aimed at using natural compounds as inhibitors of this class of enzymes. Some natural products were identified as human isoforms, presenting as common characteristics a bulk hydrophobic moiety and an α , β -unsaturated carbonylic system able to perform a thiol-Michael reaction. In this work, we evaluated two chemically related natural products: 1,4-dihydroxy-2-(3',7'-dimethyl-1'-oxo-2'E,6'-octadienyl) benzene (**C1**) and 4-hydroxy-2-(3',7'-dimethyl-1'-oxo-2'E,6'-octadienyl) benzoic acid (**C2**), both were isolated from branches *Piper crassinervium* (Piperaceae), over the peroxidase activity of AhpC from *Pseudomonas aeruginosa* (PaAhpC) and *Staphylococcus epidermidis* (SeAhpC). By biochemical assays we show that although both compounds can perform the Michael addition reaction, only compound **C2** was able to inhibit the PaAhpC peroxidase activity but not SeAhpC, presenting IC₅₀ = 20.3 μ M. SDS-PAGE analysis revealed that the compound was not able to perform a thiol-Michael addition, suggesting another inhibition behavior. Using computer-assisted simulations, we also show that an acidic group present in the structure of compound **C2** may be involved in the stabilization by polar interactions with the Thr and Arg residues from the catalytic triad and several apolar interactions with hydrophobic residues. Finally, **C2** was not able to interfere in the peroxidase activity of the isoform Prx2 from humans or even the thiol proteins of the Trx reducing system from *Escherichia coli* (EcTrx and EcTrxR), indicating specificity for *P. aeruginosa* AhpC.

Keywords: Prenylated benzoic acid, prenylated hydroquinone, long chain lipids hydroperoxides, alkyl hydroperoxide reductase C (AhpC), peroxidase activity, molecular simulations

INTRODUCTION

Bacteria resistance to antibacterial drugs may be directly related to oxidative stress since antibiotics from different classes have the common ability to generate reactive oxygen species (ROS), damaging macromolecules, especially DNA [1, 2]. ROS are also produced by host immune cells to fight pathogen infections, and studies suggest that inhibition of antioxidant enzymes is deleterious to pathogens infection and establishment [3]. Bacteria cells possess several antioxidant enzymes able to decompose ROS, including the typical 2-Cys peroxiredoxins, so called AhpCs [4]. These enzymes are very abundant in bacteria and are considered virulence factors to some species, indicating they play an important role in pathogenesis [3, 5-10]. AhpC is the abbreviation used for alkyl hydroperoxide reductase subunit C, referring to its discovery, since the bacterial strains carrying deletions of *ahpC* gene were very sensitive to organic hydroperoxides [11]. Later studies revealed that AhpC are also very reactive to several kinds of hydroperoxide, such as hydrogen peroxide (H_2O_2) and peroxynitrite (NOO^-) [12-14].

The peroxidatic mechanism of typical 2-Cys Prx is based on a conserved catalytic triad (TC) composed of a reactive cysteine residue (peroxidatic cysteine - Cys_P), an Arg, and a Thr or Ser. The mechanistic importance of the catalytic triad residues has been well studied experimentally, and it is based on an intricate hydrogen bonds network involved in the hydroperoxide decomposition [15, 16]. The Arg and Thr/Ser are able to stabilize the Cys_P in the thiolate state ($\text{Cys}_\text{P}\text{-S}^-$) by hydrogen bonds, decreasing its pK_a . These residues are also involved in the orientation and activation of the hydroperoxide molecule (R-OOH) by hydrogen bonds network, allowing the optimal reactivity of Cys_P through a $\text{S}_\text{N}2$ mechanism [15, 16]. Together, this molecular mechanism culminates in Cys_P oxidation to cysteine sulfenic acid ($\text{Cys}_\text{P} - \text{SOH}$) and the release of the leaving group (R-OH) (**Fig 1**). Computer-assisted

simulations of the reaction of different Prxs with H₂O₂ gave further insights into the mechanism of catalysis at a molecular level, supporting the catalytic mechanism proposed [17, 18].

The typical 2-Cys Prx are obligate homodimers. After hydroperoxide decomposition, the Cys_P forms an intramolecular disulfide with a second cysteine residue (resolving Cys; Cys_R) found in the adjacent subunit. Despite that, the minimum catalytic unit of 2-Cys Prx has been homodimer, when these enzymes are in the reduced state (Cys_P-S⁻) they are found as α 2[5] decamers, forming a structure that resembles a doughnut [20]. In enzymes containing Thr as part of the catalytic triad, the peroxide decomposition with consequent oxidation of the cysteines to disulfide triggers conformational changes that result in the decamer disruption in dimers [19-21]. Conversely to 2-Cys Prx containing Ser in the CT, the disulfide formation occurs without significant quaternary structure transition [22, 23]. The reducing agent of typical 2-Cys Prx are represented by the thioredoxin system or by the AhpF which are able to reduce the Prx disulfide, restoring the reactive state [23, 24].

Despite the apparent AhpC importance to the establishment and survival of pathogenic bacteria in hosts, few studies aimed at finding inhibitors to these enzymes [25, 26]. Some isoforms of typical Prx 2-Cys are overexpressed in some human genetic diseases, including cancer and biological processes as inflammation, and some inhibitory macro and micro-molecules, such as antibodies and natural or synthetic low molecular weight compounds, have already been characterized as eukaryotic counterparts [27, 28-34]. A common feature shared among low molecular weight compounds is a very bulky carbon skeleton, which may resemble natural oxidant substrates as organic hydroperoxides. Among the natural compounds identified to eukaryotic isoforms, three of them are *ent*-kauranes diterpenes (adenanthin, JM-202, and parvifoline AA). The inhibitory action of these compounds is based on the alkylation of the catalytic cysteines through a thiol-Michael addition involving an α,β-unsaturated carbonylic system from the inhibitors [29, 30, 33].

As part of our continuous studies concerning the identification of natural inhibitors of peroxiredoxins, we here isolated two chemically related natural compounds – 1,4-dihydroxy-2-(3',7'-dimethyl-1'-oxo-2'E,6'-octadienyl) benzene (**C1**) and 4-hydroxy-2-(3',7'-dimethyl-1'-oxo-2'E,6'-octadienyl) benzoic acid (**C2**) from branches of *Piper crassinervium* (Piperaceae). These chemicals were tested over the AhpCs containing Thr or Ser as part of the catalytic triad, using recombinant enzymes from *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*, opportunistic Gram -negative and -positive bacteria involved in hospital acquired infections. Using biochemical approaches, we identified compound **C2** as a novel natural product with inhibitory properties over PaAhpC but not over SeAhpC, that presents IC₅₀ in the order of 20.3 μ M. We also demonstrated that the inhibitory properties were not due to the thiol-Michael addition mechanism over the catalytic cysteines, since the intramolecular disulfide formation of the enzyme was preserved. We also evaluated the inhibitory effect of the compound **C2** over the human isoform Prx2 and the thiol proteins Trx and TrxR from bacteria, and no significant inhibition was detected in the tested conditions, suggesting specificity to PaAhpC. Finally, we executed computer assisted molecular simulations to investigate a possible mode of binding, which revealed that compound **C2** is maintained in the active site by several non-polar interactions with residues of the PaAhpC active site microenvironment comprising three subunits of the decamer. Curiously **C2** possess physicochemical characteristics that resemble organic peroxides found in biological systems that are derived from aromatic compounds or long chain molecules, such as nitrogenous bases and lipids hydroperoxides and, ultimately, may mimics these kinds of biological substrates. Altogether, our results reveal a novel class of inhibitory natural compounds able to exert activity over the bacterial PaAhpC.

EXPERIMENTAL

General

Chromatographic separation procedures were performed using silica gel (Merck, 230–400 mesh) and silica gel 60 PF₂₅₄ (Merck) for column separation and for analytical (0.50mm) TLC, respectively. NMR spectra were recorded on a Varian Inova 500 spectrometer, operating at 500 MHz (¹H nuclei) and 125 MHz (¹³C nuclei). CDCl₃ (Aldrich) was used as a solvent whereas tetramethylsilane (TMS) was employed as an internal standard. ESI-HRMS were recorded on a Bruker Daltonics MicroTOF QII spectrometer acquired with ESI (electrospray ionization) in both positive and negative ion mode. All solvents and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Thermo Scientific (Logan, UT, USA).

Plant Material

Branches of *P. crassinervium* were collected at Parque Estadual Fontes do Ipiranga, São Paulo State, Brazil and identified by Dr. Guilherme M. Antar from the Instituto de Biociências of Universidade de São Paulo (IB-USP), receiving the registration code SISGEN A4123E4. A voucher specimen (SPF 218827) was deposited in the Herbarium of IB-USP, São Paulo, SP, Brazil.

Extraction and isolation of the constituents

Dried and powdered branches (85 g) were exhaustively extracted with *n*-hexane at room temperature. The solvent of combined *n*-hexane was evaporated under reduced pressure to afford 747 mg of crude extract. Part of this material (670 mg) was chromatographed over a silica-gel column, eluted with increasing amounts of EtOAc in *n*-hexane, to afford pure **C1** (79.5 mg) and **C2** (44.2 mg).

Recombinant proteins

P. aeruginosa AhpC (PaAhpC), *S. epidermidis* AhpC (SeAhpC), and the Trx system from *Escherichia coli* (EcTrx and EcTrxR) were previously obtained [22]. The expression of the human Prx2 (HsPrx2) and *Saccharomyces cerevisiae* Trx system (ScTrx1 and ScTrxR1) were performed as previously described by Truzzi and colleagues [35].

Protein expression and purification

Transformed *E. coli* BL21 (DE3) cells containing pET15b-*pa_ahpc*, pET15b-*se_ahpc*, pET15b-*ec_trx*, pET15b-*ec_trxr*, pET28b-*hs_prx2*, pET15b-*sc_trx1* and pPROEX-*sctrxr1* were grown overnight at 37 °C in 100 mL of medium LB (10% tryptone, 5% NaCl and 5% yeast extract) containing 100 µg/mL of ampicillin and then transferred to 1 L of fresh LB containing ampicillin (100 µg/mL) and grown to OD₆₀₀ = 0.6–0.8. Recombinant protein expression was induced by IPTG addition to a final concentration of 1 mM for 3 hours at 37 °C. The cells were harvested by centrifugation at 4.000 × g/4 °C/20 min, and the pellet was suspended with start buffer (20 mM sodium phosphate pH 7.4; 300 mM NaCl; 20 mM imidazole) containing PMSF (1 mM). Cell disruptions were performed by sonication (30% amplitude/ 24 cycles of 5 seconds and resting for 15 seconds in an ice bath, and nucleic acids were removed using streptomycin sulfate ([Final] = 1%). The suspensions were clarified by filtration (45µm membrane; Merck–Millipore) and following centrifugation (45 min/4°C/12.000 g) and the protein extracts free of nucleic acids were collected.

Determination of enzyme concentrations

Protein concentrations were measured by spectrophotometry at $\lambda = 280$ nm by the molar extinction coefficient using the ProtParam tool (<http://web.expasy.org/protparam>). The enzyme abbreviations, Uniprot codes and enzyme properties are presented in table 1.

Table 1. Molar extinction coefficients, molecular weight and Uniprot code of the proteins used in this work.

Enzyme	ϵ 280 nm ($M^{-1} cm^{-1}$)	Molecular weight (kDa)	Uniprot entry
PaAhpC	21095	20.54	Q9I6Z3
ScAhpC	25556	21.08	Q8CMQ2
EcTrx	14105	12.63	P0AA25
EcTrxR	19160	35.45	P0A9P4
HsPrx2	21555	21.89	P32119
ScTrx1	10095	11.23	P22217
ScTrxR1	27640	34.23	P29509

Abbreviations: PaAhpC = *P. aeruginosa* AhpC, ScAhpC = *S. epidermidis* AhpC, EcTrx = *E. coli* Trx1; EcTrxR = *E. coli* TrxR1, HsPrx2 = *H. sapiens* Prx2, ScTrx1 = *S. cerevisiae* Trx1 and ScTrxR1 = *S. cerevisiae* TrxR1.

Treatment of the enzymes with compounds C1 and C2

Enzyme samples [100 μ M] were reduced with 5mM dithiothreitol (DTT) for 30 minutes at 25 °C and desalted using PD10 columns (Cytiva Life Sciences, Marlborough, MA, USA), quantified, and the protein concentration were adjusted to 10 μ M and treated with 50 M

equivalents of compounds **C1** or **C2** for one hour at room temperature. As control of cysteine alkylation/enzyme inactivation, the Prx were treated with N-ethylmaleimide (NEM) 1h/RT. Finally, the treated proteins were desalted again to remove excess of the compounds and quantified again for use in subsequent assays.

NADPH coupled oxidation assay to test inhibitory properties of compounds C1 and C2

The inhibitory properties of compounds **C1** and **C2** on PaAhpC and SeAhpC peroxidase activity were tested using the *E. coli* Trx system by the coupled NADPH oxidation assay as previously described [22]. Briefly: 3 μ M of PaAhpC or SeAhpC was added to a mix containing 6 μ M of EcTrx, 0.9 μ M of EcTrxR, 150 μ M of NADPH, in 50 mM HEPES pH 7.4 buffer, 1 mM DTPA and 100 μ M sodium azide, and then incubated for 5 minutes at 37°C. The reactions were initiated by the addition of H₂O₂ (500 μ M) and NADPH decay were monitored spectrophotometrically at 340 nm. As negative controls are represented by reactions without AhpC are (negative control), or AhpC pretreated with NEM (40 μ M) (inhibition control), proteins without pretreatment (positive control), and proteins pretreated either with compounds **C1** and **C2**. To evaluate the inhibitory activity of compound **C2** over the human peroxiredoxin isoform were used human HsPrx2 (5 μ M) and the heterologous yeast Trx system (ScTrx1, 10 μ M and ScTrxR1, 0.2 μ M).

Analysis of protein sequences and crystallographic structures

Protein alignments were performed using Clustal Ω [36], and the graphical representations were generated using JalView [37]. Molecular crystallographic structures representations were made using the PyMol (<https://pymol.org/2/>).

Molecular Docking Approaches

Molecular dockings were performed using the coordinates 4MA9 from *S. typhimurium* AhpC obtained from the Protein Data Bank. Three-dimensional structures of compounds **C1** and **C2** were constructed using MolView [38]. Docking simulations were performed using AutoDock Vina [39, 40], gridding the active site region ($20 \times 20 \times 20 \text{ \AA}$), using the deprotonates Cys_P (thiolate state). Were generated 15 positions per simulation. Each ligand orientation was analyzed using UCSF Chimera tool [41] to determine the best pose using as criterion the distance of the reactive Cys_P and the α,β -unsaturated group of the compounds with maximum distance of 5.0 Å and also the position compared with ligands found in the crystallographic structures of Prx isoforms active site as H₂O₂ (PDB code = 3A2V), benzoate (2V32 1HD2; 1OC3; 2V41; 1H4O) and tert-butylbenzene-diol (4K7O). The protein-ligand analysis interactions of the best poses were performed using the LigPlot+.

RESULTS

Chemical characterization of compounds **C1** and **C2**

Compounds **C1** and **C2**, isolated as white amorphous solids, displayed $[\text{M} - \text{H}]^-$ ion peaks in ESI-HRMS at m/z 259.1328 and 287.1322, respectively. ¹H NMR spectra indicated the presence of 1,2,4-trisubstituted aromatic ring based on the signals at δ 6.84 (d, $J = 8.9$ Hz, H-6), 6.98 (dd, $J = 8.9$ and 3.0 Hz, H-5) and 7.21 (d, $J = 3.0$ Hz, H-3) for compound **C1**, and at δ 7.03 (d, $J = 8.7$ Hz, H-6), 8.15 (dd, $J = 8.7$ and 1.6 Hz, H-5) and 8.58 (d, $J = 1.6$ Hz, H-3) for compound **C2**. These different signals suggest the occurrence of hydroquinone and 4-hydroxybenzoic unities in compounds **C1** and **C2**, respectively, as previously reported in

metabolites isolated from leaves of *P. crassinervium* [42]. Despite these differences, ¹H NMR spectra of both compounds showed similar signals attributed to 1-oxo-geranyl moiety at δ 1.62 (H-9'), 1.72 (s, H-8'), 2.18 (s, H-10'), 2.30 (m, H-4'), 2.20 (m, H-5'), 5.12 (t, *J* = 7.3 Hz), and 6.66 (s, H-2'). ¹³C NMR spectra of compounds **C1** and **C2** exhibited signals attributed to sp² carbons of geranyl moiety at δ 196.1/195.8 (C-1'), 115.0/118.8 (C-2'), 161.4/163.8 (C-3') 124.2/122.7 (C-6'), and 132.9/133.0 (C-7'), as well as three methyl groups at δ 25.7 (C-8'), 17.8 (C-9'), and 20.1/20.4 (C-10'); and two methylene carbons at δ 41.6/41.9 (C-4') and 26.2/26.3 (C-5'). In the case of ¹³C NMR spectrum of compound **C1**, the remaining six peaks were observed at δ 119.2 (C-1), 119.7 (C-2), 120.6 (C-3), 123.0 (C-4), 147.3 (C-5), and 157.4 (C-6), peaks that were attributed to carbons of a hydroquinone system. For compound **C2**, seven signals of sp² carbons, assigned to 4-hydroxybenzoic unit, were observed at δ 119.8 (C-1), 167.7 (C-2), 118.9 (C-3), 137.0 (C-4), 120.2 (C-5), 133.1 (C-6), and 170.5 (COOH) (**Figs S1-S6**). Comparison with literature data allowed the identification of compounds **C1** and **C2** as 1,4-dihydroxy-2-(3',7'-dimethyl-1'-oxo-2'*E*,6'-octadienyl) benzene and 4-hydroxy-2-(3',7'-dimethyl-1'-oxo-2'*E*,6'-octadienyl) benzoic acid [42, 43], respectively (**Fig 2**).

Evaluation of inhibitory properties of compounds **C1** and **C2**

To assess the inhibitory activity of compounds **C1** and **C2**, we employed the NADPH oxidation assay [29] using the recombinant AhpC from *P. aeruginosa* (PaAhpC) or *S. epidermidis* (SeAhpC) and the heterologous Trx system from *E. coli*. Representative results of the PaAhpC, SeAhpC, EcTrx, and EcTrxR expression and purification procedures are shown in **Fig S7 A-D**. As inhibition control, reduced samples of PaAhpC or SeAhpC were treated with NEM, a well-known and powerful cysteine alkylating agent that is able to annihilate peroxiredoxin activity [44]. As expected for the samples subjected to NEM treatment, no peroxidase activity was detected to both enzymes as indicated by the initial rates of hydrogen

peroxide decomposition when compared to the enzyme without treatment (**Fig 3 and Table 2**). Regarding the tested natural products, compound **C1** was not able to interfere significantly on the peroxidase activity of both AhpCs (**Fig 3A and 3C; Table 2**). Conversely, while the compound **C2** nearly abolished PaAhpC enzyme activity, no significant effect was observed on SeAhpC (**Fig 3B and 3D; Table 2**). We also compared the residual amounts of NADPH after 300 seconds of reaction, and the results confirmed that compound **C2** was able to inhibit the peroxidase activity of PaAhpC but not of SeAhpC (**Fig S8**).

Table 2. Initial rates of hydrogen peroxide decomposition by PaAhpC or SeAhpC after treatment with compounds **C1** or **C2**.

S ample	No enzyme (Negative control)	Enzyme + NEM (Inhibition control)	Enzyme (Positive control)	En zyme + C1	Enzyme + C2
Pa AhpC v_0 (μMs^{-1})	0.02 ± 0.01	0.04 ± 0.01	0.52 ± 0.02	0.46 ± 0.01	0.13 ± 0.03
Se AhpC v_0 (μMs^{-1})	0.01 ± 0.01	0.03 ± 0.01	0.70 ± 0.07	0.65 ± 0.02	0.60 ± 0.01

Abbreviations: PaAhpC = *P. aeruginosa* AhpC and SeAhpC = *S. epidermidis* AhpC.

The difference in the inhibitory properties of compound **C2** over the AhpCs of *P. aeruginosa* and *S. epidermidis* may rely on differences in enzyme structure. It has been shown that PaAhpC and SeAhpC present distinct properties including redox structural switches (dimer $\rightarrow$ decamer transition) and peroxidase activity over distinct hydroperoxides which were

correlated to the single amino acid substitution in the catalytic triad [22], suggesting that structural peculiarities may impact the interaction with the substrates.

Determination of IC₅₀ of compound C2 to PaAhpC

Since the compound **C2** was able to inhibit the peroxidase activity of PaAhpC, we proceeded to determine the apparent IC₅₀ of the enzyme. For this, previously reduced enzymes samples were treated with different stoichiometric ratios of compound **C2** (5, 10, 25, 50, and 100 molar equivalents). Excess was removed and the PaAhpC was evaluated by the NADPH oxidation assay (**Fig 4A**). Our results revealed that the amount of compound **C2** necessary to inhibit the PaAhpC was approximately 20 μ M (**Fig 4B**), a high value for an inhibitor but similar to the amount of the compound adenanthin (15 μ M), another natural inhibitor to the human Prx2 [29].

Evaluation of specificity over the Prx human isoform and oxidoreductases thiol proteins

Since compound **C2** did not exert a significant inhibitory effect on SeAhpC, we evaluated if the compound was able to inhibit other thiol proteins. Initially, we evaluated the inhibitory effect over Prx2 from humans (HsPrx2), the host of *P. aeruginosa*, and Trx and TrxR from bacteria, since some inhibitors described to eukaryotic Prx also inhibit the two latter thiol proteins [26, 48, 49].

We expressed and purified the HsPrx2 and used the heterologous Trx system from *S. cerevisiae* (ScTrx1 and ScTrxR1) (**Fig S7 E-G**) to perform the peroxidase activity assays by NADPH oxidation assay, as described previously by Truzzi and coworkers [35]. The need for the heterologous yeast system lies in the fact that human TrxR is a seleno protein able to

decompose hydroperoxides [50, 51], and the use of the human Trx system would make the results unreliable. The NADPH oxidation assay was performed using the same experimental conditions applied to the Prx bacterial isoforms, and the results revealed that compound **C2** was not able to inhibit the human isoform HsPrx efficiently, since only a very slight decrease in peroxidase activity was detected (**Fig 5A, Table 3**).

Table 3. Initial rates of peroxide decomposition after treatment with compound **C2** of HsPrx2, EcTrx and EcTrxR.

S ample	No enzyme (Negative control)	Enzyme + NEM (Inhibition control)	Enzyme (Positive control)	Enzyme + C2
H sPrx2 v_0 (μMs^{-1})	0.02 ± 0.01	0.02 ± 0.01	0.62 ± 0.03	0.59 ± 0.01
E cTrx v_0 (μMs^{-1})	0.01 ± 0.01	0.03 ± 0.01	0.51 ± 0.01	0.50 ± 0.02
E cTrxR v_0 (μMs^{-1})	0.01 ± 0.01	0.03 ± 0.01	0.51 ± 0.01	0.49 ± 0.01

Abbreviations: HsPrx2 = *H. sapiens* Prx2, EcTrx = *E. coli* Trx1 and EcTrxR = *E. coli* TrxR.

Concerning the thiol proteins from *E. coli*, compound **C2** was not able to significantly affect the activity of EcTrx or EcTrxR (**Fig 5B**). Only a very slight decay of Trx activity was detected and no inhibition was observed for EcTrxR. We then compared the residual amounts of NADPH after 300 seconds of reaction, and the results show that **C2** was not able to affect the enzymatic activity of HsPrx2, EcTrx, and EcTrxR (**Fig S9**). These results indicate there is

a selectivity of compound **C2** for PaAhpC when compared to other thiol proteins. This finding is important since some Prx inhibitors are able to inhibit other thiol enzymes, including different Prx isoforms and the Trx system enzymes [25, 26, 29, 30, 48, 49].

Mode of PaAhpC inhibition

To better understand the mode of inhibition, we used PaAhpC pretreated with compound **C2** (50 μM), the excess was removed and the enzyme was challenge with different concentrations of hydroperoxides (from 50 to 750 μM). Although the **C2** concentration of 50 μM is able to strongly inhibit the peroxidase activity of PaAhpC, if the mode of inhibition is a competitive behavior, the growing concentrations of peroxides may be able to shift the compound **C2** away from the active site and restore the peroxidase PaAhpC activity. Our results revealed that only a residual activity was observed (**Fig 6A**). Since the amount of inhibitor was not able to overwhelm the activity of all the enzyme population, the portion of the enzyme that had not reacted with the inhibitor would maintain its normal kinetics. The determination of kinetic parameters revealed that no significant effect was observed in the K_m . On the other hand, parameters as K_{cat} and V_{max} were expressively impacted (**Fig 6B; Table 4**). This behavior is compatible with an irreversible inhibitor as expected by a thiol-Michael addition chemistry [29, 52-54].

Table 4. Enzymatic parameters determined to cumene hydroperoxide (CHP) to PaAhpC pretreated without treatment or pre-treated with compound **C2**.

Sample	K_m (M^{-1})	K_{cat} (s^{-1})	V_{max}
PaAhpC + CHP	65.93	0.2243	0.672 9
PaAhpC/ C2 + CHP	61.58	0.0765	0.114 8

Investigation of Michael addition adducts formation

Some natural products identified as inhibitors of human Prx exert their biological activity via thiol-Michael addition reaction by alkylating the catalytic cysteines [29, 30, 33]. Compounds **C1** and **C2** tested in this work possess functional groups able to perform a Michael addition, i.e., an α,β -unsaturated carbonyl group. However, while compound **C1** showed no significant inhibitory effect, compound **C2** was able to inhibit PaAhpC, suggesting that the form of inhibition may occur by other means.

To evaluate if the inhibitory mechanism of compound **C2** to PaAhpC occurs through the thiol-Michael addition, we performed non-reducing SDS-PAGE, taking advantage of the difference in gel migration of the protein in different oxidation states. When oxidized to disulfide, AhpC enzymes migrate as a dimer (~ 44 kDa) due to intermolecular disulfide bond formation. On the other hand, the reduced enzyme appears as a monomer (~ 22 kDa) [55]. Consequently, it is possible to evaluate not only the redox state of these enzymes but also if the inhibitor was able to react with cysteine residues involved in catalysis. If one of the two cysteines is covalently modified by an inhibitor, no intermolecular disulfide is expected to be formed if the enzyme is challenge with hydroperoxides, and the proteins are detected as monomers.

Prior to peroxide treatment, PaAhpC samples were reduced by DTT (**Fig 7, lane 2**), the excess was removed (**Fig 7, lane 3**), and the PaAhpC proteins were oxidized with hydrogen peroxide (**Fig 7, lane 4**). As expected, PaAhpC reduced by DTT were detected as monomers, while the oxidized samples were detected as dimers. As internal control of the inhibition of the disulfide formation, PaAhpC sample was previously treated with NEM and then oxidized by hydrogen peroxide. The NEM treatment prevented disulfide formation and the proteins

migrated as monomers (**Fig 7, lane 5**). Regarding samples pre-treated with compound **C2** (**Fig 7, lanes 6 and 7**), the PaAhpC samples were also detected as dimers, even at very high concentration (e.g. 100 molar equivalents), revealing the formation of the intermolecular disulfide and indicating the mode of inhibition does not occur by the Michael addition mechanism over the protein thiols. This suggests that compound **C2** is able to inhibit PaAhpC in a manner distinct from those already described for other Prx inhibitors from eukaryotes [29, 30, 33] and bacteria [25, 26]. Although our data suggests that the PaAhpC cysteine is not alkylated by compound **C2**, another reactive groups as amine (-NH₂) and alcohol (-OH) can perform the Michael addition chemistry (e.g. aza- and oxa-Michael addition, respectively) [56, 57]. Both groups are present in the side chains of the PaAhpC catalytic triad residues Arg and Thr and are involved in substrate targeting and stabilization (**Fig 1**) and the modification of either group by **C2** may have a strong impact in enzyme activity.

Molecular Docking Approaches

To assess molecular interactions between compound **C2** and PaAhpC, molecular docking simulations were performed using the structure of AhpC from *S. typhimurium*, since AhpC from *P. aeruginosa* (PaAhpC) has no determined crystallographic structure. The *P. aeruginosa* and *S. typhimurium* isoforms are highly related, presenting 75% of similarity and 60% identity (**Fig 8**).

Docking approaches were performed using the decameric structure once the decamer is in the active form, whose geometry is optimal for the productive binding of the peroxide substrates. The AhpC active site environment is composed of residues of three protomers: the obligate dimer and the monomer of the neighboring dimer (**Fig 9A**).

The solutions were distributed mostly in the microenvironment of the active site in close proximity to the residues involved in catalysis (**Fig 9B**). The best pose was selected based on

the free energy of Gibbs ($\Delta G = -7.2$ kcal/mol) and superposition with ligands found in the active site of Prx crystallographic structures, such as the oxidant substrate (hydrogen peroxide in *Aeropyrum pernix* Prx) [58] and compounds with structural similarities to **C2** compound (benzoic acid found in the *Arenicola marina* Prx6 and *Homo sapiens* Prx5 and tert-butylbenzene-diol present *H. sapiens* Prx5) [46, 47, 59] (**Fig 9C**).

Stabilization of compound **C2** mostly occurred by hydrophobic interactions and van der Waals forces interactions from the main or side chains (MC or SC) of the interacting from obligate dimer: P^{39'} (SC), T^{43'}(SC), F^{44'}(MC), V^{45'} (MC) (N^{45'}, in PaAhpC), C_P^{46'} (MC), L^{116'}(SC), R^{119'}(SC), E^{138'}(SC) (N¹³⁸, in PaAhpC), P^{178''} (SC/MC), and S^{179''} (MC); and from the monomer of the adjacent dimer: F^{20*}(SC), H^{75*} (SC), and F^{76*}(SC) (the prime (') and quotation (") symbols denotes residues form the obligate dimer, and the asterisk (*) denotes the adjacent protomer dimer) (**Fig 9D**).

The functional importance of some residues identified in the docking procedures has been determined. It has already been shown that the Phe/Tyr residue at this position in peroxiredoxins is involved in the decamer stabilization of the enzyme, through an unconventional hydrogen bond with the catalytic triad Thr [22, 23, 60, 61]. Additionally, Glu¹³⁸ would be related to conformational changes during the catalytic cycle [62]. Except for the Val⁴⁵ and Glu¹³⁸ of StAhpC that are substituted by Asn in PaAhpC, a residue with similar physicochemical properties, all the remaining interacting residues are strictly conserved among the two peroxiredoxins, which corroborates the feasibility of the docking analysis.

An important aspect about the mode of inhibition, which was irreversible, may have been related to the adoption of Michael with other groups such as the O and N atoms from the side chain of the catalytic triad Thr of Arg residues. The α,β -unsaturated carbonylic system is in close proximity to both reactive group residues of these amino acids, which are also more exposed than Cys_P and involved in interactions to guide the substrates to catalytic cysteines

(Fig 1). Nonetheless, these assumptions lack experimental support and the determination of PaAhpC structures with compound **C2** is needed to reach any meaningful conclusions.

DISCUSSION

We tested two biosynthetic related natural products isolated from *P. crassinervium* as inhibitor of the AhpC from bacteria: 4-hydroxy-2-(3',7'-dimethyl-1'-oxo-2'E,6'-octadienyl) benzoic acid (**C1**), and 4-hydroxy-2-(3',7'-dimethyl-1'-oxo-2'E,6'-octadienyl) benzoic acid (**C2**). Both compounds possess three common structural aspects: 1) benzene rings, 2) an α,β unsaturated carbonylic system, and 3) a flexible tail (oxo-geranyl unity) that resembles long-chain fatty acids. The sole difference is a hydroxyl group at C-4 position in compound **C1** which is substituted by a carboxylic acid in compound **C2** (Fig 2). Therefore, it is reasonable to assume that the carboxyl group is involved in the inhibitory properties of compound **C2**. In fact, benzoic acids and derivatives have been found in some crystallized in different peroxiredoxin structures, suggesting that the carboxylic acid in the aromatic ring is a factor that may favor the stabilization of molecules in the active site [45-47].

The hydrophobic nature, including the presence of one aromatic ring, places compounds **C1** and **C2** with other chemically related molecules identified for this group of proteins [29, 30, 32, 33, 63, 64]. Although the oxo-geranyl unity has not been observed in previously described inhibitors, it resembles substrates such as long-chain fatty acids hydroperoxides (oleic and linoleic acid peroxides, for example) which are believed to be biological substrates of some thiol peroxidases including Prx [65, 66]. The docking approaches presented here reveal that the tail may be stabilized in the active site pocket by several hydrophobic interactions with non-polar residues. This feature appears to be important and can be exploited in the search for natural inhibitors for Prx. Unlike other inhibitors that also present an α,β -unsaturated carbonyl system,

neither compound **C1** and **C2** perform a thiol-Michael addition reaction over the reactive cysteines, revealing a mode of inhibition distinct from those previously characterized with other natural compounds to the human isoforms (29, 30). This is an important aspect since most of the Prx inhibitors described to date perform an inhibition by means of chemical reactions involving a covalent bond between ligand and the thiol of the cysteines but with low specificity, affecting other thiol proteins and being very toxic to the cells [25, 48, 49]. Conversely, the compound **C2** shows high specificity over PaAhpC when compared to SeAhpC, HsPrx, or even other thiol proteins as the Trx system which are strongly inhibited by other Prx inhibitors [26, 48, 49]. The specificity over PaAhpC is a very positive finding, since this enzyme is a *P. aeruginosa* virulence factor, and this pathogenic bacterium is related with nosocomial infections and high mortality rates [67, 68]. In summary, our results demonstrate the inhibitory activity of a new class of natural products that possesses distinct characteristics from other compounds identified as peroxiredoxin inhibitors. This contribution on molecules able to exert inhibitory activity on Prx of bacteria can allow the selection of natural products or even lead to the synthesis of inhibitors, aiming to increase the specificity and inhibitory activity over the Prx in order to combat infectious and genetic diseases.

Acknowledgments

The authors M.A.O., V.I.M.C. and G.N.S. are members of the CEPID REDOXOMA from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (grant number 2013/07937-8). This work was also supported by grants from FAPESP to M.A.O. (2017/19942-7), M.H.T. (2017/20291-0) and J.H.G.L (2021/02789-7). V.I.M.C. is recipient of fellowship from FAPESP (2020/02868-1). We also thank CNPq for the fellowships.

Author Contributions

Conceived and designed the experiments: Marcos Antonio de Oliveira and João Henrique Ghilardi Lago.

Experiments: Vitoria Isabela Montanhero Cabrera, Gabrielle Nascimento Sividanes and Natalia Fernanda Quintiliano.

Analyzed the data: Marcos Antonio de Oliveira, João Henrique Ghilardi Lago, Vitoria Isabela Montanhero Cabrera, Gabrielle Nascimento Sividanes and Natalia Fernanda Quintiliano.

Wrote the manuscript: Marcos Antonio de Oliveira, João Henrique Ghilardi Lago and Vitoria Isabela Montanhero Cabrera.

All authors revised, edited, and approved the final version of the manuscript.

Data Availability: All relevant data are within the manuscript and its Supporting Information files.

Competing interests: The authors have declared that no competing interests exist.

References

1. Dwyer DJ, Belenky PA, Yang JH, MacDonald IC, Martell JD, Takahashi N, et al. Antibiotics induce redox-related physiological alterations as part of their lethality. *Proc Natl Acad Sci U S A*. 2014;111(20):E2100-9. Epub 2014/05/08. doi: 10.1073/pnas.1401876111. PubMed PMID: 24803433; PubMed Central PMCID: PMC4034191.
2. Lam PL, Wong RS, Lam KH, Hung LK, Wong MM, Yung LH, et al. The role of reactive oxygen species in the biological activity of antimicrobial agents: An updated mini review. *Chem Biol Interact*. 2020;320:109023. Epub 2020/02/26. doi: 10.1016/j.cbi.2020.109023. PubMed PMID: 32097615.

3. Rocha LS, Silva BPD, Correia TML, Silva RPD, Meireles DA, Pereira R, et al. Peroxiredoxin AhpC1 protects *Pseudomonas aeruginosa* against the inflammatory oxidative burst and confers virulence. *Redox Biol.* 2021;46:102075. Epub 2021/07/28. doi: 10.1016/j.redox.2021.102075. PubMed PMID: 34315109.
4. de Oliveira MA, Tairum CA, Netto LES, de Oliveira ALP, Aleixo-Silva RL, Cabrera VIM, et al. Relevance of peroxiredoxins in pathogenic microorganisms. *Appl Microbiol Biotechnol.* 2021. Epub 2021/07/15. doi: 10.1007/s00253-021-11360-5. PubMed PMID: 34258640.
5. Baek WK, Lee HS, Oh MH, Koh MJ, Kim KS, Choi SH. Identification of the *Vibrio vulnificus* ahpC1 gene and its influence on survival under oxidative stress and virulence. *J Microbiol.* 2009;47(5):624-32. Epub 2009/10/24. doi: 10.1007/s12275-009-0130-x. PubMed PMID: 19851736.
6. Brenot A, King KY, Caparon MG. The PerR regulon in peroxide resistance and virulence of *Streptococcus pyogenes*. *Mol Microbiol.* 2005;55(1):221-34. Epub 2004/12/23. doi: 10.1111/j.1365-2958.2004.04370.x. PubMed PMID: 15612930.
7. Charoenlap N, Shen Z, McBee ME, Muthupalani S, Wogan GN, Fox JG, et al. Alkyl hydroperoxide reductase is required for *Helicobacter cinaedi* intestinal colonization and survival under oxidative stress in BALB/c and BALB/c interleukin-10^{-/-} mice. *Infect Immun.* 2012;80(3):921-8. Epub 2011/12/21. doi: 10.1128/IAI.05477-11. PubMed PMID: 22184416; PubMed Central PMCID: PMC3294669.
8. Dons LE, Mosa A, Rottenberg ME, Rosenkrantz JT, Kristensson K, Olsen JE. Role of the *Listeria monocytogenes* 2-Cys peroxiredoxin homologue in protection against oxidative and nitrosative stress and in virulence. *Pathog Dis.* 2014;70(1):70-4. Epub 2013/08/10. doi: 10.1111/2049-632X.12081. PubMed PMID: 23929591.
9. Olczak AA, Seyler RW, Jr., Olson JW, Maier RJ. Association of *Helicobacter pylori* antioxidant activities with host colonization proficiency. *Infect Immun.* 2003;71(1):580-3. Epub 2002/12/24. doi: 10.1128/iai.71.1.580-583.2003. PubMed PMID: 12496216; PubMed Central PMCID: PMC143418.
10. Wilson T, de Lisle GW, Marcinkeviciene JA, Blanchard JS, Collins DM. Antisense RNA to ahpC, an oxidative stress defence gene involved in isoniazid resistance, indicates that AhpC of *Mycobacterium bovis* has virulence properties. *Microbiology.* 1998;144 (Pt 10):2687-95. Epub 1998/11/05. doi: 10.1099/00221287-144-10-2687. PubMed PMID: 9802010.
11. Jacobson FS, Morgan RW, Christman MF, Ames BN. An alkyl hydroperoxide reductase from *Salmonella typhimurium* involved in the defense of DNA against oxidative

damage. Purification and properties. J Biol Chem. 1989;264(3):1488-96. Epub 1989/01/25. PubMed PMID: 2643600.

12. Parsonage D, Karplus PA, Poole LB. Substrate specificity and redox potential of AhpC, a bacterial peroxiredoxin. Proc Natl Acad Sci U S A. 2008;105(24):8209-14. Epub 2008/01/01. doi: 10.1073/pnas.0708308105. PubMed PMID: 18165315; PubMed Central PMCID: PMCPMC2448816.

13. Parsonage D, Nelson KJ, Ferrer-Sueta G, Alley S, Karplus PA, Furdul CM, et al. Dissecting peroxiredoxin catalysis: separating binding, peroxidation, and resolution for a bacterial AhpC. Biochemistry. 2015;54(7):1567-75. Epub 2015/01/31. doi: 10.1021/bi501515w. PubMed PMID: 25633283; PubMed Central PMCID: PMCPMC4489686.

14. Zhang B, Gu H, Yang Y, Bai H, Zhao C, Si M, et al. Molecular Mechanisms of AhpC in Resistance to Oxidative Stress in *Burkholderia thailandensis*. Front Microbiol. 2019;10:1483. Epub 2019/07/25. doi: 10.3389/fmicb.2019.01483. PubMed PMID: 31338075; PubMed Central PMCID: PMCPMC6626918.

15. Hall A, Parsonage D, Poole LB, Karplus PA. Structural evidence that peroxiredoxin catalytic power is based on transition-state stabilization. J Mol Biol. 2010;402(1):194-209. Epub 2010/07/21. doi: 10.1016/j.jmb.2010.07.022. PubMed PMID: 20643143; PubMed Central PMCID: PMCPMC2941395.

16. Ferrer-Sueta G, Manta B, Botti H, Radi R, Trujillo M, Denicola A. Factors affecting protein thiol reactivity and specificity in peroxide reduction. Chem Res Toxicol. 2011;24(4):434-50. Epub 2011/03/12. doi: 10.1021/tx100413v. PubMed PMID: 21391663.

17. Nagy P, Karton A, Betz A, Peskin AV, Pace P, O'Reilly RJ, et al. Model for the exceptional reactivity of peroxiredoxins 2 and 3 with hydrogen peroxide: a kinetic and computational study. J Biol Chem. 2011;286(20):18048-55. Epub 2011/03/10. doi: 10.1074/jbc.M111.232355. PubMed PMID: 21385867; PubMed Central PMCID: PMCPMC3093878.

18. Zeida A, Reyes AM, Lebrero MC, Radi R, Trujillo M, Estrin DA. The extraordinary catalytic ability of peroxiredoxins: a combined experimental and QM/MM study on the fast thiol oxidation step. Chem Commun (Camb). 2014;50(70):10070-3. Epub 2014/07/22. doi: 10.1039/c4cc02899f. PubMed PMID: 25045760; PubMed Central PMCID: PMCPMC4336542.

19. Hall A, Nelson K, Poole LB, Karplus PA. Structure-based insights into the catalytic power and conformational dexterity of peroxiredoxins. Antioxid Redox Signal.

2011;15(3):795-815. Epub 2010/10/26. doi: 10.1089/ars.2010.3624. PubMed PMID: 20969484; PubMed Central PMCID: PMC3125576.

20. Wood ZA, Poole LB, Hantgan RR, Karplus PA. Dimers to doughnuts: redox-sensitive oligomerization of 2-cysteine peroxiredoxins. *Biochemistry*. 2002;41(17):5493-504. Epub 2002/04/24. doi: 10.1021/bi012173m. PubMed PMID: 11969410.

21. Wood ZA, Poole LB, Karplus PA. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science*. 2003;300(5619):650-3. Epub 2003/04/26. doi: 10.1126/science.1080405. PubMed PMID: 12714747.

22. Tairum CA, Santos MC, Breyer CA, de Oliveira ALP, Cabrera VIM, Toledo-Silva G, et al. Effects of Serine or Threonine in the Active Site of Typical 2-Cys Prx on Hyperoxidation Susceptibility and on Chaperone Activity. *Antioxidants (Basel)*. 2021;10(7). Epub 2021/07/03. doi: 10.3390/antiox10071032. PubMed PMID: 34202406.

23. Tairum CA, Santos MC, Breyer CA, Geyer RR, Nieves CJ, Portillo-Ledesma S, et al. Catalytic Thr or Ser Residue Modulates Structural Switches in 2-Cys Peroxiredoxin by Distinct Mechanisms. *Sci Rep*. 2016;6:33133. Epub 2016/09/16. doi: 10.1038/srep33133. PubMed PMID: 27629822; PubMed Central PMCID: PMC5024103.

24. Tairum CA, Santos MC, Breyer CA, Oliveira AL, Cabrera VIM, Toledo-Silva G, et al. Effects of Serine or Threonine in the Active Site of Typical 2-Cys Prx on Hyperoxidation Susceptibility and on Chaperone Activity. *Antioxidants (Basel)*. 2021;In press.

25. Bottcher T, Sieber SA. Showdomycin as a versatile chemical tool for the detection of pathogenesis-associated enzymes in bacteria. *J Am Chem Soc*. 2010;132(20):6964-72. Epub 2010/05/04. doi: 10.1021/ja909150y. PubMed PMID: 20433172.

26. dos Santos MC, Tairum CA, Cabrera VIM, Guimaraes Cauz AC, Ribeiro LF, Toledo Junior JC, et al. Adenanthin Is an Efficient Inhibitor of Peroxiredoxins from Pathogens, Inhibits Bacterial Growth, and Potentiates Antibiotic Activities. *Chem Res Toxicol*. 2022. Epub 2022/05/11. doi: 10.1021/acs.chemrestox.2c00049. PubMed PMID: 35537067.

27. Shichita T, Hasegawa E, Kimura A, Morita R, Sakaguchi R, Takada I, et al. Peroxiredoxin family proteins are key initiators of post-ischemic inflammation in the brain. *Nat Med*. 2012;18(6):911-7. Epub 2012/05/23. doi: 10.1038/nm.2749. PubMed PMID: 22610280.

28. Lin Y, Wang J, Zheng W, Luo Q, Wu K, Du J, et al. Organometallic ruthenium anticancer complexes inhibit human peroxiredoxin I activity by binding to and inducing oxidation of its catalytic cysteine residue. *Metallomics*. 2019;11(3):546-55. Epub 2019/01/30. doi: 10.1039/c8mt00352a. PubMed PMID: 30693924.

29. Liu CX, Yin QQ, Zhou HC, Wu YL, Pu JX, Xia L, et al. Adenanthin targets peroxiredoxin I and II to induce differentiation of leukemic cells. *Nat Chem Biol.* 2012;8(5):486-93. Epub 2012/04/10. doi: 10.1038/nchembio.935. PubMed PMID: 22484541.
30. Shi XJ, Ding L, Zhou W, Ji Y, Wang J, Wang H, et al. Pro-Apoptotic Effects of JDA-202, a Novel Natural Diterpenoid, on Esophageal Cancer Through Targeting Peroxiredoxin I. *Antioxid Redox Signal.* 2017;27(2):73-92. Epub 2016/11/02. doi: 10.1089/ars.2016.6703. PubMed PMID: 27650197; PubMed Central PMCID: PMC5510680.
31. Wei W, Ma C, Cao Y, Yang L, Huang Z, Qin D, et al. Identification of H7 as a novel peroxiredoxin I inhibitor to induce differentiation of leukemia cells. *Oncotarget.* 2016;7(4):3873-83. Epub 2015/12/31. doi: 10.18632/oncotarget.6763. PubMed PMID: 26716647; PubMed Central PMCID: PMC54826176.
32. Ye Q, Zhang Y, Cao Y, Wang X, Guo Y, Chen J, et al. Frenolicin B Targets Peroxiredoxin 1 and Glutaredoxin 3 to Trigger ROS/4E-BP1-Mediated Antitumor Effects. *Cell Chem Biol.* 2019;26(3):366-77 e12. Epub 2019/01/22. doi: 10.1016/j.chembiol.2018.11.013. PubMed PMID: 30661989; PubMed Central PMCID: PMC6557261.
33. Zhu H, Wang B, Kong L, An T, Li G, Zhou H, et al. Parvifoline AA Promotes Susceptibility of Hepatocarcinoma to Natural Killer Cell-Mediated Cytolysis by Targeting Peroxiredoxin. *Cell Chem Biol.* 2019;26(8):1122-32 e6. Epub 2019/05/28. doi: 10.1016/j.chembiol.2019.04.003. PubMed PMID: 31130519.
34. Liu W, Guo W, Zhu Y, Peng S, Zheng W, Zhang C, et al. Targeting Peroxiredoxin 1 by a Curcumin Analogue, AI-44, Inhibits NLRP3 Inflammasome Activation and Attenuates Lipopolysaccharide-Induced Sepsis in Mice. *J Immunol.* 2018;201(8):2403-13. Epub 2018/09/07. doi: 10.4049/jimmunol.1700796. PubMed PMID: 30185517.
35. Truzzi DR, Coelho FR, Paviani V, Alves SV, Netto LES, Augusto O. The bicarbonate/carbon dioxide pair increases hydrogen peroxide-mediated hyperoxidation of human peroxiredoxin 1. *J Biol Chem.* 2019;294(38):14055-67. Epub 2019/08/02. doi: 10.1074/jbc.RA119.008825. PubMed PMID: 31366734; PubMed Central PMCID: PMC6755790.
36. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol.* 2011;7:539. Epub 2011/10/13. doi: 10.1038/msb.2011.75. PubMed PMID: 21988835; PubMed Central PMCID: PMC3261699.

37. Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ. Jalview Version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics*. 2009;25(9):1189-91. Epub 2009/01/20. doi: 10.1093/bioinformatics/btp033. PubMed PMID: 19151095; PubMed Central PMCID: PMC2672624.
38. Smith TJ. MolView: a program for analyzing and displaying atomic structures on the Macintosh personal computer. *J Mol Graph*. 1995;13(2):122-5, 15. Epub 1995/04/01. doi: 10.1016/0263-7855(94)00019-o. PubMed PMID: 7619787.
39. Eberhardt J, Santos-Martins D, Tillack AF, Forli S. AutoDock Vina 1.2.0: New Docking Methods, Expanded Force Field, and Python Bindings. *J Chem Inf Model*. 2021. Epub 2021/07/20. doi: 10.1021/acs.jcim.1c00203. PubMed PMID: 34278794.
40. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem*. 2010;31(2):455-61. Epub 2009/06/06. doi: 10.1002/jcc.21334. PubMed PMID: 19499576; PubMed Central PMCID: PMC3041641.
41. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem*. 2004;25(13):1605-12. Epub 2004/07/21. doi: 10.1002/jcc.20084. PubMed PMID: 15264254.
42. Danelutte AP, Lago JH, Young MC, Kato MJ. Antifungal flavanones and prenylated hydroquinones from *Piper crassinervium* Kunth. *Phytochemistry*. 2003;64(2):555-9. Epub 2003/08/29. doi: 10.1016/s0031-9422(03)00299-1. PubMed PMID: 12943774.
43. Sakamoto S, Kuroyanagi M, Ueno A, Sekita S. Triterpenoid saponins from *Sophora subprostrata*. *Phytochemistry*. 1992;31(4):1339-42. Epub 1992/04/01. doi: 10.1016/0031-9422(92)80286-n. PubMed PMID: 1368049.
44. Stacey MM, Peskin AV, Vissers MC, Winterbourn CC. Chloramines and hypochlorous acid oxidize erythrocyte peroxiredoxin 2. *Free Radic Biol Med*. 2009;47(10):1468-76. Epub 2009/09/01. doi: 10.1016/j.freeradbiomed.2009.08.022. PubMed PMID: 19716412.
45. Aguirre C, ten Brink T, Guichou JF, Cala O, Krimm I. Comparing binding modes of analogous fragments using NMR in fragment-based drug design: application to PRDX5. *PLoS One*. 2014;9(7):e102300. Epub 2014/07/16. doi: 10.1371/journal.pone.0102300. PubMed PMID: 25025339; PubMed Central PMCID: PMC4099364.
46. Declercq JP, Evrard C, Clippe A, Stricht DV, Bernard A, Knoop B. Crystal structure of human peroxiredoxin 5, a novel type of mammalian peroxiredoxin at 1.5 Å

resolution. J Mol Biol. 2001;311(4):751-9. Epub 2001/08/24. doi: 10.1006/jmbi.2001.4853. PubMed PMID: 11518528.

47. Smeets A, Loumaye E, Clippe A, Rees JF, Knoops B, Declercq JP. The crystal structure of the C45S mutant of annelid *Arenicola marina* peroxiredoxin 6 supports its assignment to the mechanistically typical 2-Cys subfamily without any formation of toroid-shaped decamers. Protein Sci. 2008;17(4):700-10. Epub 2008/03/25. doi: 10.1110/ps.073399308. PubMed PMID: 18359859; PubMed Central PMCID: PMC2271159.

48. Muchowicz A, Firczuk M, Chlebowska J, Nowis D, Stachura J, Barankiewicz J, et al. Adenanthin targets proteins involved in the regulation of disulphide bonds. Biochem Pharmacol. 2014;89(2):210-6. Epub 2014/03/19. doi: 10.1016/j.bcp.2014.02.022. PubMed PMID: 24630929.

49. Soethoudt M, Peskin AV, Dickerhof N, Paton LN, Pace PE, Winterbourn CC. Interaction of adenanthin with glutathione and thiol enzymes: selectivity for thioredoxin reductase and inhibition of peroxiredoxin recycling. Free Radic Biol Med. 2014;77:331-9. Epub 2014/10/08. doi: 10.1016/j.freeradbiomed.2014.09.025. PubMed PMID: 25289458.

50. Bjornstedt M, Hamberg M, Kumar S, Xue J, Holmgren A. Human thioredoxin reductase directly reduces lipid hydroperoxides by NADPH and selenocystine strongly stimulates the reaction via catalytically generated selenols. J Biol Chem. 1995;270(20):11761-4. Epub 1995/05/19. doi: 10.1074/jbc.270.20.11761. PubMed PMID: 7744824.

51. Queiroz RF, Stanley CP, Wolhuter K, Kong SMY, Rajivan R, McKinnon N, et al. Hydrogen peroxide signaling via its transformation to a stereospecific alkyl hydroperoxide that escapes reductive inactivation. Nat Commun. 2021;12(1):6626. Epub 2021/11/18. doi: 10.1038/s41467-021-26991-5. PubMed PMID: 34785665; PubMed Central PMCID: PMC8595612.

52. Garuti L, Roberti M, Bottegoni G. Irreversible protein kinase inhibitors. Curr Med Chem. 2011;18(20):2981-94. Epub 2011/06/10. doi: 10.2174/092986711796391705. PubMed PMID: 21651479.

53. Leung-Toung R, Zhao Y, Li W, Tam TF, Karimian K, Spino M. Thiol proteases: inhibitors and potential therapeutic targets. Curr Med Chem. 2006;13(5):547-81. Epub 2006/03/07. doi: 10.2174/092986706776055733. PubMed PMID: 16515521.

54. Liu S, Hanzlik RP. Structure-activity relationships for inhibition of papain by peptide Michael acceptors. J Med Chem. 1992;35(6):1067-75. Epub 1992/03/20. doi: 10.1021/jm00084a012. PubMed PMID: 1552501.

55. Tairum CA, Jr., de Oliveira MA, Horta BB, Zara FJ, Netto LE. Disulfide biochemistry in 2-cys peroxiredoxin: requirement of Glu50 and Arg146 for the reduction of yeast Tsa1 by thioredoxin. *J Mol Biol.* 2012;424(1-2):28-41. Epub 2012/09/19. doi: 10.1016/j.jmb.2012.09.008. PubMed PMID: 22985967.
56. Chu HW, Sethy B, Hsieh PW, Horng JT. Identification of Potential Drug Targets of Broad-Spectrum Inhibitors with a Michael Acceptor Moiety Using Shotgun Proteomics. *Viruses.* 2021;13(9). Epub 2021/09/29. doi: 10.3390/v13091756. PubMed PMID: 34578337; PubMed Central PMCID: PMCPMC8473112.
57. Nising CF, Brase S. The oxa-Michael reaction: from recent developments to applications in natural product synthesis. *Chem Soc Rev.* 2008;37(6):1218-28. Epub 2008/05/24. doi: 10.1039/b718357g. PubMed PMID: 18497934.
58. Nakamura T, Kado Y, Yamaguchi T, Matsumura H, Ishikawa K, Inoue T. Crystal structure of peroxiredoxin from *Aeropyrum pernix* K1 complexed with its substrate, hydrogen peroxide. *J Biochem.* 2010;147(1):109-15. Epub 2009/10/13. doi: 10.1093/jb/mvp154. PubMed PMID: 19819903.
59. Evrard C, Capron A, Marchand C, Clippe A, Wattiez R, Soumillon P, et al. Crystal structure of a dimeric oxidized form of human peroxiredoxin 5. *J Mol Biol.* 2004;337(5):1079-90. Epub 2004/03/30. doi: 10.1016/j.jmb.2004.02.017. PubMed PMID: 15046979.
60. Liebthal M, Kushwah MS, Kukura P, Dietz K-J. Single Molecule Mass Photometry Reveals Dynamic Oligomerization of Plant and Human Peroxiredoxins for Functional Conservation and Diversification. *bioRxiv.* 2021.
61. Loberg MA, Hurtig JE, Graff AH, Allan KM, Buchan JA, Spencer MK, et al. Aromatic Residues at the Dimer-Dimer Interface in the Peroxiredoxin Tsa1 Facilitate Decamer Formation and Biological Function. *Chem Res Toxicol.* 2019;32(3):474-83. Epub 2019/02/01. doi: 10.1021/acs.chemrestox.8b00346. PubMed PMID: 30701970; PubMed Central PMCID: PMCPMC7036284.
62. Perkins A, Nelson KJ, Williams JR, Parsonage D, Poole LB, Karplus PA. The sensitive balance between the fully folded and locally unfolded conformations of a model peroxiredoxin. *Biochemistry.* 2013;52(48):8708-21. Epub 2013/11/02. doi: 10.1021/bi4011573. PubMed PMID: 24175952; PubMed Central PMCID: PMCPMC3932808.
63. Brindisi M, Brogi S, Relitti N, Vallone A, Butini S, Gemma S, et al. Structure-based discovery of the first non-covalent inhibitors of *Leishmania major* trypanothione peroxidase by high throughput docking. *Sci Rep.* 2015;5:9705. Epub 2015/05/08. doi: 10.1038/srep09705. PubMed PMID: 25951439; PubMed Central PMCID: PMCPMC4423475.

64. Haraldsen JD, Liu G, Botting CH, Walton JG, Storm J, Phalen TJ, et al. Identification of Conoidin a as a Covalent Inhibitor of Peroxiredoxin II. *Org Biomol Chem*. 2009;7:3040-8. Epub 2009/01/01. doi: 10.1039/B901735F. PubMed PMID: 21359112; PubMed Central PMCID: PMCPMC3043594.
65. Alegria TG, Meireles DA, Cussiol JR, Hugo M, Trujillo M, de Oliveira MA, et al. Ohr plays a central role in bacterial responses against fatty acid hydroperoxides and peroxynitrite. *Proc Natl Acad Sci U S A*. 2017;114(2):E132-E41. Epub 2016/12/29. doi: 10.1073/pnas.1619659114. PubMed PMID: 28028230; PubMed Central PMCID: PMCPMC5240686.
66. Reyes AM, Hugo M, Trostchansky A, Capece L, Radi R, Trujillo M. Oxidizing substrate specificity of *Mycobacterium tuberculosis* alkyl hydroperoxide reductase E: kinetics and mechanisms of oxidation and overoxidation. *Free Radic Biol Med*. 2011;51(2):464-73. Epub 2011/05/17. doi: 10.1016/j.freeradbiomed.2011.04.023. PubMed PMID: 21571062.
67. Berube BJ, Rangel SM, Hauser AR. *Pseudomonas aeruginosa*: breaking down barriers. *Curr Genet*. 2016;62(1):109-13. Epub 2015/09/27. doi: 10.1007/s00294-015-0522-x. PubMed PMID: 26407972; PubMed Central PMCID: PMCPMC4724561.
68. Reynolds D, Kollef M. The Epidemiology and Pathogenesis and Treatment of *Pseudomonas aeruginosa* Infections: An Update. *Drugs*. 2021;81(18):2117-31. Epub 2021/11/08. doi: 10.1007/s40265-021-01635-6. PubMed PMID: 34743315; PubMed Central PMCID: PMCPMC8572145.

Figure legends

Fig 1. Molecular interactions and peroxide decomposition reactions of 2-Cys Prx. The thiolate nucleophilicity of peroxidatic cysteine (Cys_P) is increased by hydrogen bonds with the catalytic triad Arg and Thr residues **(i)**. The hydroperoxide (Hpx) is trapped by the Arg hydrogen bond which is able to target the Hpx to the Prx active site **(ii)**. The shift of the Arg and Thr hydrogen bonds from the Cys_P thiolate to the substrate stabilizes the Hpx and increase the thiolate reactivity enabling the S_N2 mechanism **(iii)**. After the catalytic reaction, the Cys_P is oxidized to cysteine sulfenic acid (Cys_P-SOH) and the release of the leaving group (R-OH, meaning a water molecule in the case of hydrogen peroxide, or the alcohol derivative, in the case of organic hydroperoxides) is assisted by polar interaction with Arg **(iv)**. The cysteine sulfenic acid formation triggers structural changes which allow the condensation among Cys_P-SOH with Cys_{SR}-SH **(v)** resulting in the disulfide formation and concomitant release of a water molecule **(vi)**. The asterisk (*) in Cys_{SR}, denotes the adjacent subunit of the homodimer.

Fig 2. Chemical structures of compounds **C1** and **C2**, isolated from branches of *P. crassinervium*. The α,β -unsaturated carbonyl system are represented in red.

Fig 3. NADPH oxidation assay of PaAhpC and SeAhpC after treatment with compounds **C1** and **C2**. To perform the assays PaAhpC and SeAhpC samples were previously reduced with DTT (5 mM/1h). The DTT excess was removed, and the enzymes were treated with 40 molar equivalents of **C1** (**A** and **C**, ■) or **C2** (**B** and **D**, ■) for 1hour at room temperature. The compounds excess was removed, and the peroxidase activity was accessed by the NADPH oxidation in reactions containing: 3 μ M of PaAhpC or SeAhpC, 6 μ M EcTrx, 0.9 μ M EcTrxR and 150 μ M NADPH in buffer 50 mM HEPES (pH = 7.4), 100 μ M DTPA and 1 mM sodium

azide. Reactions were incubated at 37°C for 5 minutes before being initiated by the addition of H₂O₂ (500 µM) and monitored spectrophotometrically at 340 nm, 37°C, for 5 minutes. Reactions containing PaAhpC or SeAhpC without natural compounds treatment (■) were used as positive controls for peroxidase activity, and reactions without AhpC (■) were used as negative controls. PaAhpC or SeAhpC samples previously treated with NEM were used as control of protein inhibition (■). All experiments were performed at least three times in triplicate.

Fig 4. IC₅₀ determination of compound **C2** for PaAhpC. **(A)** Aliquots of PaAhpC enzyme were reduced with 5 mM DTT for 1 hour and then desalted to remove excess of reductant. Aliquots containing 10 µM of the reduced and desalted enzymes were treated varying the concentration of compound **C2** (5, 10, 25, 50 and 100 µM for 1 hour at room temperature and subsequently desalted again. Next, 3µM of the treated proteins were added to reactions containing 6 µM EcTrx, 0.9 µM EcTrxR, 150 µM NADPH, 50 mM HEPES (pH 7.4), 100 µM DTPA and 1 mM sodium azide. The reactions were incubated at 37°C for 5 minutes before being initiated by the addition of 500 µM H₂O₂ and monitored spectrophotometrically at 340 nm, 37°C, for 5 minutes. Reactions without treatment with compound **C2** were used as positive controls (■), and reactions without the addition of AhpC were used as negative controls (■). **(B)** The initial rates (v_0) of each reaction were calculated, transformed into percentual values, and plotted to calculate the IC₅₀ values of compound **C2** for PaAhpC. All experiments were performed at least three times in triplicate.

Fig 5. Evaluation of inhibition properties of compound **C2** over HsPrx2 and the bacterial thiol proteins EcTrx and EcTrxR. The enzymes HsPrx2, EcTrx and EcTrxR were reduced with DTT and desalted, then treated with 50 molar equivalents of compound **C2** for one hour at RT

(■). The proteins were desalted again to remove excess compound and their peroxidase activity was analyzed by NADPH oxidation assay. (A) For Prx2, reactions were performed containing 5 μ M of Prx2, 10 μ M of ScTrx1, 0.3 μ M of ScTrxR1, 150 μ M of NADPH, 50 mM of HEPES (pH = 7.0), 100 μ M of DTPA and 1 mM of sodium were used. For EcTrx (B) and EcTrxR (C) reactions consisted of 3 μ M of PaAhpC, 6 μ M of EcTrx, 0.9 μ M of EcTrxR, 150 μ M of NADPH, 50 mM of HEPES (pH = 7.4), 100 μ M of DTPA and 1 mM sodium azide were used. Prior to experiments the reactions were incubated at 37°C/5 min and reactions were started by the addition of H₂O₂ (500 μ M) and monitored spectrophotometrically at 340 nm, 37°C, for 5 minutes. The positive controls contained the proteins HsPrx2, EcTrx or EcTrxR without prior treatment with compound C2 (■) and the negative control without the addition of PaAhpC (EcTrx or EcTrxR) (■). The enzymes samples previously treated with NEM were used as control of protein inhibition (■). The experiments were performed three times using triplicates.

Fig 6. Inhibition characteristics of compound C2 over PaAhpC with growing concentrations of organic hydroperoxide. (A) NADPH oxidation assay of PaAhpC samples previously reduced with DTT and desalted, then treated with 20 μ M of compound C2 for one hour at RT previously treated. The reactions contained with 3 μ M of PaAhpC, 6 μ M of EcTrx, 0.9 μ M of EcTrxR, 150 μ M of NADPH, 50 mM of HEPES (pH = 7.4), 100 μ M of DTPA and 1 mM sodium azide were used. Prior to experiments the reactions were incubated at 37°C/5 min and reactions were started by the addition of growing concentration of cumene hydroperoxide - CHP (50 - 750 μ M) and monitored spectrophotometrically at 340 nm, 37°C, for 5 minutes. As negative control was used reactions without the enzyme (■) and as positive control were used PaAhpC samples without prior treatment with compound C2 (■). The experiments were performed three times using triplicates. (B) Plot of the rates of NADPH

oxidation by samples of pretreated with compound **C2** (PaAhpC/**C2**; red dots and line) or without inhibitor treatment (PaAhpC/**C2**; black dots and line).

Fig 7. Evaluation of AhpC intermolecular disulfide formation by non-reducing SDS-PAGE. The molecular weight marker (Sigma Marker) was applied in the lane 1. Samples with 10 μ M of PaAhpC were reduced with DTT 5 mM/1 h/RT (lanes 2), the excess was removed (lanes 3) and proteins were treated with 2 molar equivalents of H_2O_2 /30 min/RT (lanes 4). As a positive control of the disulfide formation inhibition, PaAhpC samples previously reduced by DTT and desalted were treated with NEM (40 molar equivalents/1 h/RT) and then oxidized with hydrogen peroxide (30 eq.) (lanes 5). The DTT reduced and desalted samples were treated 40 and 100 molar equivalents of compound **C2** and oxidized with hydrogen peroxide (30 eq.) (lanes 6 and 7). The experiments were performed three times presenting similar results.

Fig 8. Alignment of the amino acid sequences of AhpCs from *S. typhimurium* and *P. aeruginosa*. The alignment of AA sequences was performed using Clustal Ω and the figure was generated with the Jalview. The abbreviations used and access codes for Uniprot (www.uniprot.org) are: AhpC of *S. typhimurium* = StAhpC (Uniprot: P0A251) and AhpC of *P. aeruginosa* = PaAhpC (Uniprot: Q02UU0).

Fig 9. Decameric structure of AhpC and docking results and best pose selection based in peroxiredoxin ligands found in pdb database. (A) *S. typhimurium* AhpC (PDB = 4MA9) in reduced decameric state. The enzyme is composed by five obligate homodimers and the two upper homodimers are represented by molecular surface and colored in purple/beige and green/pink. The other dimers of the decamer are represented in cartoon and colored in light gray. The black box denotes the active site microenvironment located at the dimers interface.

The catalytic triad residues Thr, Cys_P and Arg are colored in red, orange and blue, respectively.

(B) Molecular docking results for compound **C2**. The molecules cluster of the compound **C2** are represented by lines and colored in pink. **(C)** Superposition of the compound **C2** best hit to StAhpC with ligands found in Prx structures deposited in the pdb database: H₂O₂ (PDB code = 3A2V; color = red), benzoate (2V32; 1HD2; 1OC3; 2V41; 1H4O; yellow) and tert-butylbenzene-diol (4K7O; white). The **C2** is colored in pink with the atoms of α,β -unsaturated carbonyl system highlighted in white. The other molecules are represented in lines with different colors. The oxygens are in red. The catalytic triad (Thr, Cys_P and Arg) is represented by sticks. **(D)** Diagram of interacting residues of AhpC with compound **C2** docked in the StAhpC active site. The dashed green lines represent hydrogen bonds and hydrophobic contacts are represented by red arcs with radiating spikes. The binding analysis was performed using the LigPlot⁺.

Figure 1

[Click here to access/download;Figure;Fig_1.tiff](#)

Canada colada

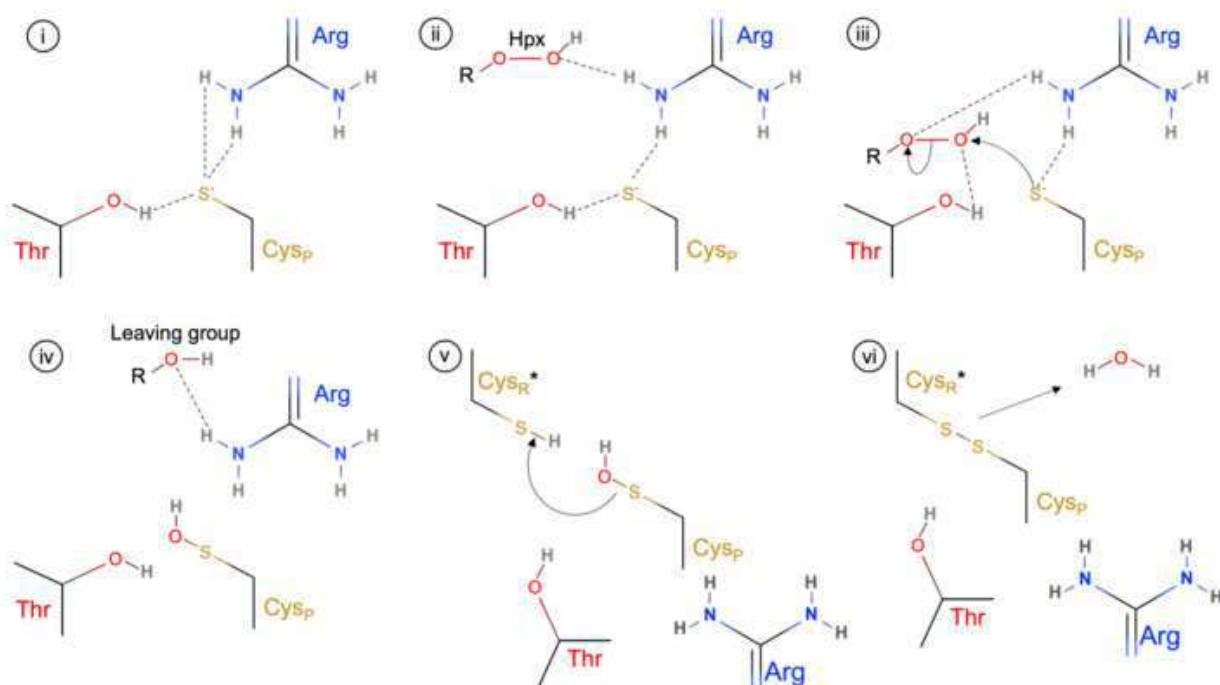


Figure 2

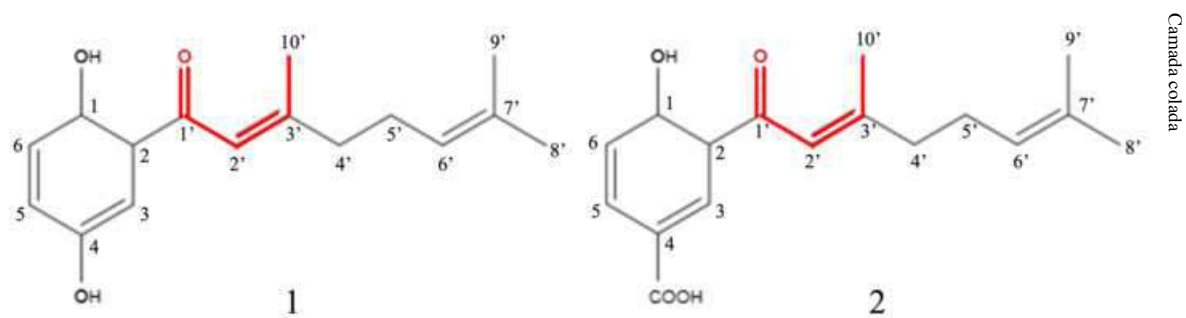
[Click here to access/download;Figure;Fig_2.tiff](#)

Figure 3

[Click here to access/download;Figure;Fig_3.tiff](#)

Canada colada

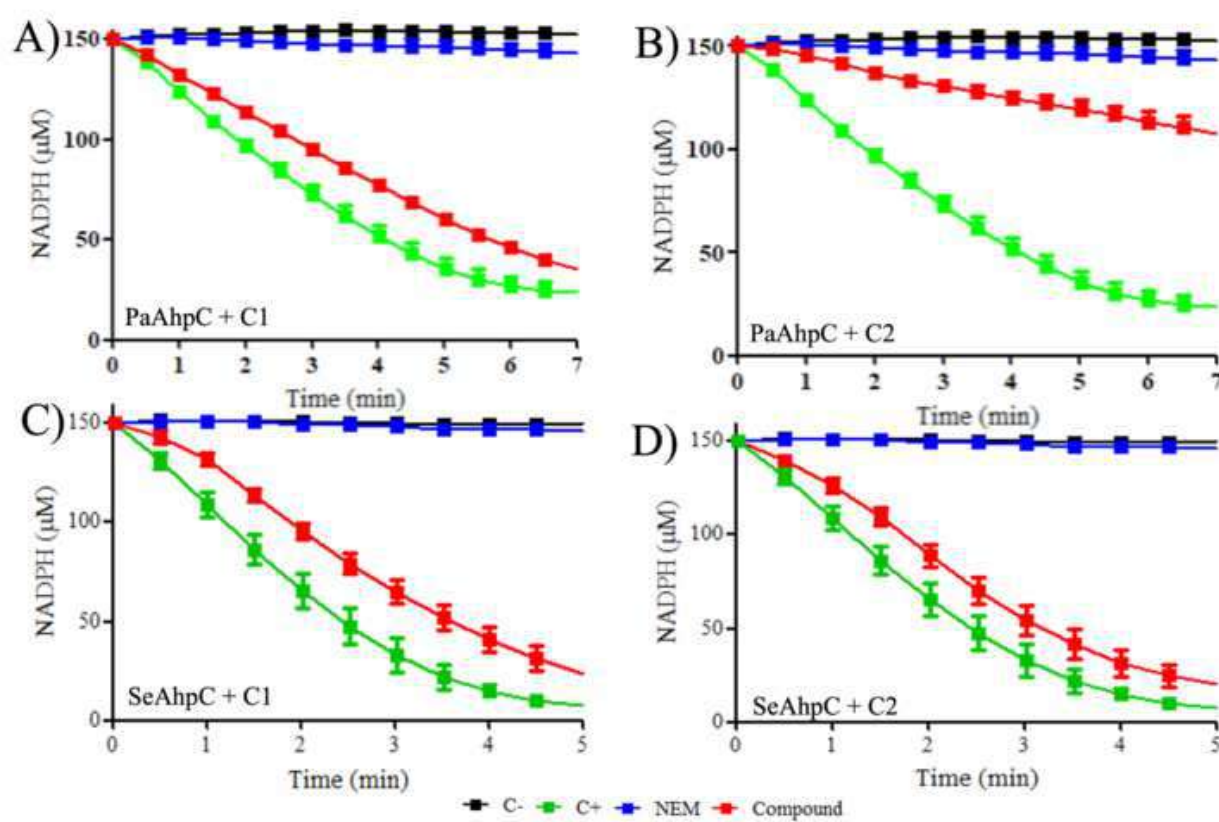
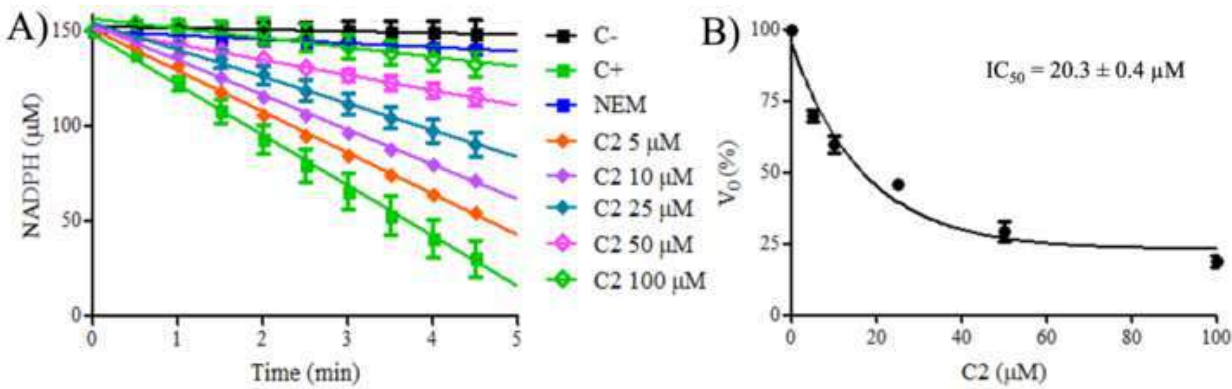


Figure 4 [Click here to access/download;Figure;Fig_4.tiff](#)



Camada colada

Figure 5

Camada colada

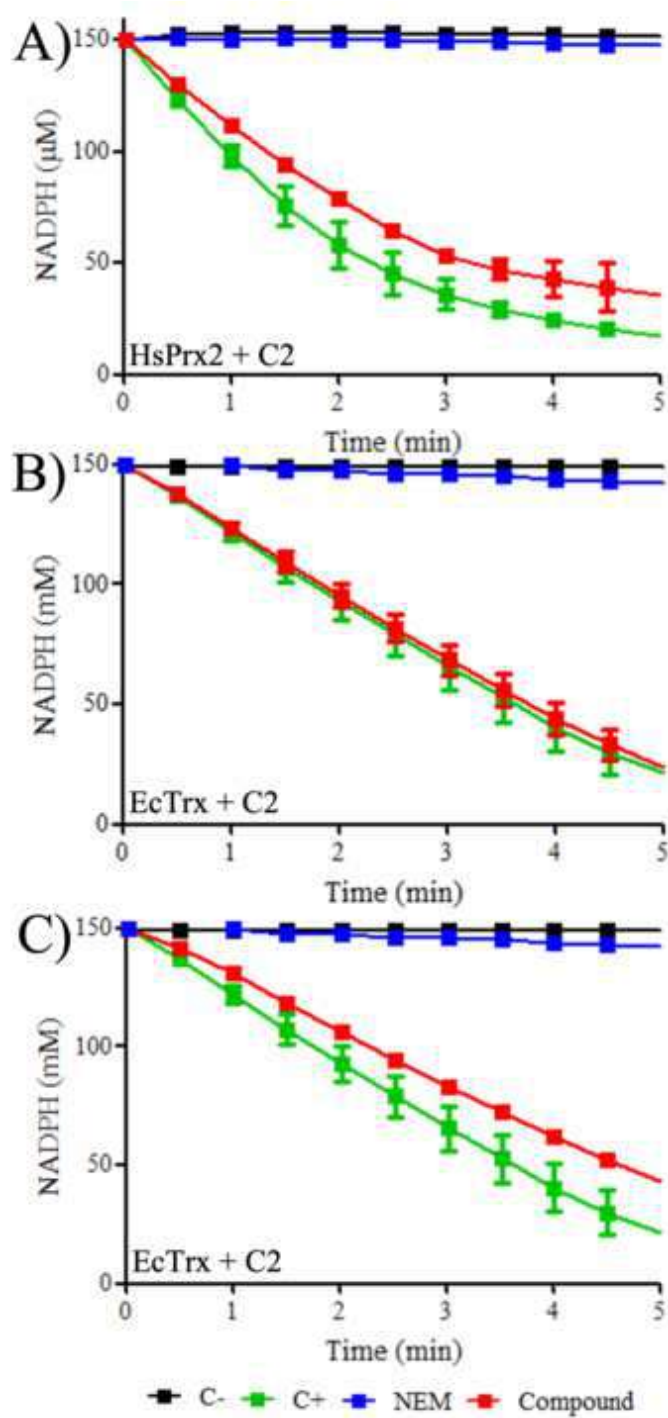
[Click here to access/download;Figure;Fig_5.tiff](#)

Figure 6

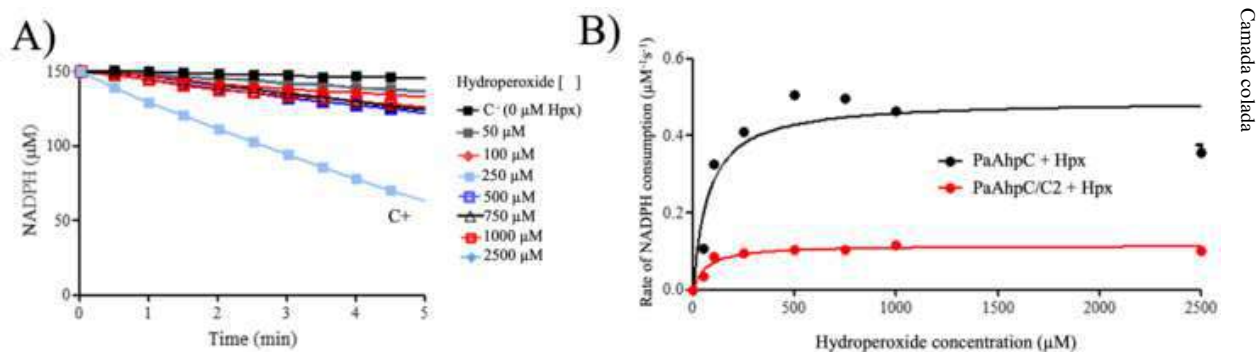
[Click here to access/download;Figure;Fig_6.tiff](#)


Figure 7

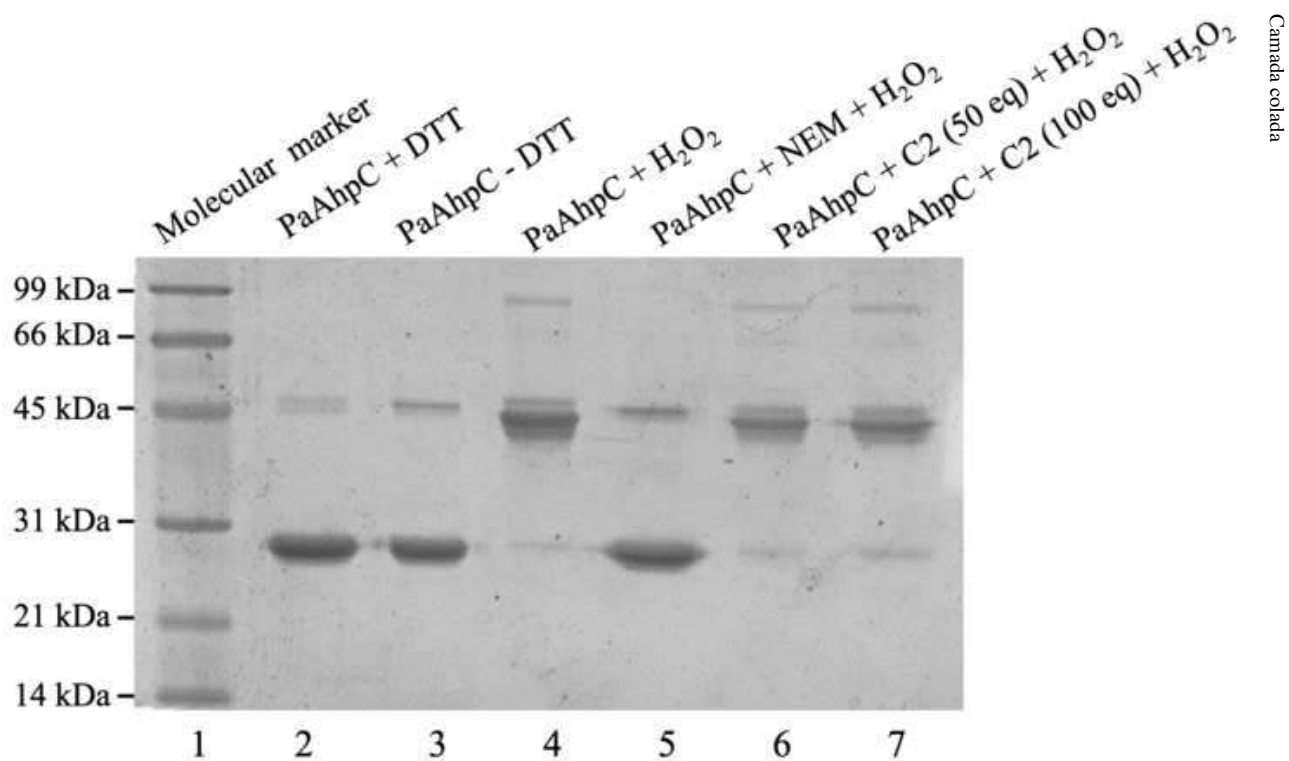
[Click here to access/download;Figure;Fig_7.tiff](#)

Figure 8

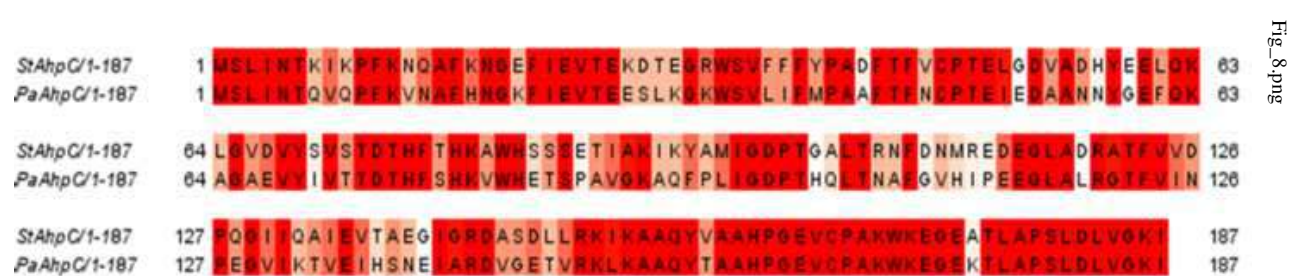
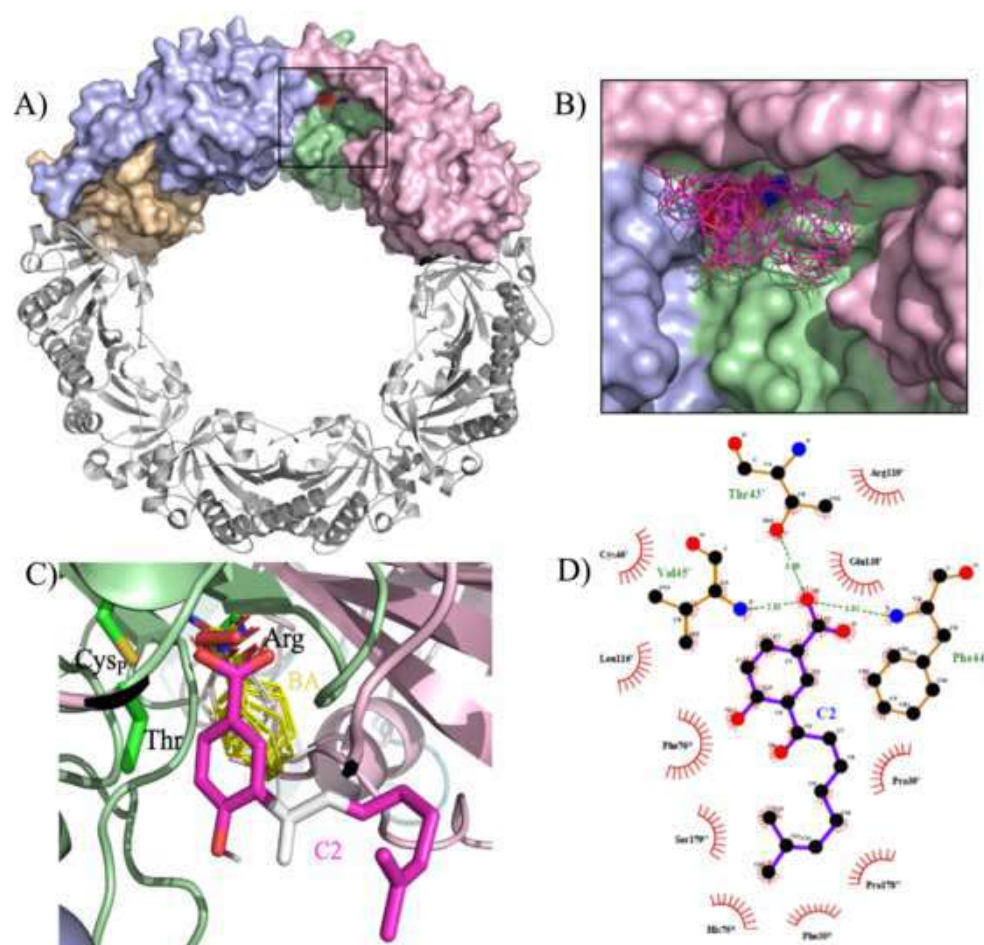
[Click here to access/download;Figure;Fig_8.tiff](#)

Figure 9

[Click here to access/download;Figure;Fig_9.tiff](#)


Canada colada

APÊNDICE 2



Article

Effects of Serine or Threonine in the Active Site of Typical 2-Cys Prx on Hyperoxidation Susceptibility and on Chaperone Activity

Carlos A. Tairum ^{1,2,†}, Melina Cardoso Santos ^{1,†}, Carlos Alexandre Breyer ^{1,†}, Ana Laura Pires de Oliveira ¹, Vitoria Isabela Montanhero Cabrera ¹, Guilherme Toledo-Silva ³, Gustavo Maruyama Mori ⁴, Marcos Hikari Toyama ¹, Luis Eduardo Soares Netto ^{2,*} and Marcos Antonio de Oliveira ^{1,*}

¹ Instituto de Biociências, Universidade Estadual Paulista, UNESP, São Vicente 01049-010, Brazil; tairumjr@usp.br (C.A.T.); melina.css@hotmail.com (M.C.S.); carlosbreyer@gmail.com (C.A.B.); alp.oliveira@unesp.br (A.L.P.d.O.); vitoria.isabela.montanhero@gmail.com (V.I.M.C.); marcos.toyama@unesp.br (M.H.T.)

² Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, São Paulo 01049-010, Brazil

³ Laboratório de Biomarcadores de Contaminação Aquática e Imunoquímica, Departamento de Bioquímica, Universidade Federal de Santa Catarina, Florianópolis 88040-900, Brazil; guilherme.toledo@ufsc.br

⁴ Laboratório de Ecologia Molecular, Instituto de Biociências, Universidade Estadual Paulista, UNESP, São Vicente 01049-010, Brazil; gustavo.mori@unesp.br

* Correspondence: nettoles@ib.usp.br (L.E.S.N.); marcos.a.oliveira@unesp.br (M.A.d.O.)

† These authors contributed equally to this work.



check for updates

Citation: Tairum, C.A.; Santos, M.C.; Breyer, C.A.; de Oliveira, A.L.P.; Cabrera, V.I.M.; Toledo-Silva, G.; Mori, G.M.; Toyama, M.H.; Netto, L.E.S.; de Oliveira, M.A. Effects of Serine or Threonine in the Active Site of Typical 2-Cys Prx on Hyperoxidation Susceptibility and on Chaperone Activity. *Antioxidants* **2021**, *10*, 1032.

<https://doi.org/10.3390/antiox10071032>

Academic Editor: Bruce Morgan

Received: 5 May 2021

Accepted: 10 June 2021

Published: 25 June 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: Typical 2-Cys peroxiredoxins (2-Cys Prx) are ubiquitous Cys-based peroxidases, which are stable as decamers in the reduced state, and may dissociate into dimers upon disulfide bond formation. A peroxidatic Cys (C_P) takes part of a catalytic triad, together with a Thr/Ser and an Arg. Previously, we described that the presence of Ser (instead of Thr) in the active site stabilizes yeast 2-Cys Prx as decamers. Here, we compared the hyperoxidation susceptibilities of yeast 2-Cys Prx. Notably, 2-Cys Prx containing Ser (named here Ser-Prx) were more resistant to hyperoxidation than enzymes containing Thr (Thr-Prx). In silico analysis revealed that Thr-Prx are more frequent in all domains of life, while Ser-Prx are more abundant in bacteria. As yeast 2-Cys Prx, bacterial Ser-Prx are more stable as decamers than Thr-Prx. However, bacterial Ser-Prx were only slightly more resistant to hyperoxidation than Thr-Prx. Furthermore, in all cases, organic hydroperoxide inhibited more the peroxidase activities of 2-Cys Prx than hydrogen peroxide. Moreover, bacterial Ser-Prx displayed increased thermal resistance and chaperone activity, which may be related with its enhanced stability as decamers compared to Thr-Prx. Therefore, the single substitution of Thr by Ser in the catalytic triad results in profound biochemical and structural differences in 2-Cys Prx.

Keywords: 2-Cys Prx; catalytic triad; organic hydroperoxides; oligomerization; hyperoxidation; chaperone

1. Introduction

Typical 2-Cys peroxiredoxins (2-Cys Prx) of the AhpC/Prx1 sub-family are peroxidases widely distributed in prokaryotes and eukaryotes that reduce hydroperoxides with high efficiency [1–4]. These enzymes are very abundant, representing 0.1–1.0% of all soluble proteins in the cells [5–8] and capable of decomposing distinct hydroperoxides [9,10]. Additionally, typical 2-Cys Prx are mediators of redox signaling and can act as molecular chaperones [11–15].

The 2-Cys Prx use a fully conserved N-terminal cysteine residue (peroxidatic cysteine-C_P), which is responsible for the nucleophilic attack on the hydroperoxide and then becomes oxidized into a sulfenic acid (C_P-SOH), which can condensate with a second Cys residue (resolving cysteine, C_R-SH) forming an intermolecular disulfide (C_P-S-S-C_R). In most cases,

these disulfide bonds are reduced by thioredoxin (Trx) [16–19]. The high reactivity of the C_P is related to the microenvironment in which it is found. C_P is in close proximity to other two fully conserved residues (Thr/Ser and Arg), forming polar interactions that increase the electrophilicity of H₂O₂, and facilitate the nucleophilic attack by the thiolate on C_P [20]. As expected, C_P [21–23], as well as Thr/Ser and Arg [3,23–25], are essential for the efficient reduction of hydroperoxides. Thus, these three residues (C_P, Thr/Ser, and Arg) compose the so-called catalytic triad [3,22,26].

Under high hydroperoxide concentrations, C_P can undergo further oxidation generating hyperoxidized species (C_P-SO₂H or C_P-SO₃H) rather than forming the disulfide bond, inactivating Prx turnover by Trx [27,28]. However, the enzyme sulfiredoxin (Srx) can reduce C_P-SO₂H to C_P-SOH in a process dependent on ATP hydrolysis for typical 2-Cys Prx [27,28]. Hyperoxidized 2-Cys Prx are frequently associated with a molecular chaperone (holdase) function and may impact signaling pathways, like cell growth and circadian rhythm [29–32]. In addition to C_P hyperoxidation, high temperatures can also confer chaperone function to these enzymes [33–36].

A remarkable feature of 2-Cys Prx is the structural rearrangement that are triggered by C_P oxidation. Reduced 2-Cys Prx enzymes (C_P-S[−]) are stabilized as decamers [(α₂)₅] and can assume the so called fully folded (FF) state (Figure 1A), in which C_P is located in the first turn of an α-helix near to N-terminal region of the protein and far away from C_R (10–16 Å), which is located in the C-terminus of these enzymes. Upon oxidation, partial unfolding of the α-helix containing C_P-SOH (the so-called locally unfolded = LU state) allows its approximation to C_R with the consequent intermolecular disulfide bond formation [37]. In the disulfide form, the 2-Cys Prx decamers tend to dissociate into low molecular weight (LMW) species, mainly dimers [38–40] (Figure 1B). Moreover, when C_P is hyperoxidized or when subjected to heat shock, 2-Cys Prx can switch from decamers to higher molecular weight (HMW) species [32,33,35,41,42] (Figure 1C). Therefore, two reversible structural switches (FF–LU and decamers dissociation) are inter-connected and the stabilities of distinct conformers are influenced by thermal and/or redox factors.

The transitions and stability of distinct oligomers also rely in structural peculiarities of these enzymes. In the 2-Cys Prx from *Escherichia coli* (EcAhpC) mutations close to the C-terminal region precludes the decamer formation [43]. Factors as pH, ionic strength, and protein concentration can influence directly the quaternary state of 2-CysPrx, indicating that the oligomeric rearrangements are directly related to specialized functions of these enzymes in different organisms and cell compartments [39,44–47]. In that regard, we have shown that the presence of Thr or Ser in the catalytic triad influences the stabilities of distinct oligomeric states that 2-Cys Prx can assume [3]. Yeast Tsa1 and Tsa2 contain Thr or Ser in the active site, respectively. Both enzymes are decamers in the reduced state, but Tsa1 is mainly a dimer when oxidized and some are intermediate species (e.g., tetramers), while Tsa2 remains as decamer even after oxidation [3]. In addition, the mutation of catalytic Thr to Ser stabilizes Tsa1 in the decameric form even when oxidized [3]. The substitution of catalytic triad Thr to Ser also stabilizes typical 2-Cys Prx from *Salmonella typhimurium* and *Enterococcus faecalis* as decamers [48,49].

Here, we described that the presence of Thr and Ser in the active site also affected the sensibility of 2-Cys Prx to hyperoxidation. Previously, other structural features were described to confer sensibility of C_P to hyperoxidation, namely a GGLG motif (close to the active site) and a YF motif (found in a α-helix in the C-terminal portion) that are present almost uniquely in eukaryotic enzymes [40]. Posteriorly, the presence of the motif A (D-X₈-N/G-X₁₀-H-X₂₇-S/G) and of the motif B (T-X₃-S/T) were associated with increased resistance of 2-Cys Prx to hyperoxidation [50]. We show that yeast 2-Cys Prx (AhpC/Prx1 subfamily) with Ser in the catalytic triad (named here Ser-Prx) are more resistant to hyperoxidation than those containing Thr (named here Thr-Prx). Moreover, analysis of all the sequences of typical 2-Cys Prx present in the NCBI GenBank revealed that occurrence of Ser-Prx is rare in multicellular eukaryotes and more frequent in eubacteria. Therefore, we describe that bacterial Ser-Prx were also more stable as decamers and

displayed more resistance to thermal inactivation than bacterial Thr-Prx. Furthermore, we found that cumene hydroperoxide (CHP) was more efficient than H_2O_2 to inactivate both Ser-Prx and Thr-Prx. Finally, we showed that bacterial Ser-Prx retained peroxidase activity and acquired molecular chaperone activity more readily than Thr-Prx after treatment at high temperatures.

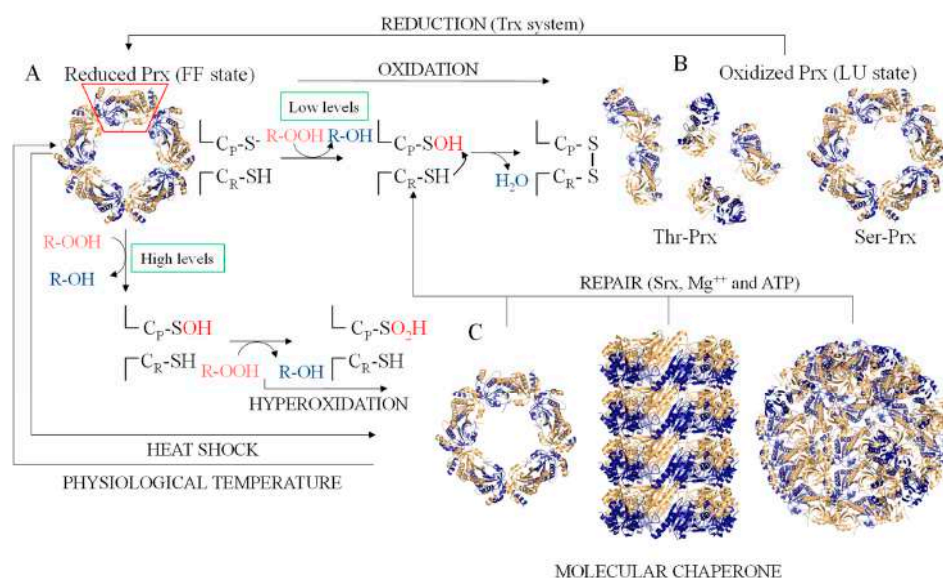


Figure 1. Structural and functional transitions of 2-Cys Prx. (A) In reduced (C_P-S^-) and in the fully folded (FF) state, 2-Cys Prx are decamers formed by association of five homodimers ($\alpha_2[\zeta]$). The red trapeze denotes one of the homodimers that compose the decamer. The monomers of each homodimer are depicted in dark blue and light orange, represented in cartoon. At low hydroperoxides level, C_P is oxidized to sulfenic acid (C_P-SOH) and then the condensation with C_R occurs, resulting in the formation of an intermolecular disulfide bond and locking the 2-Cys Prx in the locally unfolded (LU) state. (B) In 2-Cys Prx containing Thr (Thr-Prx), the disulfide formation results in decamer dissociation, while in 2-Cys Prx containing Ser (Ser-Prx), the decamers are stable even in the disulfide form. (C) At high levels of hydroperoxides, C_P-SOH can react with another(s) hydroperoxide molecule(s) resulting in hyperoxidized species (C_P-SO_2H or C_P-SO_3H) and in the functional switch from peroxidase to chaperone. This functional switch may be accompanied by the appearance of species with molecular weight higher than the decamer. This process can be reversed by Srx, with ATP hydrolysis. The functional transition from peroxidase to chaperone can also be triggered by high temperatures and the peroxidase activity is restored at physiological temperatures. The 2-Cys Prx representations were made using the crystallographic coordinates of *Saccharomyces cerevisiae* enzyme (PDB = 3SBC).

2. Materials and Methods

2.1. Amplification, Cloning and Sequencing

Yeast wild type proteins (Tsa1, Tsa2, Trx1, and TrxR1) were obtained as previously described [3,51]. Bacterial AhpC and the corresponding thiol reductive systems were amplified by PCR from the genomic DNA of the following species/strains: *Escherichia coli*/ATCC25922, and *Pseudomonas aeruginosa*/ATCC29853. The *ahpC* gene from *Staphylococcus epidermidis*/ATCC12228 was synthesized by GenScript, based on the sequence SE_2357 from KEGG database, with optimized codons for expression in *E. coli*, and flanked with *Nde* I and *Bam* HI restriction sites. Amplified DNA fragments and synthetic gene were digested with *Nde* I and *Bam* HI and cloned into pET15b vector previously digested with the same enzymes. The resulting recombinant vectors were sequenced in an ABI 3730 DNA

Analyzer (Applied Biosystems) using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and then were used to transform *E. coli* BL21 (DE3) or *E. coli* Tuner (DE3) strain (Sigma-Aldrich, Taufkirchen, Germany).

2.2. Site Directed Mutagenesis

The mutagenesis protocols were performed using Quick Change II Kit (Stratagene), following manufacturer's instructions. The products were used to transform *E. coli* XL1-Blue strain (Agilent Technologies, Santa Clara, CA, USA) and single colonies were grown on Luria Bertani (LB) medium for 16 h. Then, plasmids were extracted, purified, and sequenced. Plasmids containing the expected single substitutions were used to transform *E. coli* BL21 (DE3) or *E. coli* BL21 Tuner (DE3) expression strains (Sigma-Aldrich). The BL21 (DE3) strain containing the plasmid pET15b/*tsa1*^{t44s} was previously obtained [3]. All strains used in this work are summarized in Table 1.

Table 1. List of strains used in this work.

Species	Strain/Plasmid	Source
<i>E. coli</i>	ATCC25922	ATCC *
<i>E. coli</i>	BL21 DE3/pET15b-ahpc (Pa)	This work
<i>E. coli</i>	BL21 DE3/pET15b-ahpc (Se)	This work
<i>E. coli</i>	BL21 DE3/pET15b-trx (Ec)	This work
<i>E. coli</i>	BL21 DE3/pET15b-trxr (Ec)	This work
<i>E. coli</i>	BL21 DE3/pET15b-tsa1 (Sc)	[52]
<i>E. coli</i>	BL21 DE3 Tuner/pET15b-tsa2 (Sc)	[3]
<i>E. coli</i>	BL21 DE3/pET15b-tsa1t44s (Sc)	[3]
<i>E. coli</i>	BL21 DE3 Tuner/pET15b-tsa2s44t (Sc)	This work
<i>E. coli</i>	BL21 DE3/pET15b-trx1 (Sc)	[51]
<i>E. coli</i>	BL21 DE3/pET15b-trxr1 (Sc)	[51]
<i>E. coli</i>	XL1-Blue	Agilent
<i>P. aeruginosa</i>	ATCC29853	ATCC *
<i>S. epidermidis</i>	ATCC12228	ATCC *

* American Type Culture Collection; genes: Ec = *E. coli*, Pa = *P. aeruginosa*, Sc = *S. cerevisiae* and Se = *Staphylococcus epidermidis*.

2.3. Oligonucleotides

The oligonucleotides used to cloning and mutagenesis are shown in Table 2.

Table 2. The oligonucleotides sequences were defined based on NCBI genomic DNA sequences NC_000913.3 (*E. coli* ahpc, *trx* and *trxr*), NC_002516.2 (*P. aeruginosa* ahpc), NZ_CP035288.1 (*S. epidermidis* ahpc) and NC_001136.10 (*S. cerevisiae* tsa2).

Oligonucleotide	Sequence
Ec_Trx_F	5' <u>CGC</u> GAT CCA TAT GAT GAG CGA TAA AAT TAT TCA CCT GAC T 3'
Ec_Trx_R	5' <u>CGC</u> AAG CTT GGA TCC TTA CGC CAG GTT AGC GTC GAG GAA 3'
Ec_TrxR_F	5' <u>CGC</u> GAT CCA TAT GAT GGG CAC GAC CAA ACA CAG TAA ACT G 3'
Ec_TrxR_R	5' <u>CGC</u> AAG CTT GGA TCC TTA TTT TGC GTC AGC TAA ACC ATC 3'
Pa_AhpC_F	5' <u>CGC</u> GAT CCA TAT GAT GTC CCT GAT CAA CAC TCA AGT CCA A 3'
Pa_AhpC_R	5' <u>CGC</u> AAG CTT GGA TCC TTA GAT CTT GCC GAC CAG GTC CAG G 3'
Pa_AhpC_T43S_F	5' GCT GCC TTC TCC TTC AAC TGC 3'
Pa_AhpC_T43S_R	5' GCA GTT GAA GGA GAA GGC AGC 3'
Se_AhpC_S46T_F	5' GCG GAC TTC ACC TTT GTT TGC 3'
Se_AhpC_S46T_R	5' GCA AAC AAA GGT GAA GTC CGC 3'
Tsa2S44T_F	5' ATT GGC TTT TAC TTT TGT CTG TC 3'
Tsa2S44T_R	5' GAC AGA CAA AAG TAA AAG CCA AT 3'

* Underlined bases indicate *Nde* I and *Bam* HI cloning adapters.

2.4. Expression and Purification of Recombinant Proteins

Single colonies of *E. coli* BL21 (DE3) strain containing the expression plasmids were inoculated in LB medium (20 mL) containing 0.1 mg ampicillin/mL overnight at 37 °C/250 rpm, transferred to 1 L of fresh LB medium, and cultured further until OD₆₀₀ reached 0.6–0.8. Then the expressions of all proteins were induced by the addition of 0.3 mM IPTG at 37 °C/3 h/250 rpm in orbital shaker. Tsa2 and Tsa2^{S44T} were expressed using similar protocols; however, *E. coli* Tuner (DE3) was used instead.

Cells were harvested by centrifugation and pellets were resuspended in start buffer (50 mM sodium phosphate buffer, pH 7.4, 50 mM NaCl, 20 mM imidazole, and 2 mM PMSF) and disrupted by sonication. The cell extracts were kept in ice during streptomycin sulfate 1% treatment for 20 min. The supernatants clarified by centrifugation were homogenized by filtration and purified by IMAC using HisTrap column (GE Healthcare, Piscataway, NJ, USA). Imidazole was removed by gel filtration using PD10 Desalting column (GE Healthcare, Piscataway, NJ, USA) and the purity of recombinant proteins was verified by SDS–PAGE. The yeast Trx1 was purified by boiling method as described previously [53].

2.5. NADPH Oxidation Assay

The NADPH to NADP⁺ oxidation was monitored at A_{340nm} in 100 µL reaction mixtures containing 50 mM HEPES pH 7.4, 100 µM DTPA, 1 mM sodium azide, 1 µM Prx (Tsa1, Tsa2, or reciprocal mutants), 2 µM Trx1, 0.3 µM TrxR1, and 150 µM NADPH. In some cases, yeast enzymes were replaced by bacterial enzymes (*Pseudomonas aeruginosa* and *Staphylococcus epidermidis* AhpC; *Escherichia coli* Trx and TrxR) at the concentrations described in the legend of the figures. The reactions were initiated by H₂O₂ or CHP addition and the reaction mixtures were incubated at 30 °C or 37 °C.

2.6. Western Blot Analysis to Evaluate the C_P Hyperoxidation

To confirm if 2-Cys Prx were inactivated by C_P hyperoxidation, we collected samples after NADPH enzymatic assays. Proteins were pre-alkylated with NEM (50 mM) to avoid the formation of artifactual disulfides before non-reducing SDS–PAGE. Samples were then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) and were incubated with an anti-Prx-SO₃ (dilution 1:2500; AbFrontier, Seoul, Korea; #LF-PA0004) diluted 1000 times in TBST containing 3% skimmed milk and incubated on a rocking platform for 16 h at 4 °C. A HRP-conjugated secondary antibody (GE Healthcare—Little Chalfont, UK) was employed and samples were detected by chemiluminescence detection (ECL Prime Western Blot, GE Healthcare—Little Chalfont, UK). Images were obtained by ChemiDoc equipment (Bio-Rad—Hercules, CA, EUA).

2.7. In Silico Screening of the Typical 2-Cys Prx and Phylogenetic Analyses

The sequences of the *S. cerevisiae* Tsa1 (UniProtKB: P34760), Tsa2 (Q04120), *Salmonella typhimurium* AhpC (P0A251), and *Staphylococcus aureus* AhpC (P0A0B7) were used as eukaryotic and prokaryotic prototypes of typical 2-Cys Prx containing Thr or Ser in the catalytic triad. These amino acid sequences were used as query against TREMBL database using blastp [54] with a cutoff e-value of 10^{−3}. Then, to minimize misidentification issues, we identified Pfam domains out of the putative unique orthologs and retained only those presenting the domains found in Tsa1 and Tsa2, AhpC/Prx1 (Pfam: PF00578), redoxin (PF08534) and 1-Cys Prx_C (PF10417) with hmmscan (v.3.1b2) [55]. Sequences presenting other domains, except for SCO1–SenC (PF026300), which we observed in the *S. aureus* AhpC, were excluded. Additionally, because Prx superfamily presents a conserved active-site cysteine P-X-X-X-(T/S)-X-V-C-P-T-E motif [56], known as C_P-loop [20], we removed sequences lacking this motif. After these filtering steps, we retrieved taxonomic information of the accessions we obtained from the NCBI taxonomy database (database resources of the National Center for Biotechnology Information; June 2018).

2.8. Size Exclusion Chromatography (SEC) of Bacterial Typical 2-Cys Prx

To obtain reduced samples, 30 μ M of bacterial 2-Cys Prx were treated with TCEP 5 mM for 60 min and the TCEP excess was removed using the PD 10 column (reduced samples). To oxidize samples, reduced 2-Cys Prx (30 μ M) were treated with H_2O_2 (36 μ M) for 30 min, at room temperature. SEC was performed in an analytical Jasco-HPLC system composed by a PU 2880 Plus injector and a PDA MD 2018 detector. Samples (30 μ M in 100 mM Tris-HCl at pH 7.4) were separated by a Phenomenex BioSep-SEC-S2000 column (7.8 $\times$ 300 mm, 5 μ m, resolution range of 15 to 2000 kDa) (Phenomenex, Inc., Torrance, CA, USA), using a flow of 1 mL/min in 100 mM Tris-HCl buffer pH 7.4 monitored by absorbance (280 nm). Bovine thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B₁₂ (1.35 kDa) were used as molecular standards (Bio-Rad Laboratories, Richmond, VA, USA).

2.9. Differential Scanning Fluorimetry

The thermal unfolding of bacterial 2-Cys Prx were analyzed by using differential scanning fluorimetry (DSF) [57]. Briefly, proteins (final concentration 3 μ M in 10 mM HEPES pH 7.5, 150 mM NaCl) were mixed with 5.25 μ L of SYPRO Orange dye diluted 200 times in DMSO. Reactions mixtures were equilibrated at 25 $^\circ\text{C}$ for 30 s and then monitored from 25–95 $^\circ\text{C}$ with increments of 1 $^\circ\text{C}/\text{min}$ for 240 min using an Mx 3005P Real-Time System (Agilent Technologies, Santa Clara, CA, USA,) with excitation and emission filters set to 465 and 590 nm, respectively. The melting temperature (T_m) of AhpCs were obtained with the MxPro software and curves fit in GraphPad Prism (GraphPad Software, San Diego, CA, USA) using the Boltzmann equation to determine the midpoint of thermal denaturation (T_m). All measurements were carried out in triplicate and presented as mean $\pm$ SD.

2.10. Chaperone Activity Assay

Chaperone (holdase) activities of bacterial 2-Cys Prx were analyzed by their ability to inhibit thermally induced aggregation of citrate synthase (CS) as previously described [34]. Briefly, CS (2 μ M) was mixed with AhpC at molar ratio 20:1 in 40 mM HEPES-KOH pH 7.5. We also evaluated the differences in chaperone ability of enzymes that were previously heated (48 $^\circ\text{C}$ for 1 h). As negative control, samples without the 2-Cys Prx were used. CS aggregation was monitored by light scattering at 360 nm using a Synergy H1 microplate reader (BioTek, Winooski, VT, USA). The assay was executed at 43 $^\circ\text{C}$, with continuously shaking (fast double orbital), with reads at each 15 s for 90 min.

2.11. Analysis of Crystallographic Structures

The Prx quaternary structures were accessed from the protein structure database (www.rcsb.org accessed on 5 May 2021). The molecular 3D representations were generated using the PyMOL tool (www.pymol.org accessed on 5 May 2021).

3. Results

3.1. Presence of a Ser in the Catalytic Triad Increased Resistance of Yeast 2-Cys Prx to Hyperoxidation

Previously, we observed that the presence of a Ser residue in the catalytic triad stabilized yeast 2-Cys Prx in the decameric state even in the disulfide form [3]. Here, we investigated if the presence of a Ser or Thr in the catalytic triad might also affect the hyperoxidation susceptibilities of these enzymes.

The decrease of NADPH consumption at increasing hydroperoxides concentrations (H_2O_2 in Figure 2 and CHP in Figure 3) is consistent with 2-Cys Prx inhibition by C_P hyperoxidation. Tsa1 (a Thr-Prx) was remarkably more susceptible to inactivation (Figure 2A) when compared to Tsa2 (a Ser-Prx) (Figure 2C). At concentrations higher than 1 mM, H_2O_2 significantly inhibited Tsa1 (Figure 2A), while high doses of H_2O_2 (10 mM) did not inactivate Tsa2 (Figure 2C). Then, NADPH assays with mutant proteins (Tsa1^{T44S} and Tsa2^{S44T})

were performed. Tsa1^{T44S} (Figure 2B) was more resistant to H₂O₂ inactivation than wild type Tsa1 (Figure 2A). In contrast, Tsa2^{S44T} (Figure 2D) was more susceptible to inactivation than wild type Tsa2 (Figure 2C). Therefore, these results indicated that Thr-Prx are more susceptible to hyperoxidation than Ser-Prx.

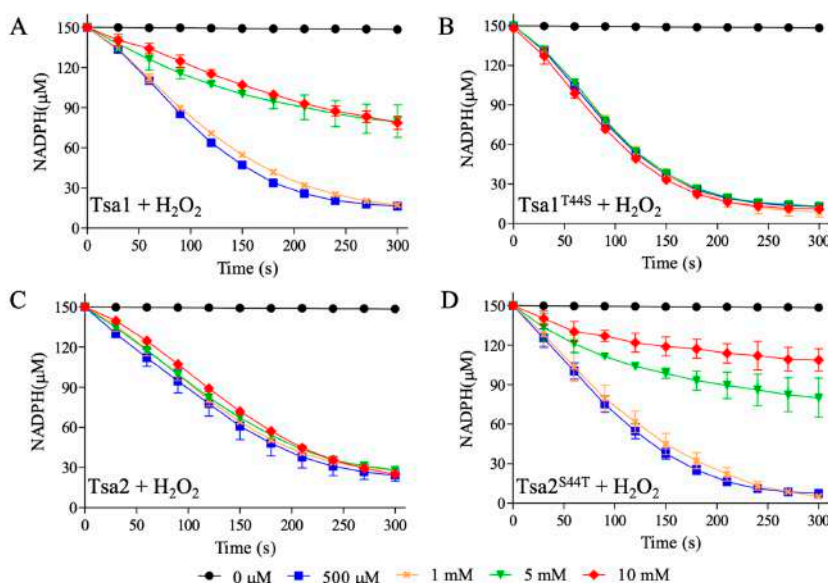


Figure 2. Tsa1, Tsa2, and mutant inhibition by H₂O₂. *S. cerevisiae* Tsa1 (A), Tsa1^{T44S} (B), Tsa2 (C), or Tsa2^{S44T} (D) were exposed to increasing amounts of H₂O₂. The assays were performed with 1 μM Prx, 2 μM Trx1, 0.3 μM TrxR1, and increased amounts of H₂O₂ (0 μM = ● black, 500 μM = ■ blue, 1 mM = × orange, 5 mM = ▼ green and 10 mM = ◆ red) in buffer containing 50 mM HEPES pH 7.4, 100 μM DTPA, 1 mM sodium azide and 150 μM NADPH. The decrease in NADPH absorbance was followed at 340 nm. The error bars represent the standard deviation. All the experiments were performed in triplicate and repeated at least three times with similar results.

We then verified the susceptibilities of these yeast 2-Cys Prx to hyperoxidation by an organic hydroperoxide (CHP). The peroxidase activities of Thr-Prx were almost abolished in all concentrations tested for Tsa1 (Figure 3A) and Tsa2^{S44T} (Figure 3D). In contrast, Ser-Prx were considerably more resistant to inactivation (Figure 3B,C).

We confirmed that 2-Cys Prx inactivation occurred due to C_P hyperoxidation, by western blot assays using anti-SO₃H antibody (Supplementary Material, Figure S1) from aliquots of samples described in Figures 2 and 3. Hyperoxidized 2-Cys Prx migrated as a dimer (~46 kDa) in non-reducing SDS-PAGE, when one of the active sites was in the disulfide form and the other with the C_P hyperoxidized (C_P-SO₃H) or as monomer, when all C_Ps were hyperoxidized.

Two aspects deserve to be highlighted from these results. As already observed [58], Tsa1 is more sensitive to hyperoxidation by CHP than to H₂O₂ (Figures 2A and 3A). We now observed that the same characteristic for Tsa2 (Figures 2C and 3C). Additionally, we verified that Ser-Prx (Tsa2 and Tsa1^{T44S}) are more resistant to hyperoxidation than Thr-Prx (Tsa1 and Tsa2^{S44T}) (Figures 2 and 3). Accordingly, *Δtsa1* yeast strains are more sensitive to organic hydroperoxides than *Δtsa1* [59].

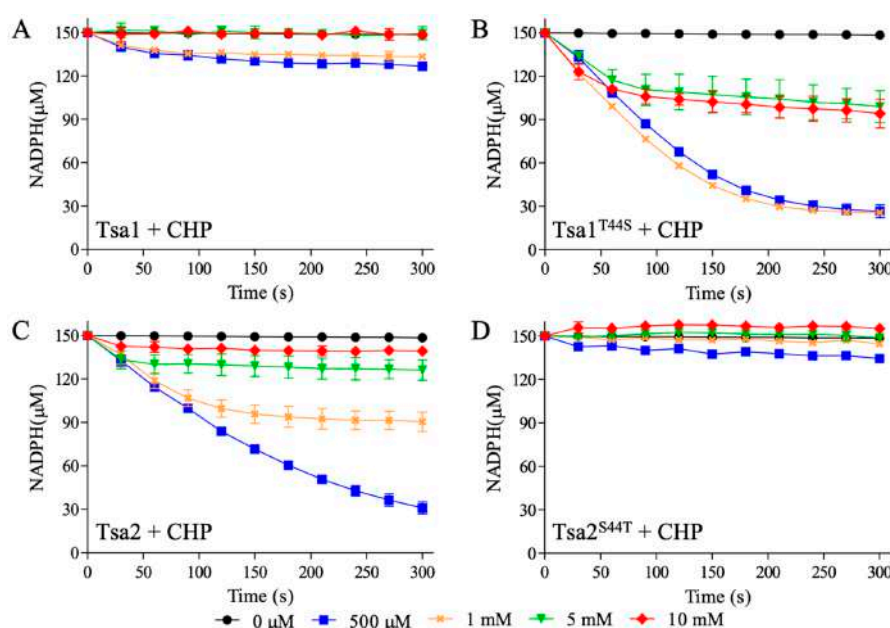


Figure 3. Inactivation of yeast Tsa1, Tsa2, and mutants by CHP. Similar to Figure 2, *S. cerevisiae* Tsa1 (A), Tsa1^{T44S} (B), Tsa2 (C), or Tsa2^{S44T} (D) were treated with growing amounts of CHP and the absorbance of NADPH was monitored at 340 nm. The experiment was executed with 1 μ M Prx, 2 μ M Trx1, 0.3 μ M TrxR1 and increased amounts of CHP (0 μ M = $\bullet$ black, 500 μ M = $\blacksquare$ blue, 1 mM = $\times$ orange, 5 mM = $\blacktriangledown$ green and 10 mM = $\blacklozenge$ red) in buffer containing 50 mM HEPES pH 7.4, 100 μ M DTPA, 1 mM sodium azide, and 150 μ M NADPH. Error bars represent the standard deviations. All the experiments were performed in triplicate and repeated at least three times with similar results.

3.2. Thr-Prx Are More Widely Distributed and Ser-Prx Is More Prevalent in Bacteria

As the presence of Ser or Thr provokes dramatic alterations in the biochemical and structural properties of yeast 2-Cys Prx, we investigated the phylogenetic distribution of Ser-Prx and Thr-Prx. We analyzed 45,939 unique protein sequences, out of which 15,034 accessions presented AhpC-TSA, Redoxin and 1-CysPrx_C domains denominations according to Pfam and no other domain fused, except for SCO1-SenC. Out of the sequences with taxonomic annotation at domain level, Thr-Prx represented the majority (14,092 sequences, approximately 93.7%), whereas the remaining (941 sequences, approximately 6.3%) are Ser-Prx (Table 3). Notably, the proportions between Thr-Prx and Ser-Prx are different in the three domains of life. Ser-Prx sequences are proportionally more representative in bacteria (7.4%) compared to eukaryotes (2.7%) and archaea (1.4%) (Figure 4). We also found 45 Thr-Prx records deposited in the NCBI database obtained by metagenome approaches and three of them were uncategorized.

Table 3. Distribution of Thr-Prx or Ser-Prx among the living groups.

Thr				Ser			
Bacteria		Eukarya		Bacteria		Eukarya	
Acidobacteria	15	Alveolata	107	FCB group *	14	Amoebozoa	1
Aquificae	16	Amoebozoa	83	Proteobacteria	15	Euglenozoa	12
Calditrichaeota	2	Apusozoa	2	PVC group §	12	Opisthokonta	75
Chrysiogenetes	1	Cryptophyta	1	Spirochaetes	2	Rhodophyta	2
Deferribacteres	5	Euglenozoa	105	Synergistetes	1		
Elusimicrobia	18	Heterolobosea	1	Terrabacteria group	803		
Environmental samples	3	Opisthokonta	2079	Unclassified	4		
FCB group *	1455	Parabasalia (parabasalids)	13				
Fusobacteria	78	Rhizaria	4				
Nitrospinae/Tectomicrobia group	11	Rhodophyta	116				
Nitrospirae	15	Stramenopiles	136				
Proteobacteria	4890	Viridiplantae	302				
PVC group	220						
Spirochaetes	149						
Synergistetes	10						
Terrabacteria group	3564						
Thermodesulfobacteria	2						
Unclassified	245						

* Superphylum that comprises Fibrobacteres, Chlorobi, and Bacteroidetes phyla, § Superphylum that comprises Planctomycetes, Verrucomicrobia, and Chlamydiae phyla.

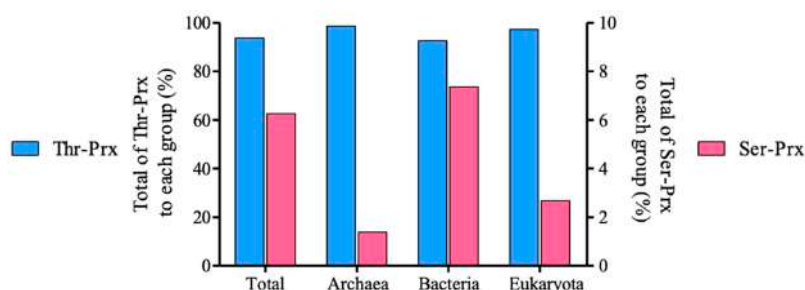


Figure 4. Distribution of the typical 2-Cys Prx containing Thr or Ser as part of the catalytic triad. The analysis of 15,034 unique sequences of typical 2-Cys from the NCBI revealed 14,092 Thr-Prx (93.7%) and 941 Ser-Prx (6.3%) sequences. Considering each domain, Ser-Prx are more abundant at bacteria (7.4%), followed by eukaryotes (2.7%), and archaea (1.4%). The left Y-axis is referent to Thr-Prx, while right Y-axis is related to Ser-Prx.

The number of sequences of Archaea 2-Cys Prx deposited in the database is very low compared to the other domains of life. Nevertheless, the number of Thr-Prx (71) from Archaea is superior to that of Ser-Prx (1). Thr-Prx are also more widely distributed in Eukarya (2949 representatives present in 12 Superphyla groups) than Ser-Prx (90 sequences found in only four groups) (Table 3). For instance, only one species presents Ser-Prx in chordates (killifish *Fundulus heteroclitus*), whereas a wide range of tetrapods presented only Thr-Prx (See Supplementary Material, Spreadsheet S1).

Concerning bacteria, 16 Superphyla groups contain Thr-Prx, with the prevalence of the Proteobacteria followed by Sphingobacteria (FCB group) and Thermodesulfobacteria groups. Conversely, Ser-Prx occurs with striking predominance in the Terrabacteria group (Table 3). Curiously, Thr-Prx are distributed similarly in Gram negative and Gram positive, whereas Ser-Prx are found mostly in Gram negative (Actinobacteria and Firmicutes).

Additionally, as *S. cerevisiae*, some bacteria (e.g., *Bacillus subtilis*) possess both Thr-Prx and Ser-Prx isoforms [60], as *S. cerevisiae*.

3.3. Quaternary Structures Characterization of Bacterial Thr-Prx and Ser-Prx

Considering the higher abundance of Ser-Prx in Bacteria, we evaluated the role of Thr/Ser in AhpC enzymes. We selected to analyze recombinant AhpCs from *Pseudomonas aeruginosa* (PaAhpC—a Thr-Prx) and AhpC from *Staphylococcus epidermidis* (SeAhpC—a Ser-Prx), as they belong to the most abundant groups of Thr-Prx (Proteobacteria) and of Ser-Prx (Terrabacteria). As with yeast 2-Cys Prx, we performed reciprocal Ser-Thr mutations and we carried out SEC analysis of bacterial 2-Cys Prx. While PaAhpC was mostly decamer in the reduced form and dimer when oxidized (Figure 5A), PaAhpC^{T44S} was predominantly decamer when oxidized or reduced (Figure 5B). Similarly, yeast Tsa1 and *Salmonella typhimurium* AhpC (both Thr-Prx) are decamers when reduced and dimers when oxidized in disulfides, while the substitution of Thr to Ser confers higher stability to the decamer even when the 2-Cys Prx are oxidized [3,39,49].

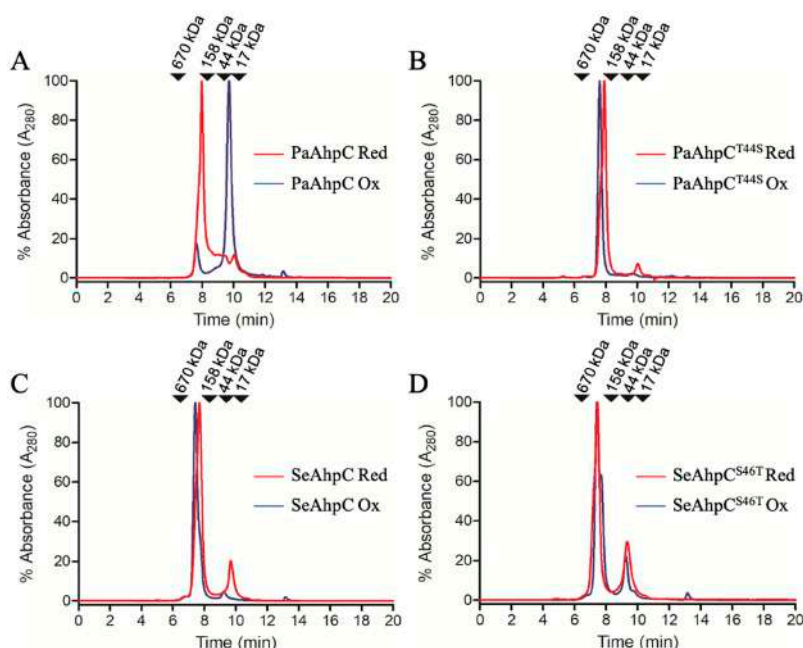


Figure 5. Size exclusion chromatography of PaAhpC and SeAhpC and Thr/Ser mutants. PaAhpC (A), PaAhpC^{T44S} (B), SeAhpC (C), and SeAhpC^{S46T} (D), at a final concentration of 30 μ M, were treated with TCEP 5 mM (red lines) or H₂O₂ 36 μ M (blue lines) for 30 min, at room temperature, before being submitted to SEC. The elution profile was monitored at a wavelength of 280 nm, at flow rate of 1 mL/min. Bovine thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa) were used as molecular standards. The experiments were performed at least three times.

Similarly to other Ser-Prx [3,48], SeAhpC was presented mostly as decamer independently of the redox state although a small amount of dimers was present in the reduced form (Figure 5C). Additionally, most of SeAhpC^{S46T} was presented as decamer and only a small fraction of the enzyme was detected as dimers, independently of the redox state (Figure 5D). It has been shown by SEC and TEM that the AhpC from *Enterococcus faecalis*, a naturally Ser-Prx, is a decamer independent of the redox state [43], similarly to yeast

Tsa2 [3]. Thus, our results showed that the presence of Thr or Ser in the catalytic triad is a relevant factor related to the redox sensitive oligomerization, but additional features also interfere with the quaternary assembly of typical 2-Cys Prx [43,45,61].

3.4. Comparative Analysis of the Inactivation of Bacterial Thr-Prx and Ser-Prx by High Hydroperoxide Concentrations and by Thermal Insult

We also investigated if the presence of Thr or Ser in the catalytic triad could affect the susceptibility of bacterial 2-Cys Prx to hyperoxidation. In the experimental conditions tested, H_2O_2 did not inhibit the NADPH consumption in the reaction mixtures containing Thr-Prx (Figure 6A,D) or Ser-Prx (Figure 6B,C) even at the highest doses (10 mM). However, when CHP was the substrate, a slight inhibition was observed to Thr-Prx (compare Figure 6E,F and Figure 6G,H). Again, we confirmed that the inhibition of peroxidase activity was a consequence of the C_P hyperoxidation by western blot analysis performed using anti- SO_3 antibody (Supplementary Material, Figure S2).

As the inhibition to bacterial Thr-Prx was less evident in relation to eukaryotic counterparts, we compared the NADPH consumption after 300 s from the data presented in Figure 2, Figure 3, and Figure 6 (Figure 7). For the yeast enzymes, we observed that NADPH consumption is quite similar between Thr-Prx and Ser-Prx at lower H_2O_2 concentrations, but it is considerably higher in samples containing Ser-Prx when 5 or 10 mM H_2O_2 were used (Figure 7A). When CHP was the substrate, the NADPH consumption was also more evident for Ser-Prx (Figure 7B). For the bacterial enzymes, there was no clear difference among the enzymes (Figure 7C). However, we could detect a lower NADPH consumption for Thr-Prx (PaAhpC and SeAhpC^{S46T}) compared to the reciprocal Ser-Prx (PaAhpC^{T43S} and SeAhpC) (Figure 7D). These results indicate that bacterial Ser-Prx present a subtle resistance to hyperoxidation to CHP, but not to H_2O_2 compared to the bacterial Thr-Prx.

We also evaluated the inactivation of Thr-Prx and Ser-Prx by thermal stress. The differential scanning fluorimetry (DSF) curve for PaAhpC presented a lag phase and the determined T_m was 58.5 ± 1.4 °C, while PaAhpC^{T44S} displayed a sudden increase in the fluorescence and the T_m of 66.1 ± 2.3 °C (Figure 8A). Therefore, replacement of the catalytic triad Thr by Ser resulted in an increase about 8 °C in the T_m (Figure 8A). For SeAhpC and SeAhpC^{S46T}, the shapes of the thermal sigmoidal profiles were very similar, although the Ser to Thr substitution resulted in a decrease of ≈ 9 °C of the T_m (61.3 ± 0.5 and 52.7 ± 0.6 °C, respectively) (Figure 8B). Therefore, the presence of Ser in the catalytic triad increased the thermal stabilities of the AhpCs.

Since the thermal stability increased in Ser-Prx, we tested if the proteins retained their peroxidase activities after the thermal pre-treatments. The Thr-Prx (PaAhpC and SeAhpC^{S46T}) were partially inactivated after being heated to 55 °C (Figure 9A,D).

However, the effect of temperature was much more noticeable for SeAhpC^{S46T}, where the pre-treatment at 55 °C abolished the peroxidase activity of the enzyme (Figure 9D). This result agreed with the determined T_m values, as the enzyme SeAhpC^{S46T} displayed the lowest T_m value. On the other hand, the Ser-Prx (PaAhpC^{T43S} and SeAhpC) retained the activity in all temperatures tested (Figure 9B,C).

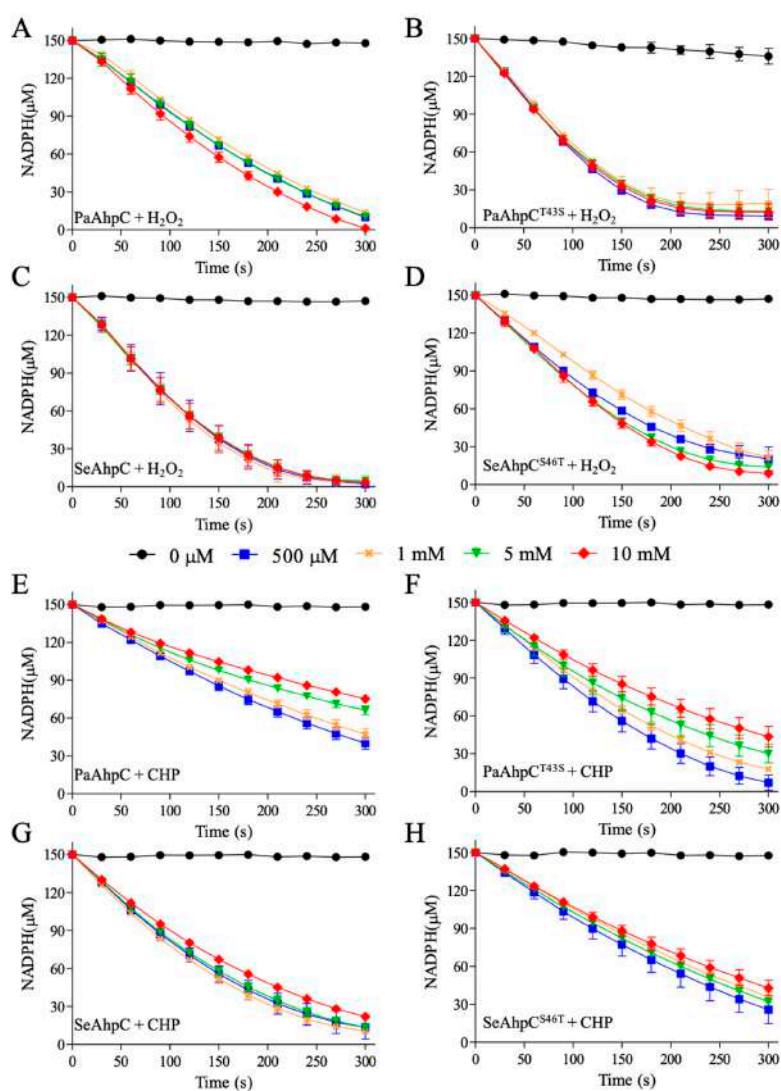


Figure 6. NADPH oxidation assay for evaluation of AhpC hyperoxidation of *P. aeruginosa* and *S. epidermidis* using the heterologous *E. coli* Trx system. Reactions were executed containing PaAhpC (A,E), PaAhpC^{T43S} (B,F), SeAhpC (C,G), or SeAhpC^{S46T} (D,H) (3 μM), EcTrx (6 μM), EcTrxR (0.9 μM), NADPH (150 μM), 50 mM HEPES pH 7.4, 1 mM sodium azide, and 100 μM DTPA. The experiments were started adding growing concentrations of H_2O_2 (A–D) or CHP (E–H) (0 μM = ● black, 500 μM = ■ blue, 1 mM = × orange, 5 mM = ▼ green and 10 mM = ◆ red). The reactions were incubated for 5 min/37 °C. The oxidation of NADPH was monitored spectrophotometrically at 340 nm. The error bars represent the standard deviations. All experiments were performed in triplicate and repeated at least three times, yielding consistent results.

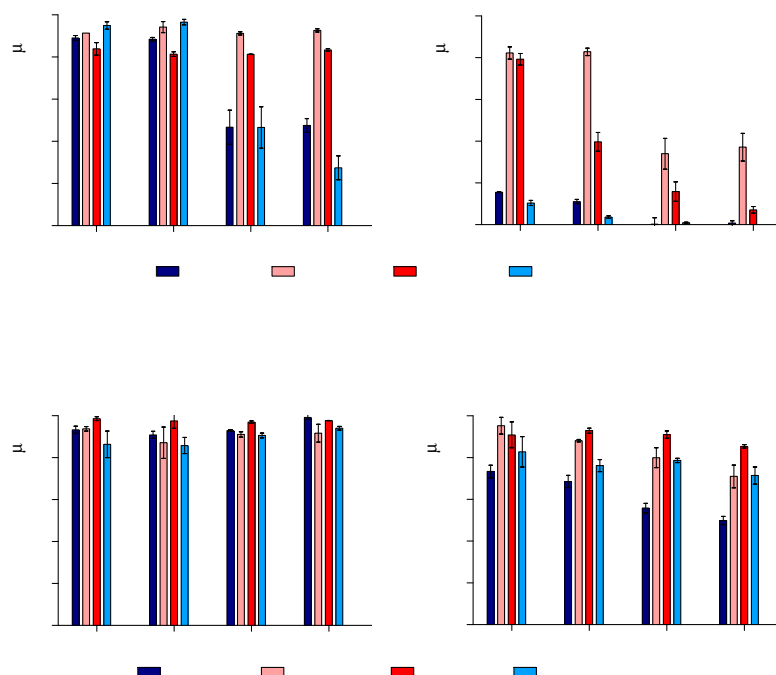


Figure 7. Evaluation of Thr-Prx and Ser-Prx inhibition. The amount of NADPH consumed after 300 s of reaction were obtained in the assays presented in Figure 2, Figure 3, and Figure 6, to H_2O_2 (A, eukaryotic Prx; C, bacterial Prx) and CHP (B, eukaryotic Prx; D, bacterial Prx). Comparing the values obtained to wild type with the reciprocal enzymes, it is possible to estimate which residue in catalytic triad makes the enzyme more resistant to hyperoxidation. Error bars represent the standard deviations.

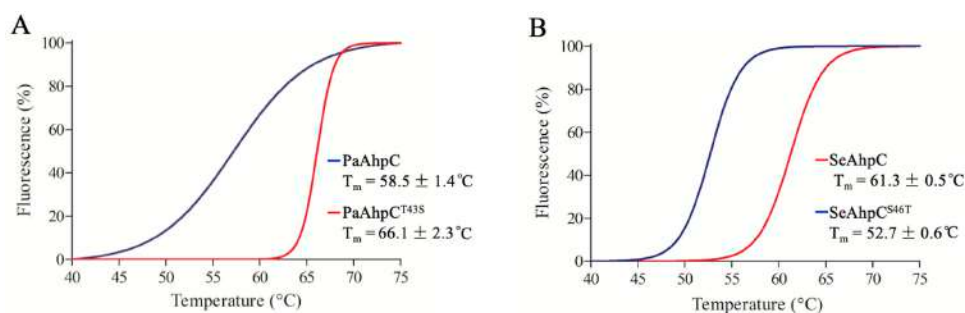


Figure 8. AhpC thermal unfolding monitored by DSF. Differential scanning fluorimetry of PaAhpC (A, blue line), PaAhpC^{T43S} (A, red line), SeAhpC (B, red line), and SeAhpC^{S46T} (B, blue line). Proteins were previously reduced with DTT (20 mM), desalted (PD10, GE Healthcare), and incubated with SYPRO Orange dye. The fluorescence scanning was carried out using a temperature gradient from 25 °C to 95 °C. The melting temperature (T_m) was determined by the Boltzmann equation to determine the midpoint of thermal denaturation. The experiments were performed in triplicate and repeated at least three times with similar results.

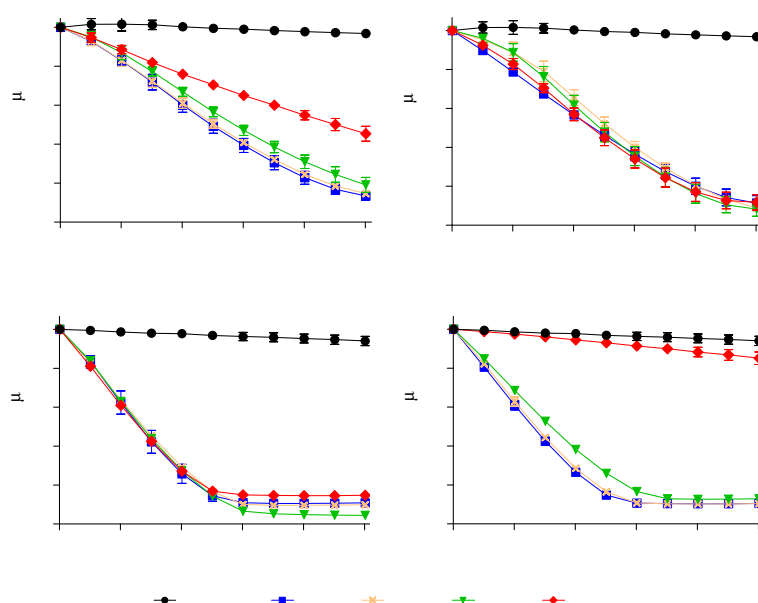


Figure 9. NADPH oxidation assay to assess the effects of thermal inactivation of AhpC enzymes. Aliquots of the PaAhpC (A), PaAhpC^{T43S} (B), SeAhpC (C) and SeAhpC^{S46T} (D) were incubated at different temperatures (37 °C = ■ blue, 42 °C = × orange, 50 °C = ▼ green and 55 °C = ◆ red) for 1 h. Then the enzymes were added to the reactions containing 6 μ M EcTrx, 0.9 μ M EcTrxR, 150 μ M NADPH, 50 mM HEPES (pH 7.0), 100 μ M DTPA, and 1mM sodium azide. The reactions were initiated by the addition of 500 μ M H₂O₂ and absorbance at 340 nm was monitored at 37 °C, for 5 min. As negative control (● black), reactions were performed without the addition of the AhpC. The standard deviations are represented by the error bars. All experiments were performed at least three times and yielded similar results using replicates.

3.5. AhpC Containing Ser Presented Enhanced Chaperone Activity

We also investigated the abilities of these AhpCs to protect citrate synthase (CS) against thermal insult. CS slowly aggregated when kept at 48 °C as indicated by increased light scattering as a function of time (Figure 10A,B, black lines). In the presence of PaAhpC, the CS aggregation decreased significantly (Figure 10A, blue line). We also tested if the pre-treatment of PaAhpC at 48 °C by 1 h was able to exert additional protection to CS, but no difference was observed in comparison to the untreated enzyme (Figure 10A, red line). In the case of SeAhpC, the sample without thermal pre-treatment presented a more pronounced protection activity (Figure 10B, blue line) than PaAhpC. Notably, pre-heated SeAhpC (48 °C/1 h) protected CS with very high efficiency, because only a negligible amount of CS aggregated (Figure 10B, red line). Therefore, our results suggest that AhpCs containing Ser in the catalytic triad are more prone to act as chaperone induced by heat shock stress than their counterparts containing Thr.

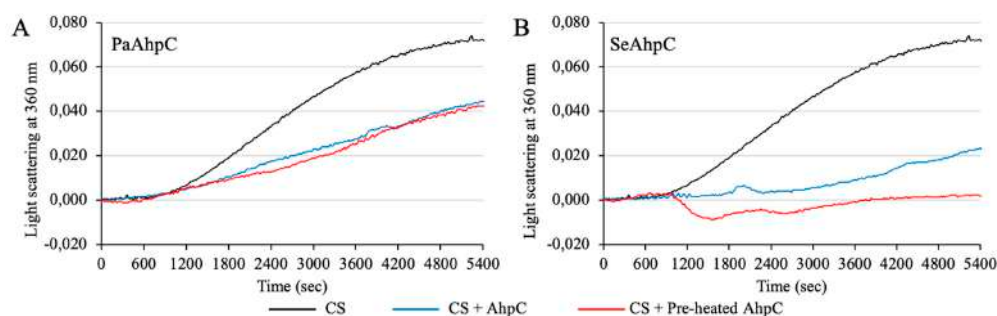


Figure 10. Comparative protection of PaAhpC and SeAhpC to citrate synthase (CS) aggregation by thermal stress. Chaperone activity of PaAhpC and SeAhpC was monitored by its ability to protect citrate synthase (CS) from thermally induced aggregation. The aggregation of 2 μ M of CS in 40 mM HEPES-KOH pH 7.5 was monitored by light scattering ($\lambda = 360$ nm) for 90 min, either alone or in the presence of 40 μ M PaAhpC (A) or SeAhpC (B). The assays were executed with the enzyme pre-heated at 48 $^{\circ}$ C for 1 h (red lines) or not (blue lines). The CS alone was used as negative control (black lines). The experiments were performed in triplicates under the same conditions.

The ability of the typical 2-Cys Prx to act as chaperone (holdase) was already described for several enzymes from eukaryotes [32,33] and from some bacterial proteins [34,62,63]. These enzymes are able to switch from peroxidase to chaperone in response to oxidative stress, by hyperoxidation of C_P or by thermal stress. Considering the high resistance of AhpC to C_P hyperoxidation, the thermal stress may be the main factor that triggers the chaperone activity, as observed by Kamariah and coworkers (2018) using *E. coli* AhpC (Thr-Prx) as model [34]. Here, we observed the same effect for SeAhpC, which is a Ser-Prx. It is tempting to speculate that chaperone activity may be facilitated in these enzymes because they are stabilized as decamers, irrespective to the oxidative state.

4. Discussion

The roles of Thr or Ser in the catalytic triad of the typical 2-Cys Prx has been considered redundant, but recently, we (and others) showed that Ser-Prx are more stable in the decameric form [3,49]. Here, we demonstrated that the presence of Ser or Thr in the active site affected also the C_P hyperoxidation susceptibilities, especially for yeast 2-Cys Prx, besides being related to thermal resistance and the chaperone activities acquisition of AhpCs. Furthermore, we showed that the vast majority of multicellular organisms have Thr on catalytic triad. Ser-Prx is more prevalent in bacteria, which might be related with diverse ecological strategies in some groups of this domain. Although the typical 2-Cys Prx from bacteria are very resistant to hyperoxidation, we demonstrated that the Thr to Ser substitution slightly increased the resistance to inactivation of AhpC by CHP.

Previously, eukaryotic 2-Cys Prx were described to be highly susceptible to oxidative inactivation in comparison with bacterial counterparts [40]. Accordingly, eukaryotic 2-Cys Prx were named as sensitive, while bacterial enzymes were referred as robust. Furthermore, authors identified the presence of GGLG and YF motifs exclusively in eukaryotic 2-Cys Prx, which were proposed to be positively selected during evolution [40]. The presence of the GGLG and YF motifs near the C_P residue would represent a steric hindrance effect, decreasing the rate of intermolecular disulfide bond formation [37]. According to this model, C_R and H_2O_2 compete for C_P -SOH. Therefore, enzymes with fast rates of condensation reaction between C_R and C_P -SOH would correspond to increased resistance to hyperoxidation. More recently, two other structural elements, named motifs A and B, were identified by conferring resistance to hyperoxidation in 2-Cys Prx [50].

Here, we analyzed the susceptibilities to hyperoxidation of sensitive (Tsa1, Tsa2) and robust (PaAhpC, SeAhpC) 2-Cys Prx carrying Thr or Ser in the catalytic triad. Remarkably, yeast Ser-Prx (Tsa1^{T44S} and Tsa2) are considerably more resistant to inactivation than Thr-

Prx (Tsa1 and Tsa2^{S44T}) by hyperoxidation (Figure 2, Figure 3, and Figure 7) and this effect is only moderate in bacterial enzymes treated with CHP (Figures 6 and 7). Noteworthy, motifs A and B previously described as resistance factors to hyperoxidation [50] are also highly similar among the yeast and bacterial counterparts (Supplementary material, Table S1 and Figure S3). Therefore, the observed differences in inactivation between Tsa1 and Tsa2 or between PaAhpC and SeAhpC are probably not related to the compositions of motifs A and B in these enzymes. In fact, the natural substitution of catalytic Thr to Ser stands as a novel trait related to resistance or susceptibility to C_P hyperoxidation.

We also analyzed the crystallographic structures of Tsa1 (PDB = 3SBC) and Tsa2 (PDB = 5DVB) to gain insights on mechanisms underlying differences in the hyperoxidation susceptibilities. In our previous work [3], we observed that the side chain of catalytic Ser presents higher mobility than the Thr in Tsa1. The additional methyl group in Thr side chain interacts with an aromatic ring in the adjacent dimer. Thus, the B-factor of active site regions in the 10 chains of Tsa2 structure are higher than observed to Tsa1. Now, we analyzed the interactions of catalytic Thr and Ser in an attempt to explain the differences in hyperoxidation resistance. We observed that the Ser⁴⁴ in some chains of Tsa2 are in position quite similar to Thr⁴⁴ in Tsa1 (Figure 11A). However, in three chains, the Ser⁴⁴ of Tsa2 are found in an alternative position compared to that one observed to Thr⁴⁴. Consequently, the motif GGLG is moved away from the C-terminal α -helix where YF motif is found (Figure 11B) and a helix upstream to the GGLG motif is also deformed. We propose that the high mobility of Ser side chain makes the interaction between GGLG and C-terminal motif YF more unstable and, therefore, facilitate the approximation between C_P and C_R and disulfide formation. In fact, our data indicated that, in yeast enzymes (displaying the GGLG/YF motifs), the differences in hyperoxidation are more pronounced between Ser-Prx and Thr-Prx. Accordingly, Peskin and colleagues (2021) [61] showed that substitution of human Prx2 C_R by amino acids that disrupts the C-terminal structure is able to affect the enzyme activity, indicating an interconnection between the C-terminal region and the active site microenvironment in eukaryotes. These data indicate that even regions located far from the active site can exert effect in the 2-Cys Prx activity.

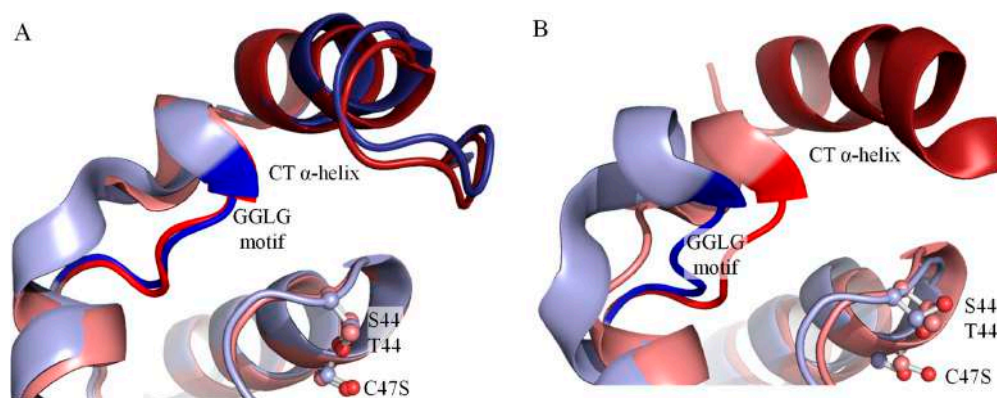


Figure 11. Active site, GGLG and YF motif regions of Tsa1 and Tsa2. (A) Superposition of Tsa1^{C47S} (3SBC = pink) and Tsa2^{C47S} (5DVB = light blue) show similar position of Thr⁴⁴ (Tsa1) and Ser⁴⁴ (Tsa2). Motif GGLG (red in Tsa1 and blue in Tsa2) and CT α -helix (dark red in Tsa1 and dark blue in Tsa2) are also in quite similar position. (B) When Ser⁴⁴ is found in an alternative conformation, GGLG motif is displaced to a further position, while CT α -helix does not appear in the crystallographic structure probably due to being more mobile and, consequently, with low resolution. Crystallographic structures are presented in cartoon and highlighted amino acids are presented as balls and sticks. Graphical models were generated by PyMOL.

In contrast, Thr/Ser substitutions in PaAhpC and SeAhpC produce less pronounced effects on the susceptibilities to hyperoxidation, possibly due to GGLG and YF motifs are absent at these enzymes. We compared the crystallographic structure of a Thr-Prx (*Salmonella typhimurium* AhpC-PDB entry 1YEP) with the only AhpC containing Ser in the PDB database (*Enterococcus faecalis*—PDB entry 5Y63), but we did not observe any major structural differences. However, it is worth to mention that disulfide formation in bacterial AhpC belonging to Thr-Prx group is considerable faster (75 s^{-1}) compared to human enzymes such as Prx2 and Prx3 (1.7 and 22 s^{-1} , respectively) [38,64].

We also observed that 2-Cys Prx are more susceptible to hyperoxidation by organic hydroperoxides than H_2O_2 (Figures 2, 3 and 6), as previously described [34], which may be related with the environment close to the active site pocket. Crystallographic observations revealed the presence of large hydrophobic portions around this region [40,43,48]. In fact, *ahpC* gene inactivation frequently render bacteria with increased sensitivity to organic peroxide and AhpC over-expression invariably leads to a gain of resistance against these oxidants [65–71]. Moreover, *Mycobacterium tuberculosis* and *Helicobacter pylori* mutants for *ahpC* gene accumulated higher levels of lipid hydroperoxides during aerobic growth, when compared to wild type strains [72].

Considering that presence of Ser in catalytic triad makes 2-Cys Prx resistant to C_p hyperoxidation, more stable as a decamer and increase its chaperone activity, we speculate that these enzymes may present complementary functions, protecting cells from severe stressful conditions. Ser-Prx might be more prone to hydroperoxides detoxification and thermal stress defense, while Thr-Prx would be better adapted to cell signaling functions. Notably, Ser-Prx were able to perform both peroxidase and chaperone activities after heat shock and possibly these molecular activities may occur simultaneously. Consistent with this idea, Ser-Prx are more frequent in bacteria, some of them pathogenic. Therefore, Ser-Prx might be considered as target for the development of specific inhibitors to prokaryotic enzymes. Finally, it is amazing to note that a single residue substitution by another with similar properties can confer remarkable functional and structural differences between very homologous enzymes.

5. Conclusions

The single natural substitution of the active site catalytic triad Thr to Ser occurs in several 2-Cys Prx from different organisms and influences the oligomeric redox state, resistance to hyperoxidation, thermostability, and chaperone activity defining two groups among the 2-Cys Prx: Ser-Prx and Thr-Prx. This substitution may have been selected during the evolutive process and can be directly related to differential functions of these enzymes in the cells.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/antiox10071032/s1>, Figure S1: Western blot to confirm C_p hyperoxidation of Tsa1 and Tsa2; Figure S2: Evaluation of AhpC hyperoxidation by western blotting; Figure S3: Sequence alignment of Tsa1, Tsa2 and bacterial typical 2-Cys Prx to evaluate the conservation of the hyperoxidation resistance motifs (named A and B) and catalytic triad Thr or Ser distribution; Spreadsheet S1: Distribution of typical 2-Cys Prx among the organisms; Table S1: Conservation of hyperoxidation resistance motifs in typical 2-Cys peroxiredoxins isoforms.

Author Contributions: Conceptualization, M.A.d.O., L.E.S.N., C.A.B., M.C.S. and C.A.T.; methodology, M.A.d.O., L.E.S.N., C.A.B., M.C.S. and C.A.T.; formal analysis, M.A.d.O., L.E.S.N., M.H.T., C.A.B., A.L.P.d.O., M.C.S., G.M.M., G.T.-S. and C.A.T.; investigation, C.A.B., M.C.S., A.L.P.d.O., G.M.M., G.T.-S., V.I.M.C. and C.A.T.; writing—original draft preparation, M.C.S., C.A.T., L.E.S.N. and M.A.d.O. All authors have read and agreed to the published version of the manuscript.

Funding: The authors M.A.d.O., M.C.S., A.L.P.d.O., C.A.T., and L.E.S.N. are members of the CEPID REDOXOMA from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (grant number 2013/07937-8). This work was also supported by grants from FAPESP to M.A.O. (2007/50930-3; 2017/19942-7) and M.H.T. (2017/20291-0). C.A.B., M.C.S., C.A.T., A.L.P.d.O., and V.I.M.C. were recipients of fellowships from FAPESP (2011/13500-6; 2017/06263-4; 2018/12628-8; 2019/04054-4; and 2020/02868-1).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The sequence of the *S. epidermidis ahpc* synthetic gene with the codons optimized for expression in *E. coli* is available at NCBI GenBank (accession code MZ133620).

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered a conflict of interest.

References

- Horta, B.B.; de Oliveira, M.A.; Discola, K.F.; Cussiol, J.R.; Netto, L.E. Structural and biochemical characterization of peroxiredoxin Qbeta from *Xylella fastidiosa*: Catalytic mechanism and high reactivity. *J. Biol. Chem.* **2010**, *285*, 16051–16065. [\[CrossRef\]](#) [\[PubMed\]](#)
- Ogusucu, R.; Rettori, D.; Munhoz, D.C.; Netto, L.E.; Augusto, O. Reactions of yeast thioredoxin peroxidases I and II with hydrogen peroxide and peroxyxynitrite: Rate constants by competitive kinetics. *Free Radic. Biol. Med.* **2007**, *42*, 326–334. [\[CrossRef\]](#) [\[PubMed\]](#)
- Tairum, C.A.; Santos, M.C.; Breyer, C.A.; Geyer, R.R.; Nieves, C.J.; Portillo-Ledesma, S.; Ferrer-Sueta, G.; Toledo, J.C., Jr.; Toyama, M.H.; Augusto, O.; et al. Catalytic Thr or Ser Residue Modulates Structural Switches in 2-Cys Peroxiredoxin by Distinct Mechanisms. *Sci. Rep.* **2016**, *6*, 33133. [\[CrossRef\]](#)
- Truzzi, D.R.; Coelho, F.R.; Paviani, V.; Alves, S.V.; Netto, L.E.S.; Augusto, O. The bicarbonate/carbon dioxide pair increases hydrogen peroxide-mediated hyperoxidation of human peroxiredoxin 1. *J. Biol. Chem.* **2019**, *294*, 14055–14067. [\[CrossRef\]](#) [\[PubMed\]](#)
- Bengtsson-Palme, J.; Alm Rosenblad, M.; Molin, M.; Blomberg, A. Metagenomics reveals that detoxification systems are underrepresented in marine bacterial communities. *BMC Genom.* **2014**, *15*, 749. [\[CrossRef\]](#)
- Chae, H.Z.; Kim, H.J.; Kang, S.W.; Rhee, S.G. Characterization of three isoforms of mammalian peroxiredoxin that reduce peroxides in the presence of thioredoxin. *Diabetes Res. Clin. Pract.* **1999**, *45*, 101–112. [\[CrossRef\]](#)
- Ghaemmaghami, S.; Huh, W.K.; Bower, K.; Howson, R.W.; Belle, A.; Dephoure, N.; O'Shea, E.K.; Weissman, J.S. Global analysis of protein expression in yeast. *Nature* **2003**, *425*, 737–741. [\[CrossRef\]](#)
- Wood, Z.A.; Schroder, E.; Robin Harris, J.; Poole, L.B. Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem. Sci.* **2003**, *28*, 32–40. [\[CrossRef\]](#)
- Cox, A.G.; Winterbourn, C.C.; Hampton, M.B. Mitochondrial peroxiredoxin involvement in antioxidant defence and redox signalling. *Biochem. J.* **2009**, *425*, 313–325. [\[CrossRef\]](#)
- Winterbourn, C.C. Reconciling the chemistry and biology of reactive oxygen species. *Nat. Chem. Biol.* **2008**, *4*, 278–286. [\[CrossRef\]](#)
- Brown, J.D.; Day, A.M.; Taylor, S.R.; Tomalin, L.E.; Morgan, B.A.; Veal, E.A. A peroxiredoxin promotes H₂O₂ signaling and oxidative stress resistance by oxidizing a thioredoxin family protein. *Cell Rep.* **2013**, *5*, 1425–1435. [\[CrossRef\]](#)
- Netto, L.E.; Antunes, F. The Roles of Peroxiredoxin and Thioredoxin in Hydrogen Peroxide Sensing and in Signal Transduction. *Mol. Cells* **2016**, *39*, 65–71.
- Rhee, S.G.; Woo, H.A. Multiple functions of peroxiredoxins: Peroxidases, sensors and regulators of the intracellular messenger H₂O₂, and protein chaperones. *Antioxid. Redox Signal.* **2011**, *15*, 781–794. [\[CrossRef\]](#) [\[PubMed\]](#)
- Stocker, S.; Maurer, M.; Ruppert, T.; Dick, T.P. A role for 2-Cys peroxiredoxins in facilitating cytosolic protein thiol oxidation. *Nat. Chem. Biol.* **2018**, *14*, 148–155. [\[CrossRef\]](#) [\[PubMed\]](#)
- Tang, H.M.; Siu, K.L.; Wong, C.M.; Jin, D.Y. Loss of yeast peroxiredoxin Tsa1p induces genome instability through activation of the DNA damage checkpoint and elevation of dNTP levels. *PLoS Genet.* **2009**, *5*, e1000697. [\[CrossRef\]](#)
- Chae, H.Z.; Chung, S.J.; Rhee, S.G. Thioredoxin-dependent peroxide reductase from yeast. *J. Biol. Chem.* **1994**, *269*, 27670–27678. [\[CrossRef\]](#)
- Jonsson, T.J.; Ellis, H.R.; Poole, L.B. Cysteine reactivity and thiol-disulfide interchange pathways in AhpF and AhpC of the bacterial alkyl hydroperoxide reductase system. *Biochemistry* **2007**, *46*, 5709–5721. [\[CrossRef\]](#) [\[PubMed\]](#)
- Netto, L.E.S.; Chae, H.Z.; Kang, S.W.; Rhee, S.G.; Stadtman, E.R. Removal of hydrogen peroxide by thiol-specific antioxidant enzyme (TSA) is involved with its antioxidant properties. TSA possesses thiol peroxidase activity. *J. Biol. Chem.* **1996**, *271*, 15315–15321. [\[CrossRef\]](#) [\[PubMed\]](#)
- Pineyro, M.D.; Arcari, T.; Robello, C.; Radi, R.; Trujillo, M. Tryparedoxin peroxidases from *Trypanosoma cruzi*: High efficiency in the catalytic elimination of hydrogen peroxide and peroxyxynitrite. *Arch. Biochem. Biophys.* **2011**, *507*, 287–295. [\[CrossRef\]](#) [\[PubMed\]](#)
- Hall, A.; Parsonage, D.; Poole, L.B.; Karplus, P.A. Structural evidence that peroxiredoxin catalytic power is based on transition-state stabilization. *J. Mol. Biol.* **2010**, *402*, 194–209. [\[CrossRef\]](#)

21. Chae, H.Z.; Kim, I.H.; Kim, K.; Rhee, S.G. Cloning, sequencing, and mutation of thiol-specific antioxidant gene of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **1993**, *268*, 16815–16821. [\[CrossRef\]](#)
22. Flohe, L.; Budde, H.; Bruns, K.; Castro, H.; Clos, J.; Hofmann, B.; Kansal-Kalavar, S.; Krumme, D.; Menge, U.; Plank-Schumacher, K.; et al. Tryparedoxin peroxidase of *Leishmania donovani*: Molecular cloning, heterologous expression, specificity, and catalytic mechanism. *Arch. Biochem. Biophys.* **2002**, *397*, 324–335. [\[CrossRef\]](#) [\[PubMed\]](#)
23. König, J.; Lotte, K.; Plessow, R.; Brockhinke, A.; Baier, M.; Dietz, K.J. Reaction mechanism of plant 2-Cys peroxiredoxin. Role of the C terminus and the quaternary structure. *J. Biol. Chem.* **2003**, *278*, 24409–24420. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Miranda-Vizuete, A.; Damdimopoulos, A.E.; Pedrajas, J.R.; Gustafsson, J.A.; Spyrou, G. Human mitochondrial thioredoxin reductase cDNA cloning, expression and genomic organization. *Eur. J. Biochem.* **1999**, *261*, 405–412. [\[CrossRef\]](#)
25. Nagy, P.; Kartón, A.; Betz, A.; Peskin, A.V.; Pace, P.; O'Reilly, R.J.; Hampton, M.B.; Radom, L.; Winterbourn, C.C. Model for the exceptional reactivity of peroxiredoxins 2 and 3 with hydrogen peroxide: A kinetic and computational study. *J. Biol. Chem.* **2011**, *286*, 18048–18055. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Portillo-Ledesma, S.; Sardi, F.; Manta, B.; Tourn, M.V.; Clippe, A.; Knoops, B.; Alvarez, B.; Coitino, E.L.; Ferrer-Sueta, G. Deconstructing the catalytic efficiency of peroxiredoxin-5 peroxidatic cysteine. *Biochemistry* **2014**, *53*, 6113–6125. [\[CrossRef\]](#)
27. Biteau, B.; Labarre, J.; Toledano, M.B. ATP-dependent reduction of cysteine-sulphinic acid by *S. cerevisiae* sulphiredoxin. *Nature* **2003**, *425*, 980–984. [\[CrossRef\]](#)
28. Jonsson, T.J.; Johnson, L.C.; Lowther, W.T. Structure of the sulphiredoxin-peroxiredoxin complex reveals an essential repair embrace. *Nature* **2008**, *451*, 98–101. [\[CrossRef\]](#)
29. Day, A.M.; Brown, J.D.; Taylor, S.R.; Rand, J.D.; Morgan, B.A.; Veal, E.A. Inactivation of a peroxiredoxin by hydrogen peroxide is critical for thioredoxin-mediated repair of oxidized proteins and cell survival. *Mol. Cell* **2012**, *45*, 398–408. [\[CrossRef\]](#)
30. Del Olmo, M.; Kramer, A.; Herzel, H. A Robust Model for Circadian Redox Oscillations. *Int. J. Mol. Sci.* **2019**, *20*, 2368. [\[CrossRef\]](#)
31. Edgar, R.S.; Green, E.W.; Zhao, Y.; van Ooijen, G.; Olmedo, M.; Qin, X.; Xu, Y.; Pan, M.; Valekunja, U.K.; Feeney, K.A.; et al. Peroxiredoxins are conserved markers of circadian rhythms. *Nature* **2012**, *485*, 459–464. [\[CrossRef\]](#) [\[PubMed\]](#)
32. Veal, E.A.; Underwood, Z.E.; Tomalin, L.E.; Morgan, B.A.; Pillay, C.S. Hyperoxidation of Peroxiredoxins: Gain or Loss of Function? *Antioxid. Redox Signal.* **2018**, *28*, 574–590. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Jang, H.H.; Lee, K.O.; Chi, Y.H.; Jung, B.G.; Park, S.K.; Park, J.H.; Lee, J.R.; Lee, S.S.; Moon, J.C.; Yun, J.W.; et al. Two enzymes in one; two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function. *Cell* **2004**, *117*, 625–635. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Kamariah, N.; Eisenhaber, B.; Eisenhaber, F.; Gruber, G. Molecular mechanism of the *Escherichia coli* AhpC in the function of a chaperone under heat-shock conditions. *Sci. Rep.* **2018**, *8*, 14151. [\[CrossRef\]](#)
35. Moon, J.C.; Hah, Y.S.; Kim, W.Y.; Jung, B.G.; Jang, H.H.; Lee, J.R.; Kim, S.Y.; Lee, Y.M.; Jeon, M.G.; Kim, C.W.; et al. Oxidative stress-dependent structural and functional switching of a human 2-Cys peroxiredoxin isotype II that enhances HeLa cell resistance to H₂O₂-induced cell death. *J. Biol. Chem.* **2005**, *280*, 28775–28784. [\[CrossRef\]](#)
36. Teixeira, F.; Tse, E.; Castro, H.; Makepeace, K.A.T.; Meinen, B.A.; Borchers, C.H.; Poole, L.B.; Bardwell, J.C.; Tomas, A.M.; Southworth, D.R.; et al. Chaperone activation and client binding of a 2-cysteine peroxiredoxin. *Nat. Commun.* **2019**, *10*, 659. [\[CrossRef\]](#)
37. Hall, A.; Nelson, K.; Poole, L.B.; Karplus, P.A. Structure-based insights into the catalytic power and conformational dexterity of peroxiredoxins. *Antioxid. Redox Signal.* **2011**, *15*, 795–815. [\[CrossRef\]](#)
38. Peskin, A.V.; Dickerhof, N.; Poynton, R.A.; Paton, L.N.; Pace, P.E.; Hampton, M.B.; Winterbourn, C.C. Hyperoxidation of peroxiredoxins 2 and 3: Rate constants for the reactions of the sulfenic acid of the peroxidatic cysteine. *J. Biol. Chem.* **2013**, *288*, 14170–14177. [\[CrossRef\]](#)
39. Wood, Z.A.; Poole, L.B.; Hantgan, R.R.; Karplus, P.A. Dimers to doughnuts: Redox-sensitive oligomerization of 2-cysteine peroxiredoxins. *Biochemistry* **2002**, *41*, 5493–5504. [\[CrossRef\]](#)
40. Wood, Z.A.; Poole, L.B.; Karplus, P.A. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* **2003**, *300*, 650–653. [\[CrossRef\]](#)
41. Angelucci, F.; Saccoccia, F.; Ardini, M.; Boumis, G.; Brunori, M.; Di Leandro, L.; Ippoliti, R.; Miele, A.E.; Natoli, G.; Scotti, S.; et al. Switching between the alternative structures and functions of a 2-Cys peroxiredoxin, by site-directed mutagenesis. *J. Mol. Biol.* **2013**, *425*, 4556–4568. [\[CrossRef\]](#)
42. Pastor-Flores, D.; Talwar, D.; Pedre, B.; Dick, T.P. Real-time monitoring of peroxiredoxin oligomerization dynamics in living cells. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 16313–16323. [\[CrossRef\]](#)
43. Dip, P.V.; Kamariah, N.; Nartey, W.; Beushausen, C.; Kostyuchenko, V.A.; Ng, T.S.; Lok, S.M.; Saw, W.G.; Eisenhaber, F.; Eisenhaber, B.; et al. Key roles of the *Escherichia coli* AhpC C-terminus in assembly and catalysis of alkylhydroperoxide reductase, an enzyme essential for the alleviation of oxidative stress. *Biochim. Biophys. Acta* **2014**, *1837*, 1932–1943. [\[CrossRef\]](#)
44. Barranco-Medina, S.; Dietz, K.J. Thermodynamics of 2-Cys peroxiredoxin assembly determined by isothermal titration calorimetry. *Methods Enzymol.* **2009**, *466*, 409–430.
45. Liebthal, M.; Kushwah, M.S.; Kukura, P.; Dietz, K.J. Single molecule mass photometry reveals dynamic oligomerization of plant and human peroxiredoxins for functional conservation and diversification. *bioRxiv* **2021**. [\[CrossRef\]](#)

46. Morais, M.A.; Giuseppe, P.O.; Souza, T.A.; Alegria, T.G.; Oliveira, M.A.; Netto, L.E.; Murakami, M.T. How pH modulates the dimer-decamer interconversion of 2-Cys peroxiredoxins from the Prx1 subfamily. *J. Biol. Chem.* **2015**, *290*, 8582–8590. [\[CrossRef\]](#) [\[PubMed\]](#)
47. Morais, M.A.B.; Giuseppe, P.O.; Souza, T.; Castro, H.; Honorato, R.V.; Oliveira, P.S.L.; Netto, L.E.S.; Tomas, A.M.; Murakami, M.T. Calcium and magnesium ions modulate the oligomeric state and function of mitochondrial 2-Cys peroxiredoxins in Leishmania parasites. *J. Biol. Chem.* **2017**, *292*, 7023–7039. [\[CrossRef\]](#) [\[PubMed\]](#)
48. Pan, A.; Balakrishna, A.M.; Nartey, W.; Kohlmeier, A.; Dip, P.V.; Bhushan, S.; Gruber, G. Atomic structure and enzymatic insights into the vancomycin-resistant *Enterococcus faecalis* (V583) alkylhydroperoxide reductase subunit C. *Free Radic. Biol. Med.* **2018**, *115*, 252–265. [\[CrossRef\]](#) [\[PubMed\]](#)
49. Nelson, K.J.; Perkins, A.; Van Swearingen, A.E.D.; Hartman, S.; Brereton, A.E.; Parsonage, D.; Salsbury, F.R., Jr.; Karplus, P.A.; Poole, L.B. Experimentally Dissecting the Origins of Peroxiredoxin Catalysis. *Antioxid. Redox Signal.* **2018**, *28*, 521–536. [\[CrossRef\]](#) [\[PubMed\]](#)
50. Bolduc, J.A.; Nelson, K.J.; Haynes, A.C.; Lee, J.; Reisz, J.A.; Graff, A.H.; Clodfelter, J.E.; Parsonage, D.; Poole, L.B.; Furdul, C.M.; et al. Novel hyperoxidation resistance motifs in 2-Cys peroxiredoxins. *J. Biol. Chem.* **2018**, *293*, 11901–11912. [\[CrossRef\]](#) [\[PubMed\]](#)
51. Oliveira, M.A.; Discola, K.F.; Alves, S.V.; Medrano, F.J.; Guimaraes, B.G.; Netto, L.E. Insights into the specificity of thioredoxin reductase-thioredoxin interactions. A structural and functional investigation of the yeast thioredoxin system. *Biochemistry* **2010**, *49*, 3317–3326. [\[CrossRef\]](#)
52. Tairum, C.A., Jr.; de Oliveira, M.A.; Horta, B.B.; Zera, F.J.; Netto, L.E. Disulfide biochemistry in 2-cys peroxiredoxin: Requirement of Glu50 and Arg146 for the reduction of yeast Tsa1 by thioredoxin. *J. Mol. Biol.* **2012**, *424*, 28–41. [\[CrossRef\]](#)
53. Gonzalez Porque, P.; Baldesten, A.; Reichard, P. Purification of a thioredoxin system from yeast. *J. Biol. Chem.* **1970**, *245*, 2363–2370. [\[CrossRef\]](#)
54. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403–410. [\[CrossRef\]](#)
55. Mistry, J.; Finn, R.D.; Eddy, S.R.; Bateman, A.; Punta, M. Challenges in homology search: HMMER3 and convergent evolution of coiled-coil regions. *Nucleic Acids Res.* **2013**, *41*, e121. [\[CrossRef\]](#) [\[PubMed\]](#)
56. Hofmann, B.; Hecht, H.J.; Flohe, L. Peroxiredoxins. *Biol. Chem.* **2002**, *383*, 347–364. [\[CrossRef\]](#) [\[PubMed\]](#)
57. Huynh, K.; Partch, C.L. Analysis of protein stability and ligand interactions by thermal shift assay. *Curr. Protoc. Protein Sci.* **2015**, *79*, 21–28. [\[CrossRef\]](#)
58. Kriznik, A.; Libiad, M.; Le Cordier, H.; Boukhenouna, S.; Toledano, M.B.; Rahuel-Clermont, S. Dynamics of a Key Conformational Transition in the Mechanism of Peroxiredoxin Sulfinylation. *ACS Catal.* **2020**, *10*, 3326–3339. [\[CrossRef\]](#)
59. Munhoz, D.C.; Netto, L.E. Cytosolic thioredoxin peroxidase I and II are important defenses of yeast against organic hydroperoxide insult: Catalases and peroxiredoxins cooperate in the decomposition of H₂O₂ by yeast. *J. Biol. Chem.* **2004**, *279*, 35219–35227. [\[CrossRef\]](#)
60. Zwick, J.V.; Noble, S.; Ellaicy, Y.K.; Coe, G.D.; Hakey, D.J.; King, A.N.; Sadauskas, A.J.; Faulkner, M.J. AhpA is a peroxidase expressed during biofilm formation in *Bacillus subtilis*. *Microbiologyopen* **2017**, *6*, e00403. [\[CrossRef\]](#)
61. Peskin, A.V.; Meotti, F.C.; Kean, K.M.; Gobl, C.; Peixoto, A.S.; Pace, P.E.; Horne, C.R.; Heath, S.G.; Crowther, J.M.; Dobson, R.C.J.; et al. Modifying the resolving cysteine affects the structure and hydrogen peroxide reactivity of peroxiredoxin 2. *J. Biol. Chem.* **2021**, in press. [\[CrossRef\]](#)
62. Chuang, M.H.; Wu, M.S.; Lo, W.L.; Lin, J.T.; Wong, C.H.; Chiou, S.H. The antioxidant protein alkylhydroperoxide reductase of *Helicobacter pylori* switches from a peroxide reductase to a molecular chaperone function. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 2552–2557. [\[CrossRef\]](#)
63. Lee, J.T.; Lee, S.S.; Mondal, S.; Tripathi, B.N.; Kim, S.; Lee, K.W.; Hong, S.H.; Bai, H.W.; Cho, J.Y.; Chung, B.Y. Enhancement of the Chaperone Activity of Alkyl Hydroperoxide Reductase C from *Pseudomonas aeruginosa* PAO1 Resulting from a Point-Specific Mutation Confers Heat Tolerance in *Escherichia coli*. *Mol. Cells* **2016**, *39*, 594–602. [\[CrossRef\]](#)
64. Parsonage, D.; Nelson, K.J.; Ferrer-Sueta, G.; Alley, S.; Karplus, P.A.; Furdul, C.M.; Poole, L.B. Dissecting peroxiredoxin catalysis: Separating binding, peroxidation, and resolution for a bacterial AhpC. *Biochemistry* **2015**, *54*, 1567–1575. [\[CrossRef\]](#)
65. Cha, M.K.; Bae, Y.J.; Kim, K.J.; Park, B.J.; Kim, I.H. Characterization of two alkyl hydroperoxide reductase C homologs alkyl hydroperoxide reductase C_{H1} and alkyl hydroperoxide reductase C_{H2} in *Bacillus subtilis*. *World J. Biol. Chem.* **2015**, *6*, 249–264. [\[CrossRef\]](#) [\[PubMed\]](#)
66. Charoenlap, N.; Eiamphungporn, W.; Chauvatcharin, N.; Utamapongchai, S.; Vattanaviboon, P.; Mongkolsuk, S. OxyR mediated compensatory expression between *ahpC* and *katA* and the significance of *ahpC* in protection from hydrogen peroxide in *Xanthomonas campestris*. *FEMS Microbiol. Lett.* **2005**, *249*, 73–78. [\[CrossRef\]](#) [\[PubMed\]](#)
67. Fukumori, F.; Kishii, M. Molecular cloning and transcriptional analysis of the alkyl hydroperoxide reductase genes from *Pseudomonas putida* KT2442. *J. Gen. Appl. Microbiol.* **2001**, *47*, 269–277. [\[CrossRef\]](#) [\[PubMed\]](#)
68. Loprasert, S.; Atichartpongkun, S.; Whangsuk, W.; Mongkolsuk, S. Isolation and analysis of the *Xanthomonas* alkyl hydroperoxide reductase gene and the peroxide sensor regulator genes *ahpC* and *ahpF-oxyR-orfX*. *J. Bacteriol.* **1997**, *179*, 3944–3949. [\[CrossRef\]](#)
69. Sherman, D.R.; Mdululi, K.; Hickey, M.J.; Arain, T.M.; Morris, S.L.; Barry, C.E., 3rd; Stover, C.K. Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. *Science* **1996**, *272*, 1641–1643. [\[CrossRef\]](#)

-
70. Wilson, T.; de Lisle, G.W.; Marcinkeviciene, J.A.; Blanchard, J.S.; Collins, D.M. Antisense RNA to *ahpC*, an oxidative stress defence gene involved in isoniazid resistance, indicates that AhpC of *Mycobacterium bovis* has virulence properties. *Microbiology* **1998**, *144*, 2687–2695. [[CrossRef](#)]
 71. Zhang, B.; Gu, H.; Yang, Y.; Bai, H.; Zhao, C.; Si, M.; Su, T.; Shen, X. Molecular Mechanisms of AhpC in Resistance to Oxidative Stress in *Burkholderia thailandensis*. *Front. Microbiol.* **2019**, *10*, 1483. [[CrossRef](#)] [[PubMed](#)]
 72. Wang, G.; Conover, R.C.; Benoit, S.; Olczak, A.A.; Olson, J.W.; Johnson, M.K.; Maier, R.J. Role of a bacterial organic hydroperoxide detoxification system in preventing catalase inactivation. *J. Biol. Chem.* **2004**, *279*. [[CrossRef](#)] [[PubMed](#)]

APÊNDICE 3

Adenanthin Is an Efficient Inhibitor of Peroxiredoxins from Pathogens, Inhibits Bacterial Growth, and Potentiates Antibiotic Activities

Melina Cardoso dos Santos, Carlos Abrunhosa Tairum, Vitoria Isabela Montanhero Cabrera, Ana Carolina Guimarães Cauz, Luiz Fernando Ribeiro, José Carlos Toledo Junior, Marcos Hikari Toyama, João Henrique Ghilardi Lago, Marcelo Brocchi, Luis Eduardo Soares Netto,* and Marcos Antonio de Oliveira*



Cite This: <https://doi.org/10.1021/acs.chemrestox.2c00049>



Read Online

ACCESS |



Metrics & More

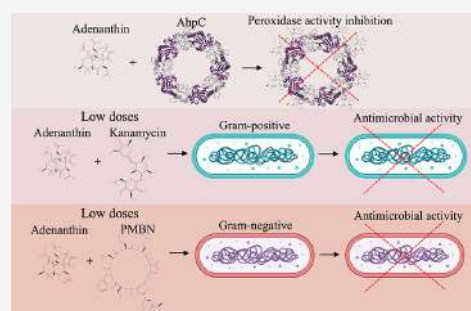


Article Recommendations



Supporting Information

ABSTRACT: The emergence and re-emergence of bacterial strains resistant to multiple drugs represent a global health threat, and the search for novel biological targets is a worldwide concern. AhpC are enzymes involved in bacterial redox homeostasis by metabolizing diverse kinds of hydroperoxides. In pathogenic bacteria, AhpC are related to several functions, as some isoforms are characterized as virulence factors. However, no inhibitor has been systematically evaluated to date. Here we show that the natural *ent*-kaurane Adenanthin (Adn) efficiently inhibits AhpC and molecular interactions were explored by computer assisted simulations. Additionally, Adn interferes with growth and potentializes the effect of antibiotics (kanamycin and PMBN), positioning Adn as a promising compound to treat infections caused by multiresistant bacterial strains.



INTRODUCTION

The high incidence of drug-resistant bacteria is a huge problem to public health around the world.¹ Several works are focused on discovering new drugs aiming to combat these strains. In this context, the search for inhibitors to antioxidant enzymes emerges as an important field, since the bacterial infection triggers oxidative defense mechanisms.² Among the enzymes that bacteria use to fight oxidative stresses generated by host defense, the Peroxiredoxins (Prx) are the main scavengers of several hydroperoxides in the cells.^{3–6} Prx enzymes can be subdivided into six classes based on structural and functional characteristics: AhpC-Prx1, BCP-PrxQ, Tpx, PrxS, Prx6, and AhpE.⁷ Among them, the AhpC-Prx1 class is widely distributed and one of the most abundant bacterial enzymes in several Gram-positive and Gram-negative species.^{2,8} The bacterial enzymes of this class are named as AhpC (Alkyl hydroperoxide reductase C), since they were initially shown to decompose alkyl hydroperoxides.⁹ Later studies revealed that AhpC are also very reactive to several kinds of hydroperoxide, such as hydrogen peroxide and peroxyxynitrite.^{10–12}

The catalytic activity of AhpC-Prx1 members is based on a conserved catalytic triad composed by a reactive cysteine residue (peroxidatic cysteine - C_p), arginine (Arg), and threonine (Thr) or serine (Ser). After hydroperoxide decomposition, the C_p forms a disulfide with a second cysteine residue (resolving Cys, C_r) found in the adjacent subunit. In

bacteria, the disulfide form of AhpC is commonly recycled by the flavoenzyme AhpF, which uses NADH as the electron donor,¹³ or by the Trx system, composed of thioredoxin (Trx) and thioredoxin reductase (TrxR) and NADPH is the electron donor.^{14–16} (Figure 1).

C_p and C_r are found in different subunits in AhpC-Prx1 and the minimum catalytic unit of these enzymes is a homodimer (Figure 1). However, five homodimers can assemble as decamers (Figure 1), which has been associated with catalytic activity and interaction with substrates.^{17–19} However, after peroxide decomposition and disulfide formation, these enzymes tend to dissociate in dimers.^{20,21} This dissociation may be related to the active site pocket position, which is part of the homodimers interface when these enzymes are in the decameric form.^{19,22}

It has already been shown that the quaternary structure transitions of AhpC-Prx1 enzymes are related to the composition of the catalytic triad.⁸ As mentioned above, the

Special Issue: Natural Products in Redox Toxicology
VSI

Received: February 9, 2022



ACS Publications

© XXXX American Chemical Society

A

<https://doi.org/10.1021/acs.chemrestox.2c00049>
Chem. Res. Toxicol. XXXX, XXX, XXX–XXX

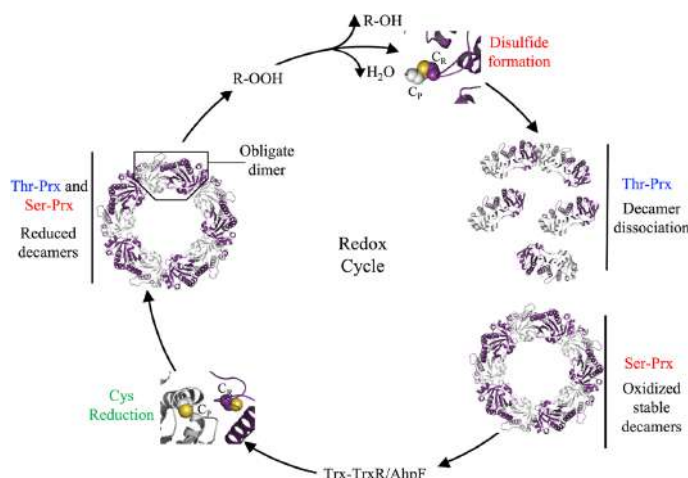


Figure 1. AhpC-Prx1 catalytic cycle. On the left, reduced AhpC-Prx1 enzyme is represented as decamer (each dimer subunit is represented by white or purple). The hydroperoxide (R-OOH) decomposition result in the intermolecular disulfide formation between C_p and C_r. The oxidized form of the Thr-AhpC enzymes dissociate into dimers and/or other oligomeric species. In turn, Ser-AhpC remains as decamers. AhpC-Prx1 disulfide reduction is performed by the Trx system (eukaryotes and some bacteria) or AhpF (only for bacteria), which render the enzymes able to decompose hydroperoxides.

catalytic triad is composed by C_p, Arg, and Thr or Ser. While AhpC-Prx1 containing Thr (Thr-AhpC) may dissociate in dimers in a redox-dependent way, the enzymes containing Ser (Ser-AhpC) are decamers independently of the redox state.^{19,22,23} Additionally, Ser-AhpC possess higher thermostability and chaperone activity when compared to Thr-AhpC.⁸ It is worth mentioning that Prx containing Ser are considerably more frequent in bacteria when compared to multicellular organisms, and some species possess both Thr-AhpC and Ser-AhpC isoforms, suggesting some functional diversification.^{24–27}

Several works indicated the importance of AhpC to pathogenic bacteria and its involvement in virulence.^{2,25,28–30} In this context, the AhpC may represent a promising target to microbicidal drugs, but no study has systematically explored inhibitors for these bacterial enzymes. Conversely, some studies involving mammalian AhpC-Prx1 have identified inhibitor molecules, including natural compounds.^{31–34} Indeed, an inhibitor named Adenanthin (Adn), a natural diterpenoid from *Isodon adenantha*, was described as an effective inhibitor of two cytosolic human isoforms of AhpC-Prx1 (Prx1 and Prx2). Adn performs a Michael addition, in which an α,β -unsaturated carbonyl system is able to covalently modify the thiol groups of catalytic cysteines.³¹ In human neoplastic cells, Adn treatment inhibits Prx isoforms and interferes with the cell growth of tumor cells, while presenting low toxicity to healthy cells^{31,35,36} and low side effects in human tumors xenograft mice models.^{36,37}

Here we show that Adn is an effective inhibitor of both Thr-AhpC and Ser-AhpC. The IC₅₀ value of Adn for *Escherichia coli* AhpC was similar to 10-fold lower than the value determined for human isoforms (Prx1 and Prx2, respectively). The inhibitory mechanism of AhpCs by Adn is similar to the one described for mammalian isoforms, involving modification of catalytic cysteine residues. To gain further insights into the Adn-AhpC interaction, we performed molecular docking analysis and verified that Adn can be stabilized mainly by

several hydrophobic interactions with the side chain of residues present at the dimer–dimer interfaces of the decamer. We also observed that only Gram-positive bacterial cell growth was inhibited by Adn. Since the bacterial cell wall is a barrier to antibiotic accessibility, we tested the association of Adn with polymyxin B nonapeptide (PMBN), a permeation compound, over *E. coli* cells (Gram-negative) and showed that the compounds association enhance their microbial activity. Finally, we described that administration of Adn combined with kanamycin maximizes the antibiotic effect even in very low doses of the antibiotics. Taken together, our data place Adn as the first inhibitor systematically characterized for AhpC and demonstrate that the use of this molecule in combination with antibacterial drugs potentiates the action of such pharmaceuticals.

MATERIALS AND METHODS

Reagents. All the chemical compounds were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) and Thermo Scientific (Logan, UT, U.S.A.). Adn was purchased from ChemFaces (Wuhan, HB, China).

Cell Culture Media. Luria–Bertani (LB): (1% tryptone; 0.5% yeast extract; 0.5% NaCl) and Mueller Hinton (MH, Kasvi, Brazil). To bacteria harboring recombinant plasmids, the selections were performed supplementing the media with ampicillin (100 μ g/mL).

Cloning, Sequencing, and Site Directed Mutagenesis. The Trx system from *Escherichia coli* (EcTrx and EcTrxR) was previously obtained.⁸ AhpC and AhpF from *E. coli* (EcAhpC and EcAhpF) were amplified by PCR from genomic DNA using the oligonucleotides (Ec_AhpC_F: 5'-CGC GAT CCA TAT GAT GTC CTT GAT TAA CAC CAA AAT TAA A-3' and Ec_AhpC_R: 5'-CGC AAG CTT GGA TCC TTA GAT TTT ACC AAC CAG GTC CAG-3'; Ec_AhpF_F: 5'-CGC GAT CCA TAT GAT GCT CGA CAC AAA TAT GAA AAC TCA A-3' and Ec_AhpF_R: 5'-CGC AAG CTT GGA TCC TTA TGC AGT TTT GGT GCG AAT CAG-3') containing adapters with sites to *Nde* I and *Bam*HI restriction enzymes (underlined bases). Amplified DNA fragments and pET15b vector were digested with *Nde* I and *Bam*HI before the ligation procedures using T4 DNA ligase (Thermo Scientific). The resulting recombinant vectors were used to transform *E. coli* DH5 α strains.

B

<https://doi.org/10.1021/acs.chemrestox.2c00049>
Chem. Res. Toxicol. XXXX, XXX, XXX–XXX

Table 1. Strains Used in this Work

species	strain/plasmid	source
<i>Bacillus subtilis</i>	ATCC 55569	ATCC ^a
<i>Escherichia coli</i>	ATCC25922	ATCC ^a
<i>E. coli</i>	BL21 DE3/pET15b-ahpc (Ec)	this work
<i>E. coli</i>	BL21 DE3/pET15b-ahpc44s (Ec)	this work
<i>E. coli</i>	BL21 DE3/pET15b-trx (Ec)	this work
<i>E. coli</i>	BL21 DE3/pET15b-trxr (Ec)	this work
<i>E. coli</i>	BL21 DE3/pET15b-ahpf (Ec)	this work
<i>E. coli</i>	DH5 α	Sigma-Aldrich
<i>E. coli</i>	XL1-Blue	Agilent
<i>Pseudomonas aeruginosa</i>	ATCC 29853	ATCC ^a
<i>Salmonella typhimurium</i> LT2	ATCC 19585	ATCC ^a
<i>Staphylococcus aureus</i>	ATCC 29213	ATCC ^a
<i>Staphylococcus epidermidis</i>	ATCC 12228	ATCC ^a

^aAmerican Type Culture Collection; Genes: Ec = *E. coli*.

After propagation, the plasmids were extracted and sequenced in an ABI 3730 DNA Analyzer (Applied Biosystems) using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

The catalytic triad Thr from EcAhpC was substituted by Ser using Quick Change II Kit (Stratagene), using the primers EcAhpC_T44S_F 5'-GCT GAC TTT TCT TTC GTA TGC-3' and EcAhpC_T44S_R 5'-GCA TAC GAA AGA AAA GTC AGC-3' following manufacturer's instructions. The products were used to transform *E. coli* XL1-Blue strain (Agilent Technologies) and single colonies were grown on Luria–Bertani (LB) medium for 16 h. Then, plasmids were extracted, purified, and sequenced. The plasmids containing the correct constructs were used to transform *E. coli* BL21 (DE3) (Sigma-Aldrich) for heterologous expression.

Bacterial Strains. All strains used in this study are summarized in Table 1.

Expression, Purification, and Quantification. *E. coli* BL21 (DE3) cells (Lucigen, Middleton, WI, U.S.A.) containing pET15b vector cloned with recombinant genes were inoculated separately into 10 mL of LB medium containing 100 μ g/mL ampicillin and grown for 16 h/37 $^{\circ}$ C/250 rpm in an orbital shaker. Subsequently, the culture was transferred to 1 L of fresh LB/Amp and grown to OD₆₀₀ $\sim$ 0.6. Then, IPTG was added to a final concentration of 0.1 mM. The expression was performed for 3 h/37 $^{\circ}$ C/250 rpm, and then the cells were harvested by centrifugation (30 min/4 $^{\circ}$ C/4.000g) and resuspended in 50 mM Tris buffer (pH 7.4), 500 mM NaCl. Cell disruptions were performed by sonication (30% amplitude) and nucleic acids were removed using streptomycin sulfate ([Final] = 1%). Next the extracts were centrifuged for 45 min/4 $^{\circ}$ C/12.500g, and the protein extracts free of nucleic acids were collected. Once the proteins were expressed containing a His-tag, purification was performed by immobilized metal affinity chromatography using His-Trap columns (GE Healthcare, Piscataway, U.S.A.) by imidazole gradient. The quality of purification was assessed by SDS-PAGE (12%) under reducing conditions. After these procedures, the proteins were desalted via gel filtration chromatography using PD10 columns (GE Healthcare, Piscataway, U.S.A.) and concentrated by centrifugation (4.000g/4 $^{\circ}$ C) using Ultracel YM-30 concentrator (Millipore) to a final concentration of $\sim$ 1–5 mg/mL. The enzyme concentrations were determined by absorbance at 280 nm, considering the molar extinction coefficients for each protein (Table 2) obtained by the ProtParam tool (<https://web.expasy.org/protparam/>).

Treatment of Proteins with Adn. EcAhpC, EcAhpC^{T44S}, EcAhpF, EcTrx1, and EcTrxR proteins at a concentration of 100 μ M were reduced with 5 mM dithiothreitol (DTT) for 30 min at room temperature. Next, proteins were desalted on a PD10 column (GE Healthcare), quantified, and treated with fixed (60 μ M or 20 molar equivalents) or varying concentrations of Adn (1.5, 3.0, 7.5, 15, or 30 μ M) for 1 h at room temperature. Finally, the treated proteins were desalted again to remove Adn excess and quantified for use in subsequent assays.

Table 2. Molar Extinction Coefficients and Molecular Weight for Wild-Type, Mutant AhpC, and Reductant Systems Proteins

protein	ϵ 280 nm (M ⁻¹ cm ⁻¹)	molecular weight (kDa)	Uniprot entry
EcAhpC	24075	21.58	P0AE08
EcAhpC ^{T44S}	24075	21.57	^a
EcAhpF	34755	57.00	P35340
EcTrx	14105	12.63	P0AA25
EcTrxR	19160	35.45	P0A9P4

^aThe enzyme containing catalytic triad Thr to Ser substitution was constructed to this work.

Assessment of Oxidoreductase Activity of the Enzymes. To evaluate the effect of Adn treatment over the oxidoreductase activity, the NADPH oxidation (EcAhpC, EcAhpC^{T44S}, EcTrx1, and EcTrxR) or the DTNB reduction assays (EcAhpF) were employed as previously described.^{8,38}

Evaluation of AhpC Disulfide Formation After Adn Treatment by Non-Reducing SDS-PAGE. To investigate if the inhibition by Adn occurs by the cysteine blocking, we verified the disulfide formation by non-reducing SDS-PAGE. Proteins were reduced by DTT and desalted prior to Adn treatment (see the topic above). Aliquots (10 μ M) of the proteins after these steps were collected and alkylated by the addition of stop buffer (50 mM Tris-HCl pH = 6.8, 2% SDS, 0.4% bromophenol blue, 10% glycerol) containing *N*-ethylmaleimide (NEM; 50 mM) to verify the effectiveness of desalting steps and DTT reduction. The Adn reaction with AhpC cysteines was also evaluated after H₂O₂ oxidation (50 μ M/30 min/RT) to induce disulfide formation. EcAhpC and EcAhpC^{T44S} samples were alkylated using iodoacetamide (IAA) before H₂O₂ oxidation as an internal control of cysteine blocking. The oxidation reactions were interrupted using stop buffer and disulfide formation was analyzed by non-reducing SDS-PAGE stained by Coomassie blue.

Adn Molecular Docking in Bacterial AhpC. Crystallographic structure coordinates of *Salmonella typhimurium* AhpC (PDB = 1N8J) were obtained from the Protein Data Bank (<https://www.rcsb.org>). The structure of Adn was obtained by PubChem (CID: 15011073). The AutoDock Vina software was utilized in all the docking experiments, with the decameric AhpC structure as the docking target. The grid boxes (30 $\times$ 30 $\times$ 30 $\text{\AA}^3$) were centered at the active sites microenvironment and 16 hits were generated to each active site, resulting in 160 poses to the decamer.³⁹ To validate the accuracy of the docking and to allow a comparison between docking scores, the ligand was redocked into the same parameters in the same targets. Each ligand orientation was analyzed using UCFS Chimera⁴⁰ to determine its viability adopting as a criterion the distance of the Adn

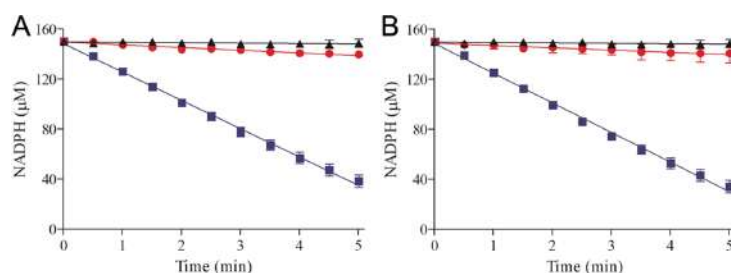


Figure 2. Evaluation of peroxidase activity inhibition of EcAhpC(A) and EcAhpC^{T44S} (B) by Adn. The proteins were previously reduced with DTT, the excess was removed, and the enzymes were treated with 20 mol equiv of Adn (●) for 1 h/RT. The excess of the compound was also removed, and the peroxidase activity was evaluated by the NADPH oxidation assay as follows: 3 μ M EcAhpC or EcAhpC^{T44S}, 6 μ M EcTrx, 0.9 μ M EcTrxR, and 150 μ M NADPH in buffer 50 mM HEPES (pH = 7.0), 100 μ M DTPA, and 1 mM sodium azide. The reactions were incubated at 37 °C for 3 min before being initiated by the addition of H₂O₂ (500 μ M) and monitored spectrophotometrically at 340 nm, 37 °C, for 5 min. Reactions containing AhpC without Adn treatment (■) were used as positive controls for peroxidase activity, and reactions without AhpC (▲) were used as negative controls. The linear fit of the data is represented in the same color as that of the markers to each reaction. All experiments were performed at least three times in triplicate.

reactive group with the reactive cysteines of the AhpCs selecting the results in which the Adn α,β -unsaturated carbonylic system were closer to reactive C_p and presented lower ligand binding energies (ΔG in kcal/mol). Positive selected results of the protein–ligand interactions were further inspected using Discovery Studio software (BIOVIA, DS, 2015) and LigPlot⁴¹.

Determination of the Minimum Inhibitory Concentration (MIC). *E. coli* (ATCC 25922) were grown in Mueller Hinton (MH) containing agar (2%) for 24 h at 37 °C and single colonies of each bacterium species were collected and diluted in saline solution (0.9%). The cell concentration was estimated by spectrophotometry (λ = 600 nm) and adjusted to $\sim 10^5$ colony forming units (CFU). Then, the cells were inoculated in MH containing varying concentrations of Adn (500, 250, 125, 62.5, 31.2, and 15.6 μ M), the cultures were incubated for 24 h at 37 °C and CFU was estimated by spectrophotometry (λ = 600 nm) (CLSI, 2015). Cell cultures without the addition of Adn were used as a positive control and culture media without cells were used as blank.

Cytotoxicity Tests of Antibiotics in Association with Adn on Bacterial Cells. Cytotoxicity tests were performed using kanamycin (Kan) (*S. aureus* and *S. epidermidis*) or polymyxin B nonapeptide (PMBN) (*E. coli*) to verify if the Adn is able to enhance the effect of antibiotics. To this purpose 10^5 CFU were inoculated in MH medium containing only Kan (1.5 μ g/mL) or associated with Adn (150 μ M). The PMBN permeabilization followed the protocol described previously by Samelin and co-workers⁴² in MH supplemented only with PMBN (0.3 μ g/mL) or in association with Adn (200 μ M). The experiments were also conducted using only Adn. All assays were incubated for 24 h at 37 °C without shaking. After this period, the cells were quantified spectrophotometrically (λ = 600 nm). Cell cultures without antibiotics or Adn were used as growth positive control, and culture medium without the addition of cells was used as blank.

Statistical Analysis. The statistical significance differences in the antimicrobial activity to the treatments with or without antibiotics (Kan or PMBN) and Adn were evaluated using one-way ANOVA and Dunnett's multiple comparison (post-test). The statistical analyses were performed using GraphPad Prism (Version 5.00) software (GraphPad Software, San Diego, CA, U.S.A.). A *p*-value <0.001 was considered as statistically significant.

RESULTS AND DISCUSSION

Adn Equally Inhibits the Peroxidatic Activities of Ser-AhpC and Thr-AhpC. Since the AhpC active site is found in the homodimer interface of decamers, the variation between Thr-Prx and Ser-Prx in quaternary structures is a feature that can affect substrate binding.^{2,8} To address this question, we

constructed an *E. coli* AhpC mutant carrying the Thr $\rightarrow$ Ser substitution (EcAhpC^{T44S}) (Supporting Information, SI, Figure S1). Adn efficiently inhibited both wild-type and mutant proteins in a similar manner (Figure 2). The determination of the initial rates of peroxide decomposition (v_0) revealed a very strong inhibition to both EcAhpC and EcAhpC^{T44S}, when compared with the untreated enzymes indicating that Adn is a potent inhibitor of the bacterial AhpC (Table 3).

Table 3. Initial Rates Determination of AhpC or Reductant Systems Treated or not with Adn by NADPH Oxidation (AhpC, Trx, and TrxR) or DTNB Reduction (AhpF) Assays

enzyme	v_0 – Adn (μ Ms ^{−1})	v_0 + Adn (μ Ms ^{−1})	variation (%)
EcAhpC	7.94 $\pm$ 0.87	0.88 $\pm$ 0.12	−89.0
EcAhpC ^{T44S}	8.20 $\pm$ 0.16	0.81 $\pm$ 0.65	−91.2
EcAhpF	13.40 $\pm$ 1.27	14.80 $\pm$ 1.47	+10.4
EcTrx	7.55 $\pm$ 0.09	1.63 $\pm$ 0.15	−79.5
EcTrxR	7.83 $\pm$ 0.63	7.95 $\pm$ 0.13	+1.0

In addition to AhpC-Prx1, Adn inhibits the mammalian reductant system, represented by the Trx and TrxR isoforms.^{43,44} However, no study to date addressed this issue for the bacterial Prx reductant system. As observed for human and yeast Trx, Adn also inhibited the activity of EcTrx1 (Figure 3A, Table 3). In contrast, EcTrxR and EcAhpF were not inhibited by Adn (Figure 3B,C, Table 3).

It is worth mentioning that like the mammalian counterpart, bacterial TrxR and AhpF are flavoenzymes. However, the human TrxR is quite different from the bacterial enzymes, presenting a higher molecular weight and a very similar structure with glutathione reductase, also containing a selenocysteine (Se-Cys) residue.⁴⁵ In fact, the Se-Cys of the human TrxR is located at a C-terminal extension of the enzyme and is highly exposed to solvent. In contrast, the catalytic Cys residues of TrxR and AhpF are buried in the structure, which might account, at least in part, for their resistance to Adn inhibition.^{46–50}

Adn Prevents the Formation of AhpC Intermolecular Disulfide Bonds. To confirm that the inhibitory mechanism of Adn in bacterial AhpC also occurs by Michael addition in catalytic cysteines, we performed non-reducing SDS-PAGE. When oxidized to disulfide, enzymes from the AhpC-Prx1 class

D

<https://doi.org/10.1021/acs.chemrestox.2c00049>
Chem. Res. Toxicol. XXXX, XXX, XXX–XXX

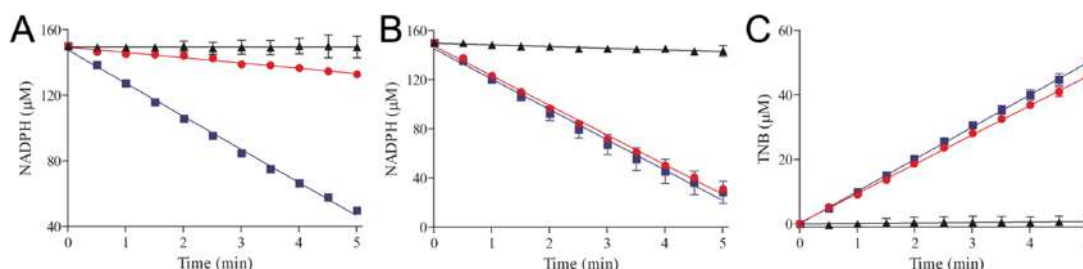


Figure 3. Evaluation of inhibition of AhpC reductase systems by Adn. To perform the assays, the enzymes previously reduced with DTT and desalted were treated with 20 mol equiv of Adn (●) for one hour at RT. The proteins were desalted again to remove the excess compound and their peroxidase activity was analyzed by NADPH oxidation assay (EcTrx1 (A) and EcTrxR (B)) or DTNB reduction (EcAhpF (C)) assays. For NADPH oxidation assays, reactions containing 3 μ M EcAhpC^{WT}, 6 μ M EcTrx, 0.9 μ M EcTrxR, 150 μ M NADPH, 50 mM HEPES (pH = 7.0), 100 μ M DTPA, and 1 mM sodium azide were used. The reactions were incubated at 37 °C for 3 min before being initiated by the addition of H₂O₂ (500 μ M) and monitored spectrophotometrically at 340 nm, 37 °C, for 5 min. DTNB reduction assay was performed in reactions containing 0.5 μ M EcAhpF, 500 μ M DTNB, 500 μ M DTPA, and 100 mM sodium phosphate (pH = 7.0). The reactions were incubated for 3 min at 37 °C, for thermo stabilization and initiated by the addition of NADPH (300 μ M). TNB formation was monitored spectrophotometrically at 412 nm/37 °C for 5 min. Positive control was made with proteins without Adn treatment (■) and reactions without EcAhpC (A and B) or EcAhpF (C) (▲) were used as negative controls. The linear fit of the data is represented in the same color of the markers to each reaction.

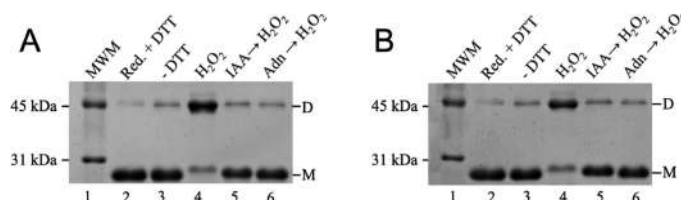


Figure 4. Evaluation of AhpC intermolecular disulfide formation by non-reducing SDS-PAGE. To both SDS-PAGE, molecular weight marker (MWM) OmniPur (Calbiochem) were applied in the lane 1. Samples with 10 μ M of EcAhpC (A) or EcAhpC^{T44S} (B) were reduced with DTT 50 mM/1 h/RT (lanes 2), the excess was removed (lanes 3) and proteins were treated with 20 μ M H₂O₂/30 min/RT (lanes 4). As a positive control of the disulfide formation inhibition, AhpC samples previously reduced by DTT and desalted were treated with iodoacetamide (IAA) (50 mM/1 h/RT) and then oxidized with hydrogen peroxide (100 μ M) (lanes 5). The DTT reduced and desalted samples were treated 200 μ M Adn and also oxidized with 100 μ M hydrogen peroxide (lanes 6). The experiments were performed two times.

migrate as a dimer (~44 kDa) as a consequence of intermolecular disulfide bond formation. However, the reduced enzyme appears as a monomer (~22 kDa).⁵¹ Thus, it is possible to evaluate not only the redox state of these enzymes, but also if the inhibitor was able to react with cysteine residues involved in catalysis. If one of the two cysteines is modified by an inhibitor, then no intermolecular disulfide is expected to be formed and the proteins are detected as monomers.

Prior to the peroxide treatment, EcAhpC and EcAhpC^{T44S} were reduced by DTT (Figure 4A,B, lanes 2), the excess was removed (Figure 4A,B, lanes 3) and the proteins were oxidized with hydrogen peroxide (Figure 4A,B, lanes 4). As expected, EcAhpC and EcAhpC^{T44S} reduced by DTT were detected as monomers, while the oxidized samples were detected as dimers (Figure 4A,B). As a positive control of cysteine alkylation, prereduced AhpC samples were treated with iodoacetamide (IAA) prior to hydrogen peroxide oxidation. As expected, the blocked enzyme migrated as monomer (Figure 4A,B, lanes 5). Finally, hydrogen peroxide treated EcAhpC and EcAhpC^{T44S} samples were detected mostly as monomers when pretreated with Adn (Figure 4A,B, lanes 6), similarly to the IAA treatment, indicating that Adn interacted with a catalytic cysteine.

Adn is a Potent Inhibitor of Bacterial AhpC. Since Adn was able to inhibit the peroxidase activity of EcAhpC and EcAhpC^{T44S}, as well as the intermolecular disulfide formation

in these proteins, we proceeded with the determination of the Adn apparent IC₅₀ to the wild type and mutant enzyme. For this purpose, previously reduced enzymes were treated with growing Adn concentrations, the excess was removed, and they were subjected to non-reducing SDS-PAGE after oxidation by hydroperoxide (Figure 5A,B) or NADPH oxidation coupled assay (Figure 5C–F).

Notably, Adn inhibited the intermolecular disulfide formation of EcAhpC and EcAhpC^{T44S} (Figure 5A,B) and the NADPH oxidation assays showed that very low doses inhibited the AhpC activity (Figure 5C,D). The apparent obtained IC₅₀ values were = 1.443 $\pm$ 0.078 for EcAhpC and = 1.356 $\pm$ 0.072 μ M for EcAhpC^{T44S} (Figure 5E,F). Noteworthy, the IC₅₀ values for EcAhpC and EcAhpC^{T44S} were similar or 10 $\times$ lower than those reported to human isoforms (Prx1 = 1.5 and Prx2 = 15 μ M).³¹

Insights on the Mode of Adn Binding to the Active Site Pocket of AhpC by Molecular Docking Analysis. To gain insights of Adn binding in the AhpC active site microenvironment, we performed molecular docking analysis. Despite no structure of the wild-type AhpC from *E. coli* has been determined to date, several structures of bacterial AhpC are available. The amino acid sequence of *S. typhimurium* AhpC (StAhpC, PDB entry: 1N8J) displays 98.4% of identity with the *E. coli* AhpC, presenting only three substitutions of residues with similar physicochemical features (Ile²⁴, Ala⁷⁰, and

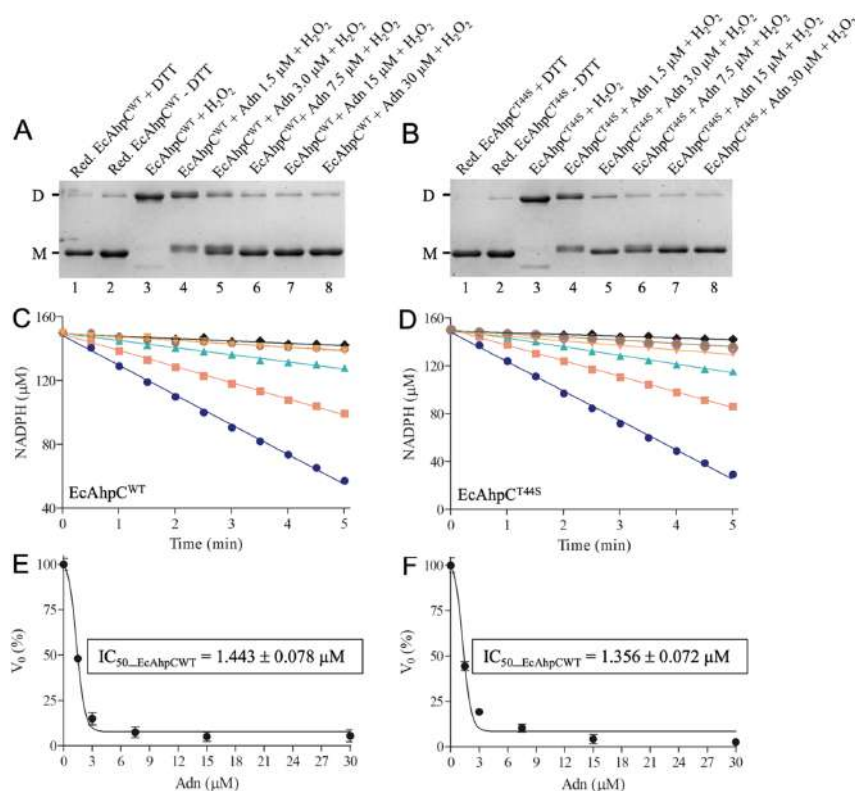


Figure 5. Inhibition of intermolecular disulfide formation by growing concentrations of Adn and IC_{50} determination for EcAhpC and EcAhpC^{T44S}. The proteins EcAhpC (A) and EcAhpC^{T44S} (B) were reduced with 5 mM DTT for 1 h (lane 1) and then desalted to remove excess of reductant (lane 2). Aliquots containing 3 μ M of the reduced and desalted enzymes were treated varying the Adn concentration (1.5, 3.0, 7.5, 15, and 30 μ M—lanes 4, 5, 6, 7 and 8, respectively) for 1 h and then oxidized with 15 μ M H₂O₂ for 30 min. The reactions were stopped by adding stop buffer (Tris 50 mM (pH = 6.8), NEM 50 mM, SDS 2%, glycerol 10%, and bromophenol blue 0.1%) and analyzed on non-reducing SDS-PAGE stained with Coomassie blue. Lane 3 represents the reduced/desalted and subsequently oxidized protein, without Adn treatment. The IC_{50} determination procedures were performed at room temperature by NADPH oxidation assay. Reduced and desalted EcAhpC^{WT} (C) and EcAhpC^{T44S} (D) were treated with growing Adn concentrations (1.5 μ M—■; 3.0 μ M—▲; 7.5 μ M—▼; 15 μ M—● and 30 μ M—● μ M for 1 h at room temperature and subsequently desalted again. Next, 3 μ M of the Adn-treated proteins were added to reactions containing 6 μ M EcTrx1, 0.9 μ M EcTrxR, 150 μ M NADPH, 50 mM HEPES (pH 7.0), 100 μ M DTPA, and 1 mM sodium azide. The reactions were incubated at 37 °C for 3 min before being initiated by the addition of 500 μ M H₂O₂ and monitored spectrophotometrically at 340 nm, 37 °C, for 5 min. Reactions without Adn treatment were used as positive controls (●), and reactions without the addition of AhpC were used as negative controls (◆). The linear fit of the data is represented in the same color of the markers to each reaction and were used to calculate the initial velocities (v_0) of each reaction. They were transformed into percentage values and plotted to calculate the IC_{50} values of Adn for EcAhpC (E) and EcAhpC^{T44S} (F). All experiments were performed at least three times in triplicate.

Ser¹⁵⁹ in *E. coli* and Val²⁴, Ser⁷⁰, and Ala¹⁵⁹ in *S. typhimurium*), all of them positioned far from the active site. Therefore, we chose the StAhpC structure to perform the docking analysis.

These simulations took into account that when reduced, AhpC-Prx1 enzymes are preferentially in decameric form (Figure 1). Indeed, it is important to notice that the active site residues are placed in the dimer–dimer interface of the decamers (Figure 6A,B). In fact, the catalytic triad Thr interacts with the aromatic ring of Phe/Tyr residues from adjacent dimer and with ligands that cocrystallized in structures of decameric Prx and are stabilized in the active site by at least three protomers of the decamer.^{17,51} Therefore, the interaction analyses covered the entire catalytic site pocket, including residues from obligate dimer and from the adjacent dimer.

From all the positive hits in the active site pocket obtained by Adn docking (Figure 6C), the best pose presented a free Gibbs energy (ΔG) equal to 5.9 kcal/mol with the Adn α,β -unsaturated carbonylic system orientated to the S_Y catalytic cysteine residue at a distance of ~ 7.14 Å (Figure 6D and SI Figure S2). The analysis of physicochemical properties of residues at this region revealed that the Adn binding occurs at a hydrophobic/uncharged region (Figure 6E). The Adn molecule is stabilized by interactions with Phe^{44'}, Glu^{114'}, Leu^{116'}, and Glu^{138'}; Pro^{178''}, Ser^{179''}, and Leu^{180''} residues (the prime (') represent amino acids found at C_p protomer and quotation (") mark residues from C_B protomer) and from the adjacent protomer (Gln13*, Phe20*, Phe76*, and His75*; the asterisk denotes amino acids residues the adjacent dimer protomer). Except for Glu^{138'} and Ser^{179''}, all molecular interactions are performed by the side chain of the residues

F

<https://doi.org/10.1021/acs.chemrestox.2c00049>
Chem. Res. Toxicol. XXXX, XXX, XXX–XXX

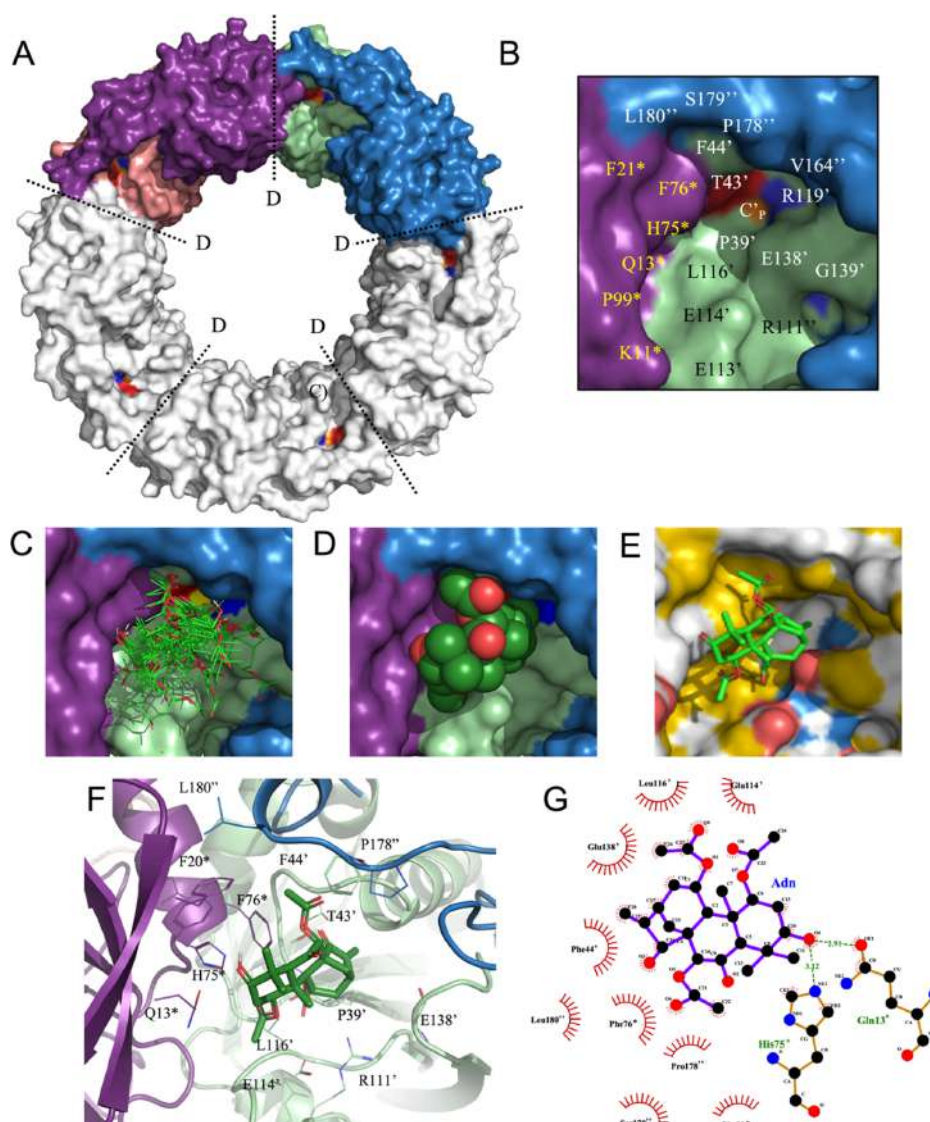


Figure 6. AhpC structure and docking analysis with Adn. (A) AhpC from *S. typhimurium* (PDB = 1N8J) in reduced decameric form are composed by five homodimers connected by the dimeric (D) interface (black dashed line). The structure surface is colored in light gray, except by the upper homodimers, that are represented in purple/pink and blue/green. The catalytic triad residues Thr, C_R, and Arg are colored in red, orange, and blue, respectively. This pattern of colors is observed to all figures, except (E) and (G). (B) Detail of the active site microenvironment. The residues of the intimate dimer protomer containing the catalytic triad are marked with prime ('), the C_R protomer with quotation ('). Amino acids from the adjacent homodimer are assigned with asterisk (*). (C) StAhpC molecular surface containing the cluster of positive hits in the active site pocket obtained by Adn docking. The Adn molecules are represented in lines with the carbon atoms in green and oxygens in red. (D) Detail of the active site containing the selected best pose represented by spheres with carbons in green and oxygens in red. (E) Adn docking result (sticks) with the active site microenvironment represented in YRB to highlight polar and hydrophobic active site characteristics (YRB representation = hydrocarbon groups without polar substitutions, yellow; glutamate and aspartate negatively charged oxygens represented in red; lysine and arginine of positively charged nitrogen groups are in blue; and all remaining atoms including the polar backbone, white). (F) AhpC represented in cartoon, Adn in sticks, and AhpC residues that interact with Adn are represented in lines. (G) Diagram of interacting residues with Adn molecule. The hydrogen bond lengths are in green and interactions as green dashed lines. Hydrophobic contacts are represented by red arcs with radiating spikes. The molecular graphics and the binding analysis were performed using the PyMol and LigPlot[†].

(Figure 6F,G). Only two polar interactions were detected in the simulations (with Gln13* and His75* residues), suggesting that the Adn hydrophobic characteristic is more important for

its binding in the active site. This is relevant since several alkyl hydroperoxides that are reduced by AhpC are hydrophobic and/or bulky,^{8–10,14,25} and these residues may also be involved

G

<https://doi.org/10.1021/acs.chemrestox.2c00049>
Chem. Res. Toxicol. XXXX, XXX, XXX–XXX

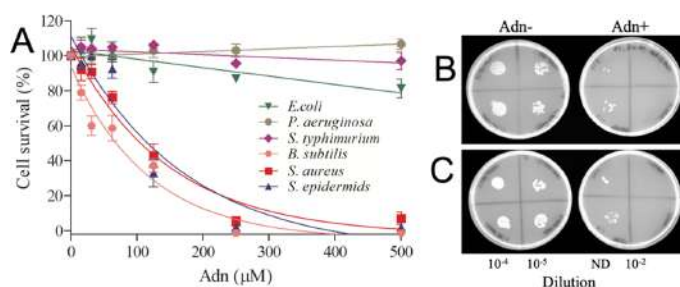


Figure 7. MIC₅₀ determination and cell survival of Gram-positive and Gram-negative strains after challenge with Adn. (A) Bacterial cells (10⁵ CFU) from *B. subtilis* (●), *S. aureus* (■), *S. epidermidis* (▲), *E. coli* (▼), *P. aeruginosa* (●), and *S. typhimurium* (◆) were inoculated in Mueller Hinton medium containing increasing concentrations of Adn and incubated for 24 h at 37 °C, and then quantified spectrophotometrically at 600 nm. *S. aureus* (B) and *S. epidermidis* (C) cells were incubated in absence (left) or presence of 500 μM Adn (right) in MH for 16h/37 °C. The untreated cells were diluted 10 000 (left) and 100 000-fold (right) and plated in MH-agar and 5 μL of the cell culture was spotted in the plate. The Adn treated cells were inoculated in MH-agar (no dilution, left; or 100-fold dilution, right), grown for 16h/37 °C and spotted in the plate as previously described. These assays were performed in duplicate three times.

in the stabilization of biological oxidizing substrates. Additionally, we found variations between bacterial and human Prx2 in the residues involved in the Adn binding (SI Figure S3), which may explain the differences of the IC₅₀ values (Figure 5).

Adn is Able to Inhibit the Growth of Gram-Positive Bacteria, but not Gram-Negative Bacterial Species. We also evaluated the effects of Adn treatment on the growth of Gram-positive (*B. subtilis*, *S. aureus*, and *S. epidermidis*) and to Gram-negative (*E. coli*, *P. aeruginosa*, and *S. typhimurium*) bacteria. In the conditions tested, the cell growth of all the Gram-positive bacteria tested was severely affected and MIC₅₀ were within the 110–150 μM range, while no significant effect was observed to the Gram-negative bacteria (Figure 7A). The Table 4 summarizes the MIC₅₀ results.

Table 4. Values of the Adn Minimal Inhibitory Concentration 50 (MIC₅₀) Determined to Different Gram-Positive and Gram-Negative Bacterial Species

species	gram	strain	MIC ₅₀ (μM)
<i>B. subtilis</i>	+	ATCC 55569	116.2 (±11.0)
<i>S. aureus</i>	+	ATCC29213	111.3 (±14.8)
<i>S. epidermidis</i>	+	ATCC 12228	146.8 (±17.5)
<i>E. coli</i>	–	ATCC25922	ND ^a
<i>P. aeruginosa</i>	–	ATCC 29853	ND ^a
<i>S. typhimurium</i>	–	ATCC 19585	ND ^a

^aND = Not determined.

In sequence, cells from *S. aureus* and *S. epidermidis* were treated with 500 μM Adn in MH medium for 16 h. After the treatment, the cells were plated in MH/agar, to determine if Adn displays bactericide or bacteriostatic properties. Our results indicated that Adn killed both bacteria, since cells preincubated with Adn did not grow in plates even after removal of this compound. In fact, Adn treated cells presented very low numbers of colonies after 24 h, while the control (without Adn treatment) presented colonies even when they were diluted 10 000 times (Figure 7B,C). Therefore, Adn presented antimicrobial activity over *S. aureus* and *S. epidermidis*.

These results are significant since the IC₅₀ values of Adn determined to EcAhpC (Figure 5E,F) are similar or lower than to human isoforms and the toxicity of Adn is very low in

normal human cells and animal models.^{31,35,36} In fact, Adn is able to kill hepatocarcinoma malignant cells at a concentration of 10 mg/kg without observable toxic effects on mice.³⁶ Therefore, the higher susceptibility of Adn on bacterial AhpC enables its use to treat infectious diseases caused by bacteria using lower doses than those needed for the tumor remission and, therefore, with low risk of side effects.

It is important to highlight that the importance of the AhpC to bacterial growth is related to hydroperoxide decomposition in physiological doses generated by the cell growth events.^{4,52} Nevertheless, since the Adn is also able to interfere with the activity of Trx (Figure 3A) and possibly other thiol proteins that share structural similarities as glutaredoxins (Grx) and disulfide bond proteins (DSB), as shown in human counterparts,^{44,53} the effects on the bacterial growth may represent inhibitory effects on other thiol proteins in addition to AhpC.

Adn Potentiates the Antimicrobial effects over Gram-Positive Bacteria by Kanamycin. We investigated if Adn could potentialize the effects of antibiotics in Gram-positive bacteria. We analyzed kanamycin (Kan), an aminoglycoside widely used for the treatment of bacterial infections. In fact, Kan is frequently used to evaluate toxic effects in combination with different compounds able to inhibit bacterial cell growth, including natural compounds.^{54–57}

Prior to these experiments, we determined the MIC to Kan for *S. aureus* and *S. epidermidis* strains (SI Figure S4). Then *S. aureus* and *S. epidermidis* bacterial strains were treated only with Kan (1.5 μg/mL), only with Adn (150 μM), or simultaneously with both compounds (Figure 8, Table 5). The results indicated that treatment only with Adn caused ~60% decrease in cell growth for *S. aureus* (statistically significant decrease $p < 0.001$), and 47% for *S. epidermidis*, while treatment using with Kan alone decreased the cell growth in ~29% for *S. aureus* and ~44% for *S. epidermidis*. When these bacterial strains were treated simultaneously with Adn and Kan, the inhibitory effect was very high, reaching ~97% for both strains, suggesting a synergistic effect promoted by Adn and Kan ($p < 0.001$) (Figure 8, Table 5). Taken together, the results demonstrate that Adn combined with Kan is able to potentiate the antimicrobial action of the pharmaceutical.

Adn Combination with Polymyxin B Nonapeptide (PMBN) Inhibits the Gram-Negative Cell Growth. It did not escape our attention that the bacteria that showed

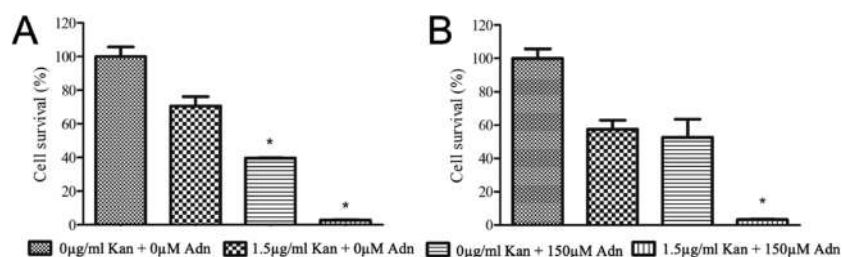


Figure 8. Antibacterial activity of kanamycin in combination with Adn on pathogenic strains of *S. aureus* and *S. epidermidis*. Bacterial cells were grown for 6 h in MH and then diluted in the same culture medium to $OD_{600} = 0.01$. The cells were then treated with 1.5 µg/mL kanamycin (Kan) or 150 µM Adn separately (columns 2 and 3), or simultaneously (column 4) for 24 h/37 °C. After the incubation period, cell density was quantified by spectrophotometry ($\lambda = 600$ nm). The assays were performed in triplicate with independent repetitions. The asterisk (*) means statistically significant differences of the cell growth between the control (without treatment) and cells treated only with Kan or Adn or treated with Kan + Adn (p value < 0.001).

Table 5. Effects of the Antibacterial Activity of Kanamycin and in Association with Adn

	cell survival (%)	
	<i>S. aureus</i>	<i>S. epidermidis</i>
no compound	100.22 (± 0.41)	99.74 (± 0.33)
Kan	70.68 (± 9.45)	57.58 (± 9.28)
Adn	39.84 (± 0.19) ^a	52.63 (± 18.94)
Kan + Adn	2.80 (± 0.49) ^a	3.28 (± 0.26) ^a

^aStatistically significant differences were determined using one-way ANOVA and Dunnett's multiple comparison (post-test).

susceptibility to Adn treatment are Gram-positive while the Gram-negative ones are resistant. It is well-known that Gram-negative bacteria can be more resistant or even insensitive to treatment with compounds that exhibit bactericidal or bacteriostatic activity because their outer membrane (OM) system provide an extra layer of protection that block the entry of compounds, including antibiotics.^{58,59} Some compounds are used to permeabilize the OM, being the most studied the polymyxin b nonapeptide (PMBN) which is also used as the last-line drug against multidrug-resistant strains due to compound toxicity.⁶⁰

To evaluate the effects of the permeabilization by PMBN and the sensibilation to Adn, *E. coli* cells were inoculated in culture medium containing growing concentrations of PMBN (SI Figure S5), aiming to establish a concentration of the compound that would not promote cell death by itself.

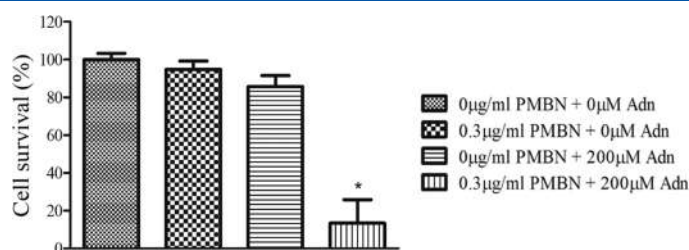


Figure 9. Evaluation of the toxicity of PMBN associated with Adn over bacterial cells. *E. coli* cells (ATCC 25922) (10^5 CFU) were inoculated in MH containing 200 µM Adn, 0.3 µg/mL PMBN or with the association of both (200 µM Adn and 0.3 µg/mL PMBN). Cells with no treatment were used as negative control. The bacterial cells were incubated for 24 h/37 °C/250 rpm in orbital shaker, and the cell densities were quantified spectrophotometrically at 600 nm. The assays were performed in triplicate with independent repetitions. The asterisk (*) indicates statistical differences in the cell growth treated with PMBN + Adn (p value < 0.001) when compared to the samples without treatment or treated only with PMBN or Adn.

1

<https://doi.org/10.1021/acs.chemrestox.2c00049>
Chem. Res. Toxicol. XXXX, XXX, XXX–XXX

activities of large, polar antibiotics exceeding the size for porin transport (>600 Da) are generally restricted by the OM.^{59,61,62} Additionally, the zwitterionic characteristics of some compounds are correlated with the increased permeability through the membranes, while higher hydrophobic compounds tend to be good substrates for bacterial drug efflux pumps.⁵⁹ In this context, compounds such as Adn, which has low polarity and a molecular weight of 500 Da, fit in drug profiles in which these bacteria are refractory. Additionally, as mentioned before, PMBN is used as the last-line drug due to the high toxicity of the compound⁶⁰ but the enhanced effect in association with Adn can represent an important advantage during treatment because a lower concentration can be used, with decreased side effects. In consequence, low concentrations of both molecules represent a promising strategy to also combat infectious diseases caused by Gram-negative bacteria and an important field of study against multidrug-resistant strains.

■ CONCLUSIONS

AhpC are frequently related to the subjugation of oxidative stress generated by the host immune system and antibiotics, and some isoforms were characterized as virulence factors, indicating important roles in infection and survival in the hosts. Nevertheless, no AhpC inhibitor had been systematically characterized to the present. Previously, Adn was characterized as an efficient inhibitor of human isoforms with very low doses and low toxicity in rats. Here we demonstrated the properties of Adn as an efficient inhibitor of bacterial AhpC, independent of the presence of Thr or Ser at catalytic triad. Computer-assisted simulations revealed that Adn is stabilized mainly by several hydrophobic interactions with amino acids from three different protomers. We have also shown that the Adn is able to decrease the growth rates of Gram-positive bacteria due to its bactericidal property. Additionally, the association of Adn with antibiotics maximizes the pharmaceutical action over Gram-positive and negative bacteria, highlighting the potential role of Adn as an antibiotic drug.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.chemrestox.2c00049>.

Representative results of recombinant proteins expression and purification; molecular graphics of the Adn superposition with ligands found in the active site of the crystallographic structures of Prx isoforms; sequence alignment of bacterial AhpC with human Prx1 and Prx2 isoforms and evaluation of bacterial minimal inhibitory concentration of kanamycin (Gram positive) and PMBN (Gram negative) (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Marcos Antonio de Oliveira – Instituto de Biociências, Universidade Estadual Paulista, UNESP, São Vicente, São Paulo 11330-900, Brazil; orcid.org/0000-0002-1192-7296; Email: marcos.a.oliveira@unesp.br

Luis Eduardo Soares Netto – Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, São Paulo, São Paulo 05508-090, Brazil; orcid.org/0000-0002-4250-9177; Email: nettoles@ib.usp.br

Authors

Melina Cardoso dos Santos – Instituto de Biociências, Universidade Estadual Paulista, UNESP, São Vicente, São Paulo 11330-900, Brazil

Carlos Abrunhosa Tairum – Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, São Paulo, São Paulo 05508-090, Brazil

Vitoria Isabela Montanhero Cabrera – Instituto de Biociências, Universidade Estadual Paulista, UNESP, São Vicente, São Paulo 11330-900, Brazil

Ana Carolina Guimarães Cauz – Departamento de Genética, Evolução, Microbiologia e Imunologia, Instituto de Biologia, Universidade Estadual de Campinas, UNICAMP, Campinas, São Paulo 13083-862, Brazil

Luiz Fernando Ribeiro – Instituto de Biociências, Universidade Estadual Paulista, UNESP, São Vicente, São Paulo 11330-900, Brazil; orcid.org/0000-0002-5105-2287

José Carlos Toledo Junior – Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, São Paulo 14040-901, Brazil

Marcos Hikari Toyama – Instituto de Biociências, Universidade Estadual Paulista, UNESP, São Vicente, São Paulo 11330-900, Brazil

João Henrique Ghilardi Lago – Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, Santo André, São Paulo 09210-580, Brazil; orcid.org/0000-0002-1193-8374

Marcelo Brocchi – Departamento de Genética, Evolução, Microbiologia e Imunologia, Instituto de Biologia, Universidade Estadual de Campinas, UNICAMP, Campinas, São Paulo 13083-862, Brazil

Complete contact information is available at:

<https://pubs.acs.org/10.1021/acs.chemrestox.2c00049>

Author Contributions

M.A.O., L.E.S.N., J.H.G.L., M.B., M.C.S., and C.A.T. conceived and designed the experiments; M.C.S., A.C.G.C., V.I.M.C., and L.F.R. executed the experiments; M.A.O., L.E.S.N., J.H.G.L., M.B., M.H.T., J.C.T.J., C.A.T., M.C.S., V.I.M.C., and L.F.R. analyzed the data; M.A.O., L.E.S.N., C.A.T. and M.C.S. wrote the paper. All authors revised, edited, and approved the final version of the manuscript.

Funding

The authors M.A.O., L.E.S.N., J.C.T.J., M.C.S., C.A.T., V.I.M.C., and L.F.R. are members of the CEPID REDOXOMA from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (grant number 2013/07937-8). This work was also supported by grants from FAPESP to M.A.O. (2017/19942-7) and M.H.T. (2017/20291-0) and J.H.G.L. from grants of the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). C.A.T., M.C.S., V.I.M.C., and L.F.R. were recipients of fellowships from FAPESP (2018/12628-8, 2017/06263-4, 2020/02868-1, and 2021/00209-3, respectively).

Notes

This article does not contain any studies with human participants or animals performed by any of the authors. The authors declare no competing financial interest.

■ ABBREVIATIONS USED

Adn, Adenanthin; AhpC, alkyl hydroperoxide resistance protein C; AhpF, alkyl hydroperoxide resistance protein F; Amp, Ampicillin; CFU, colony forming units; C_p, peroxidatic cysteine; C_R, resolving cysteine; DTNB, 5,5-dithio-bis(2-nitrobenzoic acid); DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol; H₂O₂, hydrogen peroxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HMW, high molecular weight complex; IC₅₀, half-maximal inhibitory concentration; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl β-D-1-thiogalactopyranoside; Kan, Kanamycin; LB, Luria–Bertani; MH, Mueller Hinton; MIC₅₀, minimum inhibitory concentration 50; MWM, molecular weight marker; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NEM, N-ethylmaleimide; ONOO[−], peroxynitrite; PDB, Protein Data Bank; Prx, peroxiredoxin; PMBN, polymyxin B nonapeptide; ROS, reactive oxygen species; SOH, sulfenic acid; Trx, Thioredoxin; TrxR, Thioredoxin reductase; V₀, initial velocity.

■ REFERENCES

- (1) Fisher, R. A.; Gollan, B.; Helaine, S. Persistent bacterial infections and persister cells. *Nat. Rev. Microbiol.* **2017**, *15*, 453–464.
- (2) de Oliveira, M. A.; Tairum, C. A.; Netto, L. E. S.; de Oliveira, A. L. P.; Aleixo-Silva, R. L.; Cabrera, V. I. M.; Breyer, C. A.; Dos Santos, M. C. Relevance of peroxiredoxins in pathogenic microorganisms. *Appl. Microbiol. Biotechnol.* **2021**, *105*, 5701.
- (3) Condeles, A. L.; Gomes, F.; de Oliveira, M. A.; Soares Netto, L. E.; Toledo Junior, J. C. Thiol peroxidases as major regulators of intracellular levels of peroxynitrite in live *Saccharomyces cerevisiae* cells. *Antioxidants (Basel)* **2020**, *9*, 434.
- (4) Seaver, L. C.; Imlay, J. A. Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. *J. Bacteriol.* **2001**, *183*, 7173–7181.
- (5) Storz, G.; Jacobson, F. S.; Tartaglia, L. A.; Morgan, R. W.; Silveira, L. A.; Ames, B. N. An alkyl hydroperoxide reductase induced by oxidative stress in *Salmonella typhimurium* and *Escherichia coli*: genetic characterization and cloning of *ahp*. *J. Bacteriol.* **1989**, *171*, 2049–2055.
- (6) Winterbourn, C. C.; Hampton, M. B. Thiol chemistry and specificity in redox signaling. *Free Radic Biol. Med.* **2008**, *45*, 549–561.
- (7) Poole, L. B.; Nelson, K. J. Distribution and features of the six classes of peroxiredoxins. *Mol. Cells* **2016**, *39*, 53–59.
- (8) Tairum, C. A.; Santos, M. C.; Breyer, C. A.; de Oliveira, A. L. P.; Cabrera, V. I. M.; Toledo-Silva, G.; Mori, G. M.; Toyama, M. H.; Netto, L. E. S.; de Oliveira, M. A. Effects of serine or threonine in the active site of typical 2-Cys Prx on hyperoxidation susceptibility and on chaperone activity. *Antioxidants (Basel)* **2021**, *10*, 1032.
- (9) Jacobson, F. S.; Morgan, R. W.; Christman, M. F.; Ames, B. N. An alkyl hydroperoxide reductase from *Salmonella typhimurium* involved in the defense of DNA against oxidative damage. Purification and properties. *J. Biol. Chem.* **1989**, *264*, 1488–1496.
- (10) Parsonage, D.; Karplus, P. A.; Poole, L. B. Substrate specificity and redox potential of AhpC, a bacterial peroxiredoxin. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 8209–8214.
- (11) Parsonage, D.; Nelson, K. J.; Ferrer-Sueta, G.; Alley, S.; Karplus, P. A.; Furdul, C. M.; Poole, L. B. Dissecting peroxiredoxin catalysis: separating binding, peroxidation, and resolution for a bacterial AhpC. *Biochemistry* **2015**, *54*, 1567–1575.
- (12) Zhang, B.; Gu, H.; Yang, Y.; Bai, H.; Zhao, C.; Si, M.; Su, T.; Shen, X. Molecular mechanisms of AhpC in resistance to oxidative stress in *Burkholderia thailandensis*. *Front Microbiol* **2019**, *10*, 1483.
- (13) Poole, L. B. Bacterial defenses against oxidants: mechanistic features of cysteine-based peroxidases and their flavoprotein reductases. *Arch. Biochem. Biophys.* **2005**, *433*, 240–254.
- (14) Baker, L. M.; Raudonikienė, A.; Hoffman, P. S.; Poole, L. B. Essential thioredoxin-dependent peroxiredoxin system from *Helicobacter pylori*: genetic and kinetic characterization. *J. Bacteriol.* **2001**, *183*, 1961–1973.
- (15) Parsonage, D.; Desrosiers, D. C.; Hazlett, K. R.; Sun, Y.; Nelson, K. J.; Cox, D. L.; Radolf, J. D.; Poole, L. B. Broad specificity AhpC-like peroxiredoxin and its thioredoxin reductant in the sparse antioxidant defense system of *Treponema pallidum*. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 6240–6245.
- (16) Wong, C. F.; Shin, J.; Subramanian Manimekalai, M. S.; Saw, W. G.; Yin, Z.; Bhushan, S.; Kumar, A.; Ragunathan, P.; Gruber, G. AhpC of the mycobacterial antioxidant defense system and its interaction with its reducing partner thioredoxin-C. *Sci. Rep* **2017**, *7*, 5159.
- (17) Forshaw, T. E.; Reisz, J. A.; Nelson, K. J.; Gumpena, R.; Lawson, J. R.; Jonsson, T. J.; Wu, H.; Clodfelter, J. E.; Johnson, L. C.; Furdul, C. M.; Lowther, W. T. Specificity of human sulfiredoxin for reductant and peroxiredoxin oligomeric state. *Antioxidants (Basel)* **2021**, *10*, 946.
- (18) Parsonage, D.; Youngblood, D. S.; Sarma, G. N.; Wood, Z. A.; Karplus, P. A.; Poole, L. B. Analysis of the link between enzymatic activity and oligomeric state in AhpC, a bacterial peroxiredoxin. *Biochemistry* **2005**, *44*, 10583–10592.
- (19) Tairum, C. A.; Santos, M. C.; Breyer, C. A.; Geyer, R. R.; Nieves, C. J.; Portillo-Ledesma, S.; Ferrer-Sueta, G.; Toledo, J. C., Jr.; Toyama, M. H.; Augusto, O.; Netto, L. E.; de Oliveira, M. A. Catalytic Thr or Ser residue modulates structural switches in 2-Cys peroxiredoxin by distinct mechanisms. *Sci. Rep* **2016**, *6*, 33133.
- (20) Wood, Z. A.; Poole, L. B.; Hantgan, R. R.; Karplus, P. A. Dimers to doughnuts: redox-sensitive oligomerization of 2-cysteine peroxiredoxins. *Biochemistry* **2002**, *41*, 5493–5504.
- (21) Pastor-Flores, D.; Talwar, D.; Pedre, B.; Dick, T. P. Real-time monitoring of peroxiredoxin oligomerization dynamics in living cells. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117*, 16313–16323.
- (22) Nelson, K. J.; Perkins, A.; Van Swearingen, A. E. D.; Hartman, S.; Brereton, A. E.; Parsonage, D.; Salsbury, F. R., Jr.; Karplus, P. A.; Poole, L. B. Experimentally dissecting the origins of peroxiredoxin catalysis. *Antioxid Redox Signal* **2018**, *28*, 521–536.
- (23) Pan, A.; Balakrishna, A. M.; Nartey, W.; Kohlmeier, A.; Dip, P. V.; Bhushan, S.; Gruber, G. Atomic structure and enzymatic insights into the vancomycin-resistant *Enterococcus faecalis* (V583) alkylhydroperoxide reductase subunit C. *Free Radic Biol. Med.* **2018**, *115*, 252–265.
- (24) Cha, M. K.; Bae, Y. J.; Kim, K. J.; Park, B. J.; Kim, I. H. Characterization of two alkyl hydroperoxide reductase C homologs alkyl hydroperoxide reductase C_{H1} and alkyl hydroperoxide reductase C_{H2} in *Bacillus subtilis*. *World J. Biol. Chem.* **2015**, *6*, 249–264.
- (25) Rocha, L. S.; Silva, B. P. D.; Correia, T. M. L.; Silva, R. P. D.; Meireles, D. A.; Pereira, R.; Netto, L. E. S.; Meotti, F. C.; Queiroz, R. F. Peroxiredoxin AhpC1 protects *Pseudomonas aeruginosa* against the inflammatory oxidative burst and confers virulence. *Redox Biol.* **2021**, *46*, 102075.
- (26) Wang, H. W.; Chung, C. H.; Ma, T. Y.; Wong, H. C. Roles of alkyl hydroperoxide reductase subunit C (AhpC) in viable but nonculturable *Vibrio parahaemolyticus*. *Appl. Environ. Microbiol.* **2013**, *79*, 3734–3743.
- (27) Zwick, J. V.; Noble, S.; Ellaicy, Y. K.; Coe, G. D.; Hakey, D. J.; King, A. N.; Sadauskas, A. J.; Faulkner, M. J. AhpA is a peroxidase expressed during biofilm formation in *Bacillus subtilis*. *Microbiologyopen* **2017**, *6*, e00403.
- (28) Brenot, A.; King, K. Y.; Caparon, M. G. The PerR regulon in peroxide resistance and virulence of *Streptococcus pyogenes*. *Mol. Microbiol.* **2005**, *55*, 221–234.
- (29) Dons, L. E.; Mosa, A.; Rottenberg, M. E.; Rosenkrantz, J. T.; Kristensson, K.; Olsen, J. E. Role of the *Listeria monocytogenes* 2-Cys peroxiredoxin homologue in protection against oxidative and nitrosative stress and in virulence. *Pathog Dis* **2014**, *70*, 70–74.

K

<https://doi.org/10.1021/acs.chemrestox.2c00049>
Chem. Res. Toxicol. XXXX, XXX, XXX–XXX

- (30) Olczak, A. A.; Seyler, R. W., Jr.; Olson, J. W.; Maier, R. J. Association of *Helicobacter pylori* antioxidant activities with host colonization proficiency. *Infect. Immun.* **2003**, *71*, 580–583.
- (31) Liu, C. X.; Yin, Q. Q.; Zhou, H. C.; Wu, Y. L.; Pu, J. X.; Xia, L.; Liu, W.; Huang, X.; Jiang, T.; Wu, M. X.; He, L. C.; Zhao, Y. X.; Wang, X. L.; Xiao, W. L.; Chen, H. Z.; Zhao, Q.; Zhou, A. W.; Wang, L. S.; Sun, H. D.; Chen, G. Q. Adenanthin targets peroxiredoxin I and II to induce differentiation of leukemic cells. *Nat. Chem. Biol.* **2012**, *8*, 486–493.
- (32) Shi, X. J.; Ding, L.; Zhou, W.; Ji, Y.; Wang, J.; Wang, H.; Ma, Y.; Jiang, G.; Tang, K.; Ke, Y.; Zhao, W.; Liu, H. M. Pro-apoptotic effects of JDA-202, a novel natural diterpenoid, on esophageal cancer through targeting peroxiredoxin I. *Antioxid. Redox Signal.* **2017**, *27*, 73–92.
- (33) Ye, Q.; Zhang, Y.; Cao, Y.; Wang, X.; Guo, Y.; Chen, J.; Horn, J.; Ponomareva, L. V.; Chaiswing, L.; Shaaban, K. A.; Wei, Q.; Anderson, B. D.; St Clair, D. K.; Zhu, H.; Leggas, M.; Thorson, J. S.; She, Q. B. Frenolicin B targets peroxiredoxin I and glutaredoxin 3 to trigger ROS/4E-BP1-mediated antitumor effects. *Cell Chem. Biol.* **2019**, *26*, 366–377.
- (34) Zhu, H.; Wang, B.; Kong, L.; An, T.; Li, G.; Zhou, H.; Gong, L.; Zhao, Z.; Gong, Y.; Sun, H.; Puno, P.; Li, Y. Parvifoline AA promotes susceptibility of hepatocarcinoma to natural killer cell-mediated cytotoxicity by targeting peroxiredoxin. *Cell Chem. Biol.* **2019**, *26*, 1122–1132.
- (35) Bajor, M.; Zych, A. O.; Graczyk-Jarzynka, A.; Muchowicz, A.; Firczuk, M.; Trzeciak, L.; Gaj, P.; Domagala, A.; Siernicka, M.; Zagodzón, A.; Siedlecki, P.; Kniotek, M.; O'Leary, P. C.; Golab, J.; Zagodzón, R. Targeting peroxiredoxin I impairs growth of breast cancer cells and potentially sensitises these cells to prooxidant agents. *Br. J. Cancer* **2018**, *119*, 873–884.
- (36) Hou, J. K.; Huang, Y.; He, W.; Yan, Z. W.; Fan, L.; Liu, M. H.; Xiao, W. L.; Sun, H. D.; Chen, G. Q. Adenanthin targets peroxiredoxin I/II to kill hepatocellular carcinoma cells. *Cell Death Dis* **2014**, *5*, No. e1400.
- (37) Hu, A.; Li, J.; Fang, Y.; Li, W. Adenanthin induces G2/M arrest and apoptosis in human non-small-cell lung cancer cells. *Int. J. Clin. Exp.* **2019**, 2354.
- (38) Poole, L. B.; Godzik, A.; Nayeem, A.; Schmitt, J. D. AhpF can be dissected into two functional units: tandem repeats of two thioredoxin-like folds in the N-terminus mediate electron transfer from the thioredoxin reductase-like C-terminus to AhpC. *Biochemistry* **2000**, *39*, 6602–6615.
- (39) Trott, O.; Olson, A. J. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **2009**, *31*, 455–461.
- (40) Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. UCSF Chimera—a visualization system for exploratory research and analysis. *J. Comput. Chem.* **2004**, *25*, 1605–1612.
- (41) Wallace, A. C.; Laskowski, R. A.; Thornton, J. M. LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein Eng.* **1995**, *8*, 127–134.
- (42) Salmelin, C.; Hovinen, J.; Vilpo, J. Polymyxin permeabilization as a tool to investigate cytotoxicity of therapeutic aromatic alkylators in DNA repair-deficient *Escherichia coli* strains. *Mutat. Res.* **2000**, *467*, 129–138.
- (43) Muchowicz, A.; Firczuk, M.; Chlebowska, J.; Nowis, D.; Stachura, J.; Barankiewicz, J.; Trzeciecka, A.; Klossowski, S.; Ostaszewski, R.; Zagodzón, R.; Pu, J. X.; Sun, H. D.; Golab, J. Adenanthin targets proteins involved in the regulation of disulphide bonds. *Biochem. Pharmacol.* **2014**, *89*, 210–216.
- (44) Soethoudt, M.; Peskin, A. V.; Dickerhof, N.; Paton, L. N.; Pace, P. E.; Winterbourn, C. C. Interaction of adenanthin with glutathione and thiol enzymes: selectivity for thioredoxin reductase and inhibition of peroxiredoxin recycling. *Free Radic Biol. Med.* **2014**, *77*, 331–339.
- (45) Oliveira, M. A.; Discola, K. F.; Alves, S. V.; Medrano, F. J.; Guimaraes, B. G.; Netto, L. E. Insights into the specificity of thioredoxin reductase-thioredoxin interactions. A structural and functional investigation of the yeast thioredoxin system. *Biochemistry* **2010**, *49*, 3317–3326.
- (46) Fritz-Wolf, K.; Urig, S.; Becker, K. The structure of human thioredoxin reductase 1 provides insights into C-terminal rearrangements during catalysis. *J. Mol. Biol.* **2007**, *370*, 116–127.
- (47) Lennon, B. W.; Williams, C. H., Jr.; Ludwig, M. L. Crystal structure of reduced thioredoxin reductase from *Escherichia coli*: structural flexibility in the isoalloxazine ring of the flavin adenine dinucleotide cofactor. *Protein Sci.* **1999**, *8*, 2366–2379.
- (48) Lennon, B. W.; Williams, C. H., Jr.; Ludwig, M. L. Twists in catalysis: alternating conformations of *Escherichia coli* thioredoxin reductase. *Science* **2000**, *289*, 1190–1194.
- (49) Dip, P. V.; Kamariah, N.; Subramanian Manimekalai, M. S.; Nartey, W.; Balakrishna, A. M.; Eisenhaber, F.; Eisenhaber, B.; Gruber, G. Structure, mechanism and ensemble formation of the alkylhydroperoxide reductase subunits AhpC and AhpF from *Escherichia coli*. *Acta Crystallogr. D Biol. Crystallogr.* **2014**, *70*, 2848–2862.
- (50) Wood, Z. A.; Poole, L. B.; Karplus, P. A. Structure of intact AhpF reveals a mirrored thioredoxin-like active site and implies large domain rotations during catalysis. *Biochemistry* **2001**, *40*, 3900–3911.
- (51) Tairum, C. A., Jr.; de Oliveira, M. A.; Horta, B. B.; Zara, F. J.; Netto, L. E. Disulfide biochemistry in 2-cys peroxiredoxin: requirement of Glu50 and Arg146 for the reduction of yeast Tsa1 by thioredoxin. *J. Mol. Biol.* **2012**, *424*, 28–41.
- (52) Mishra, S.; Imlay, J. Why do bacteria use so many enzymes to scavenge hydrogen peroxide? *Arch. Biochem. Biophys.* **2012**, *525*, 145–160.
- (53) Munhoz, D. C.; Netto, L. E. Cytosolic thioredoxin peroxidase I and II are important defenses of yeast against organic hydroperoxide insult: catalases and peroxiredoxins cooperate in the decomposition of H₂O₂ by yeast. *J. Biol. Chem.* **2004**, *279*, 35219–35227.
- (54) Anantharaman, A.; Rizvi, M. S.; Sahal, D. Synergy with rifampin and kanamycin enhances potency, kill kinetics, and selectivity of de novo-designed antimicrobial peptides. *Antimicrob. Agents Chemother.* **2010**, *54*, 1693–1699.
- (55) Kang, T. M.; Yuan, J.; Nguyen, A.; Becket, E.; Yang, H.; Miller, J. H. The aminoglycoside antibiotic kanamycin damages DNA bases in *Escherichia coli*: caffeine potentiates the DNA-damaging effects of kanamycin while suppressing cell killing by ciprofloxacin in *Escherichia coli* and *Bacillus anthracis*. *Antimicrob. Agents Chemother.* **2012**, *56*, 3216–3223.
- (56) Mahizan, N. A.; Yang, S. K.; Moo, C. L.; Song, A. A.; Chong, C. M.; Chong, C. W.; Abushelaibi, A.; Lim, S. E.; Lai, K. S. Terpene derivatives as a potential agent against antimicrobial resistance (AMR) Pathogens. *Molecules* **2019**, *24*, 2631.
- (57) Zacchino, S. A.; Butassi, E.; Liberto, M. D.; Raimondi, M.; Postigo, A.; Sortino, M. Plant phenolics and terpenoids as adjuvants of antibacterial and antifungal drugs. *Phytomedicine* **2017**, *37*, 27–48.
- (58) Delcour, A. H. Outer membrane permeability and antibiotic resistance. *Biochim. Biophys. Acta* **2009**, *1794*, 808–816.
- (59) Krishnamoorthy, G.; Leus, I. V.; Weeks, J. W.; Wolloscheck, D.; Rybenkov, V. V.; Zgurskaya, H. I. Synergy between active efflux and outer membrane diffusion defines rules of antibiotic permeation into Gram-negative bacteria. *mBio* **2017**, *8* DOI: 10.1128/mBio.01172-17.
- (60) Vaara, M. Polymyxin derivatives that sensitize Gram-negative bacteria to other antibiotics. *Molecules* **2019**, *24*, 249.
- (61) Lakaye, B.; Dubus, A.; Joris, B.; Frere, J. M. Method for estimation of low outer membrane permeability to beta-lactam antibiotics. *Antimicrob. Agents Chemother.* **2002**, *46*, 2901–2907.
- (62) O'Shea, R.; Moser, H. E. Physicochemical properties of antibacterial compounds: implications for drug discovery. *J. Med. Chem.* **2008**, *51*, 2871–2878.

APÊNDICE 4



Relevance of peroxiredoxins in pathogenic microorganisms

Marcos Antonio de Oliveira¹ · Carlos A. Tairum² · Luis Eduardo Soares Netto² · Ana Laura Pires de Oliveira¹ · Rogerio Luis Aleixo-Silva² · Vitoria Isabela Montanhero Cabrera¹ · Carlos A. Breyer¹ · Melina Cardoso dos Santos¹

Received: 1 February 2021 / Revised: 11 May 2021 / Accepted: 20 May 2021

© The Author(s), under exclusive licence to Springer-Verlag GmbH Germany, part of Springer Nature 2021

Abstract

The oxidative and nitrosative responses generated by animals and plants are important defenses against infection and establishment of pathogenic microorganisms such as bacteria, fungi, and protozoa. Among distinct oxidant species, hydroperoxides are a group of chemically diverse compounds that comprise small hydrophilic molecules, such as hydrogen peroxide and peroxynitrite, and bulky hydrophobic species, such as organic hydroperoxides. Peroxiredoxins (Prx) are ubiquitous enzymes that use a highly reactive cysteine residue to decompose hydroperoxides and can also perform other functions, like molecular chaperone and phospholipase activities, contributing to microbial protection against the host defenses. Prx are present in distinct cell compartments and, in some cases, they can be secreted to the extracellular environment. Despite their high abundance, Prx expression can be further increased in response to oxidative stress promoted by host defense systems, by treatment with hydroperoxides or by antibiotics. In consequence, some isoforms have been described as virulence factors, highlighting their importance in pathogenesis. Prx are very diverse and are classified into six different classes (Prx1-AhpC, BCP-PrxQ, Tpx, Prx5, Prx6, and AhpE) based on structural and biochemical features. Some groups are absent in hosts, while others present structural peculiarities that differentiate them from the host's isoforms. In this context, the intrinsic characteristics of these enzymes may aid the development of new drugs to combat pathogenic microorganisms. Additionally, since some isoforms are also found in the extracellular environment, Prx emerge as attractive targets for the production of diagnostic tests and vaccines.

Key points

- Peroxiredoxins are front-line defenses against host oxidative and nitrosative stress.
- Functional and structural peculiarities differ pathogen and host enzymes.
- Peroxiredoxins are potential targets to microbicidal drugs.

Keywords Peroxiredoxins · Oxidative stress · Antibiotic resistance · Hydroperoxides · Virulence · Structure

Introduction

Microbial pathogen infections trigger several host defense mechanisms (Medzhitov 2007; Numberger et al. 2004), such as the release of large amounts of reactive oxygen species (ROS) and nitrogen species (RNS). Among the ROS, hydrogen peroxide and superoxide are not powerful oxidants, but they can be converted to hydroxyl radical which is a highly reactive species (Halliwell and Gutteridge 2015). Furthermore, myeloperoxidase can use hydrogen peroxide to generate highly microbicidal species, such as hypochlorous acid (Winterbourn et al. 2016). Host organisms also produce nitric oxide radicals and derived oxidants (Prolo et al. 2014), such as peroxynitrite, a powerful oxidizing hydroperoxide (Radi 2018). Additionally, the oxidation of amino acids, nucleotides, and lipids generate high levels of organic

Marcos Antonio de Oliveira and Carlos A. Tairum contributed equally to this work.

✉ Marcos Antonio de Oliveira
marcos.a.oliveira@clp.unesp.br

✉ Luis Eduardo Soares Netto
nettoles@ib.usp.br

¹ Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Universidade Estadual Paulista Júlio de Mesquita Filho, Campus do Litoral Paulista, São Vicente 11330-900, Brazil

² Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, São Paulo 05508-090, Brazil

Published online: 14 July 2021

Springer

hydroperoxides (OHPs) over the course of the host response. Noteworthy, some evidences indicate that microbicidal drugs can enhance superoxide and hydrogen peroxide production (Dwyer et al. 2014; Lam et al. 2020; Repolês et al. 2020). Therefore, pathogens must remove these oxidants for establishment in a host.

Pathogenic organisms express several antioxidant enzymes to counteract oxidative insults (Staerck et al. 2017). Among them, catalases (Cat) specifically decompose hydrogen peroxide and peroxynitrite, while glutathione peroxidases (Gpx) and peroxiredoxins (Prx) can decompose several kinds of hydroperoxides, including hydrogen peroxide, peroxynitrite, and OHPs (Staerck et al. 2017). Therefore, antioxidant enzymes gain importance with the emergence of microbial strains resistant to multiple drugs, a global health threat, and the search for novel biological targets is a worldwide concern (Fisher et al. 2017). Since that some evidence indicates that microbicidal drugs can enhance superoxide and hydrogen peroxide production (Dwyer et al. 2014; Lam et al. 2020; Repolês et al. 2020), the search for antioxidant enzyme inhibitors raises as a possible alternative approach. Prx members are important targets due to their diversity and structural differences between isoforms found in pathogenic microbial organisms and hosts. Here, we aim to review several aspects of Prx enzymes such as abundance, substrate diversity, and structural/functional peculiarities that place them as potential targets for novel strategies to combat microbial infections.

Abundance and distribution of peroxiredoxins in microbial cells

Prx are the main scavengers of hydroperoxides in several organisms (Condeles et al. 2020; Seaver and Imlay 2001; Winterbourn and Hampton 2008) and especially in some microbial pathogens that lack both catalase and Gpx (Mastronicola et al. 2014; Richard et al. 2011). The significance of Prx in bacteria is highlighted by the number of different isoforms expressed, ranging from three to ten enzyme isoforms, representing some of the most abundant proteins in several bacteria. Additionally, bacterial Prx are found in the cytosol, associated with the cell membrane or with DNA, in the periplasmic space and even in biofilms (Cha et al. 1995; Enany et al. 2014; Hicks et al. 2010; Murphy et al. 2005; O'Riordan et al. 2012). It is important to mention that bacterial Prx are naturally abundant in basal conditions. Nonetheless, immune cells such as macrophages or neutrophils impose oxidative insults that can enhance Prx levels in *Salmonella typhimurium* and *Mycobacterium avium* (Francis et al. 1997; Zhu et al. 2008). Additionally, Prx are upregulated by distinct bactericidal drugs, such as amikacin, ciprofloxacin, kanamycin, tetracycline, vancomycin among others (Chen et al. 2013; Kumar et al. 2013; Li et al. 2018; Vranakis et al. 2012).

The number of Prx isoforms is also elevated in eukaryotic organisms, achieving up to six isoforms in fungi (Park et al. 2000; Rocha et al. 2018; Skrzypek et al. 2017) and three to five in protozoans (Richard et al. 2011). Several isoforms are distributed throughout diverse cellular compartments, such as the cytosol, mitochondria, nucleus, peroxisomes, protozoan glycosomes, and they are even exported outside cells (Park et al. 2000; Richard et al. 2011; Rocha et al. 2018; Urban et al. 2005; Vallejo et al. 2012). In fungi, extracellular Prx isoforms are also associated with the hypha/cell wall and biofilm (Choi et al. 2003). It is important to highlight that biofilm confers resistance to bacteria and fungi against host immune defenses and antimicrobial drugs (Lynch and Robertson 2008) and the extracellular location of some Prx in pathogenic microorganisms makes these enzymes promising targets for diagnostic tests and vaccines (Fereig et al. 2017; O'Riordan et al. 2012; Rodrigues et al. 2020).

Prx are also abundant in microbial eukaryotes, such as fungi and protozoans. *Saccharomyces cerevisiae*, a model yeast for genetic and biochemical studies, is an opportunistic pathogen in immunocompromised individuals (Souza Goebel et al. 2013). In this yeast, the abundance of Prx isoforms can attain ~ 1% of the total soluble proteins in the cell and are much more abundant than catalases and Gpx (Ghaemmaghami et al. 2003). Proteomic approaches of representative fungi and protozoans revealed a high amount of Prx in these microorganisms (de Godoy et al. 2012; Kaneva et al. 2018).

Similarly to observed in bacteria, Prx expression levels are enhanced in pathogenic fungi and protozoans in response to oxidative burst and to antimicrobial agents. In *Candida glabrata*, the expression of the Prx is strongly enhanced when insulted by the oxidative stress promoted by the host immune system (Gutierrez-Escobedo et al. 2020). In *Trypanosoma cruzi*, ectopic overexpression of Prx isoforms enhances the resistance of the pathogen against peroxynitrite and hydrogen peroxide produced by macrophages and pathogen metabolism (Piacenza et al. 2008). Concerning the antimicrobial drugs, it was observed an increased Prx expression for a wide range of antimicrobial drugs, such as amphotericin B, antimony, benznidazole, camphene thiosemicarbazide, caspofungin, flucanazole, fludioxonil, itraconazole, miltefosine, and others (Andrade et al. 2008; Das 2018; Gautam et al. 2016; Gautam et al. 2008; Shishodia et al. 2019; Silva et al. 2018; VEDIYAPPAN et al. 2010). Overall, the data presented in this section highlight the importance of Prx for microbial pathogen survival under oxidative stress conditions.

Catalytic mechanism of Prx

Prx are Cys-based peroxidases that efficiently decompose several kinds of hydroperoxides, achieving rate

constants up to $10^8 \text{ M}^{-1} \text{ s}^{-1}$ (Reyes et al. 2011; Tairum et al. 2016). The catalytic cysteine (named peroxidatic cysteine, C_P) reduces the hydroperoxide and is oxidized to sulfenic acid ($C_P\text{-SOH}$) (Hall et al. 2011). Thr (or Ser) and Arg are found with C_P in the active site of all Prx, composing a triad that is essential to the catalytic power of Prx enzymes (Fig. 1) (Hall et al. 2010; Poole and Nelson 2016). The catalytic triad Thr can be substituted by Ser in some Prx, with structural and functional consequences, but with no effect on reactivity under low concentrations of hydroperoxides (Tairum et al. 2016). Interestingly, catalytic Ser is more frequent in Bacteria than in Archaea or in Eukarya domains of life, but the evolutive reasons for this distribution are not clear to date (Tairum et al., 2021, in press).

C_P is very reactive to most hydroperoxides due to the active site microenvironment (Hall et al. 2010). Thr/Ser and Arg of the catalytic triad activate C_P and orientate the substrate in the active site for optimal reactivity (Fig. 1) through a S_N2 mechanism (Hall et al. 2010). The oxidation of C_P by hydroperoxide is common to all Prx, while the subsequent steps define two groups. Some Prx have only one catalytic cysteine (1-Cys Prx), while a second cysteine residue (named resolving cysteine, C_R) is involved in the catalysis of another group (2-Cys Prx) by forming a disulfide bond ($C_P\text{-S-S-}C_R$). 2-Cys Prx can be further divided into atypical, in which both cysteines are in the same subunit; and typical, that have a homodimer as minimal catalytic unit due to C_P and C_R are in different chains (Perkins et al. 2015).

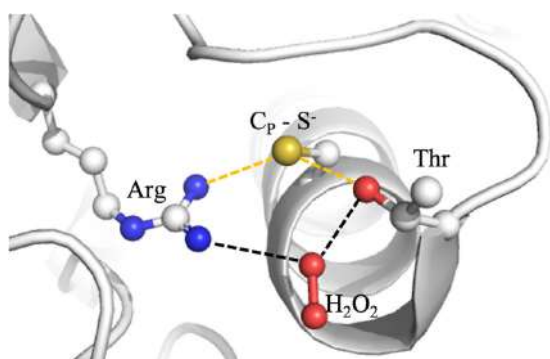


Fig. 1 Catalytic triad of Prx. Polar interactions among Arg, Thr/Ser, and C_P (yellow lines) stabilize the S^- in the deprotonated (thiolate) form. Polar interaction among Arg, Thr/Ser, and substrate (black lines) orientate the hydroperoxide towards the thiolate of C_P . The crystallographic structure of yeast Prx (PDB = 3SBC) is represented as cartoon (light gray), highlighted amino acids are represented as balls and sticks and colored as follows: carbon = light gray, oxygen = red, nitrogen = blue, and sulfur = yellow

Common aspects, structural variability, and alternative functions of Prx

All Prx enzymes display the thioredoxin-fold (Trx-fold) that is composed of four β -sheets surrounded by three α -helices, a structure that is shared with several other redox proteins from the thioredoxin super-family (Hall et al. 2011). Prx enzymes evolved from a common Trx ancestor, with the addition of secondary structural elements (Copley et al. 2004). They are subdivided into six classes: AhpC-Prx1, BCP-PrxQ, Tpx, Prx5, Prx6, and AhpE (Fig. 2a–f) based on amino acid residues in the active site and structure (Nelson et al. 2011). The AhpC-Prx1 class is mostly composed of the typical 2-Cys Prx, while the atypical 2-Cys Prx are distributed in three classes: BCP-PrxQ, Prx5, and Tpx. Proteins displaying the 1-Cys Prx mechanism are mainly present in the Prx6 group, but there are also 1-Cys Prx in the BCP-PrxQ, Prx5, and AhpE classes (Nelson et al. 2011).

The oligomeric states of these enzymes are also very diverse: monomers, dimers, decamers, and other quaternary species (Fig. 3) (Hall et al. 2011; Jang et al. 2004; Nelson et al. 2011; Tairum et al. 2016). The Prx dimers can assemble in two different ways: A-type (Fig. 3a), presenting unique

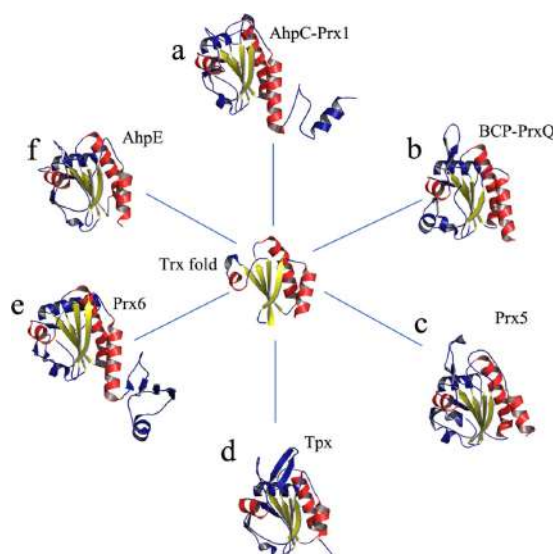


Fig. 2 The common Trx-fold and structural variations among the six Prx classes. Prx from different organisms as representatives of the six classes are derived from a common Trx-fold (PDB = 2J23) (center of image). (a) AhpC-Prx1, yeast Tsa1 (PDB = 3SBC); (b) BCP-PrxQ, *Xylella fastidiosa* BCP (PDB = 3IXR); (c) Prx5, *P. falciparum* AOP (PDB = 1XIY); (d) Tpx, *E. coli* Tpx (PDB = 3HVV); (e) Prx6, *P. aeruginosa* LsfA (PDB = 6P0W); (f) AhpE, *M. tuberculosis* AhpE (PDB = 4X0X). The structures are represented as cartoon, in which the α -helices that compose the Trx-fold are represented in red and the β -sheets are colored in yellow. Additional elements specific to each class are shown in blue. The structures are represented as monomers for clarity

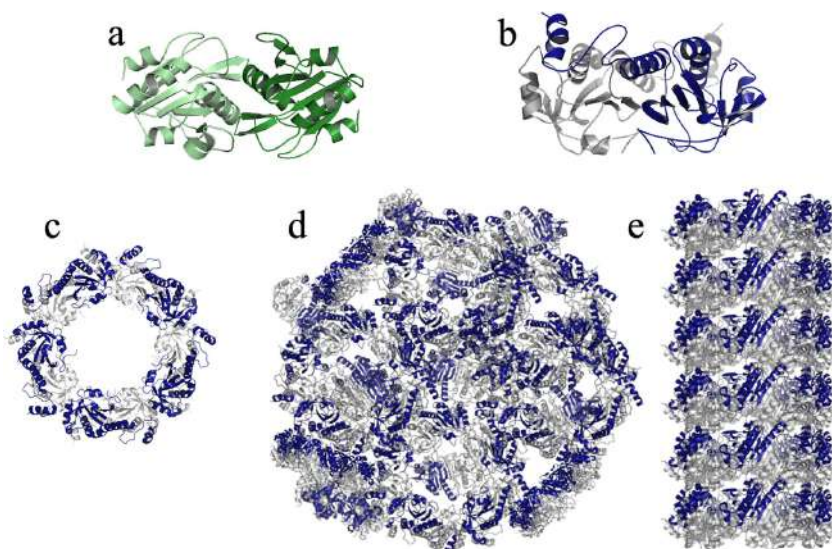


Fig. 3 Representation of the two types of Prx dimers and the quaternary structures diversity of peroxiredoxins. Structures are represented as cartoons, and each monomer is represented by a different color. **(a)** A-type dimers present in the BCP-PrxQ, Tpx, Prx5, and AhpE classes. The A-type is represented by yeast Ahp1 (PDB = 4OWY), and the monomers are colored in light green and green. **(b)** B-type dimers are found in the classes AhpC-Prx1 and Prx6 (light gray and dark blue) (PDB = 3SBC).

Some dimeric Prx from the AhpC-Prx1 and Prx6 classes can assemble into toroidal structures formed by pentamers of dimers (decamers) (PDB = 3SBC). **(c)** Under oxidative or heat stresses, some Prx can organize in high molecular weight (HMW) species as spherical **(d)** or filamentous multimers **(e)**. The HMW complexes are illustrative and were constructed using the yeast Prx coordinates (PDB = 3SBC). The monomers of the dimers are represented in dark blue and light gray for clarity

globular domain, and B-type (Fig. 3b), in which the central β -sheets of both monomers are aligned (Poole and Nelson 2016). Members of the BCP-PrxQ, Prx5, Tpx, and AhpE classes can form A-type dimers, but there is a wide variation at this organization level. Most proteins of the BCP-PrxQ class are monomers, with a few of them as A-type dimers. Representatives of the Prx5 group are detected as monomers and dimers, and some bacterial members possess Prx5 fused to a glutaredoxin molecule and can form tetramers (Kim et al. 2003; Nelson et al. 2011). The Tpx members are found only as A-type dimers. Finally, the AhpE members assemble as A-type dimers that can further associate as octamers (tetramers of dimers) (Hall et al. 2011).

The B-type dimer is present in members of the AhpC-Prx1 and Prx6 classes, representing the minimum catalytic unit of AhpC-Prx1, since C_P and C_R are present in different monomers. Interestingly, AhpC-Prx1 and Prx6 enzymes can further assemble into tetramers, octamers, decamers, dodecamers, or high molecular weight species (Poole et al. 2011; Poole and Nelson 2016). Additionally, AhpC-Prx1 enzymes with Thr in the catalytic triad possess the particularity of switching between oligomeric states in a redox-dependent manner: decamers in the reduced form (Fig. 3c) and dimers in the disulfide state (Tairum et al. 2016).

The diverse structural features among Prx classes might represent an opportunity for the characterization of inhibitors used as drugs in the combat of pathogens. Part of the diversity

of proteins of the six Prx classes resides in the amino acid composition of the active sites (Hall et al. 2011). Tpx and AhpE members are found almost exclusively in prokaryotes and BCP-PrxQ is absent in animals (Poole and Nelson 2016). For the other three classes, specific features can be taken into account to design molecules that specifically target enzymes from pathogens and not from the hosts.

The amino acid sequences of microbial Prx isoforms (bacteria, fungi, and protozoans) differ considerably from host enzymes (mammals and plants), ranging from 35 to 60% of identity (data not shown). Some of these different residues are found in the active site. These differences can be explored for obtaining specific inhibitors. To illustrate this variation, the structure of representative proteins from pathogens and hosts of the subclasses AhpC-Prx1, BCP-PrxQ, Prx5, and Prx6 were overlapped (Fig. 4). Notably, some of the differences between host and pathogen protein surfaces are close to C_P (Fig. 4a–d). AhpC-Prx1 from eukaryotes possesses a C-terminal α -helix and other specific features involved in the increased susceptibility of C_P to hyperoxidation that are absent in most prokaryotic proteins (Wood et al. 2003) (Fig. 4a). The catalytic triad Thr residue is replaced by a Ser residue in some pathogenic bacteria (*Staphylococcus* sp. and *Bacillus* sp., among others), fungi (Saccharomycetales), and protozoans (*Leishmania* sp.), but is almost absent in hosts (unpublished data). This single substitution confers considerable structural and functional differences between Prx containing Thr or Ser

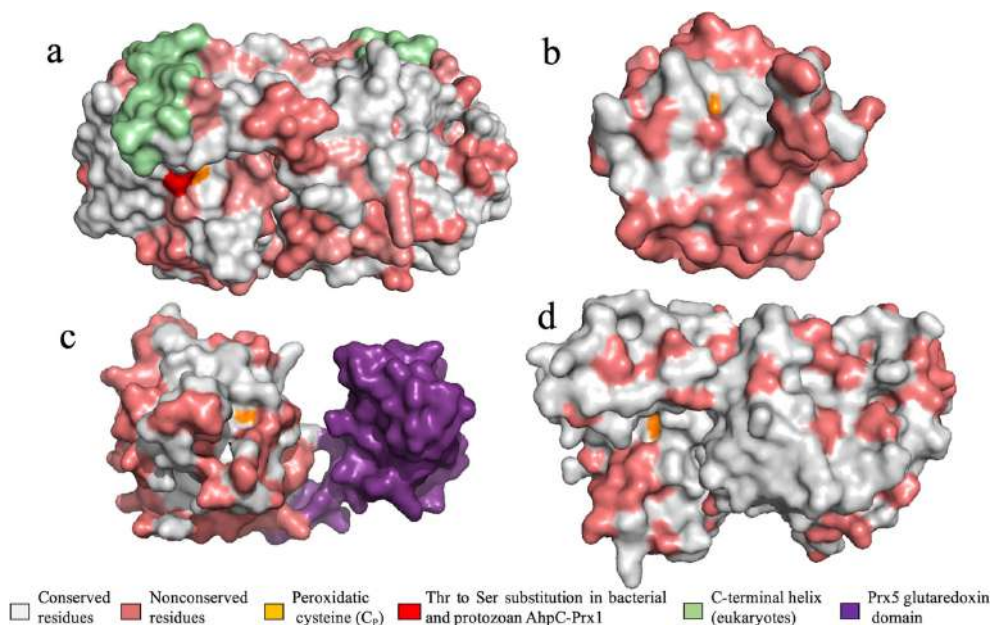


Fig. 4 Conservation of surface residues among Prx from the host and pathogen. Molecular surfaces of Prx enzymes, depicting the common amino acids between host and pathogen (white), the different ones (pink), and the active site catalytic C_p (orange). Conserved residues were considered the same amino acid or amino acids presenting similar physicochemical properties. **(a)** Molecular surface of human AhpC-Prx1 (Prx2; PDB: 1QMV) was used as template and compared to the homologue isoform from *S. typhimurium* (UniProt ID: P0A251). C-

terminal α -helix that are absent in most prokaryotes is colored in light green. Position of Thr/Ser in the active site is highlighted in red. **(b)** BCP-PrxQ from *X. fastidiosa* (PDB: 3IXR) compared with the plant *Citrus sinensis* (NCBI ID: XP_006474598.1). **(c)** The structure of Prx5 from *H. influenzae* (PDB: 1NM3) and human Prx5 (UniProt ID: P30044). The additional Grx domain present in some pathogenic bacteria is colored in purple. **(d)** Human Prx6 (PDB: 1PRX) compared with its bacterial counterpart from *P. aeruginosa* (UniProt ID: A0A0H2ZEH5)

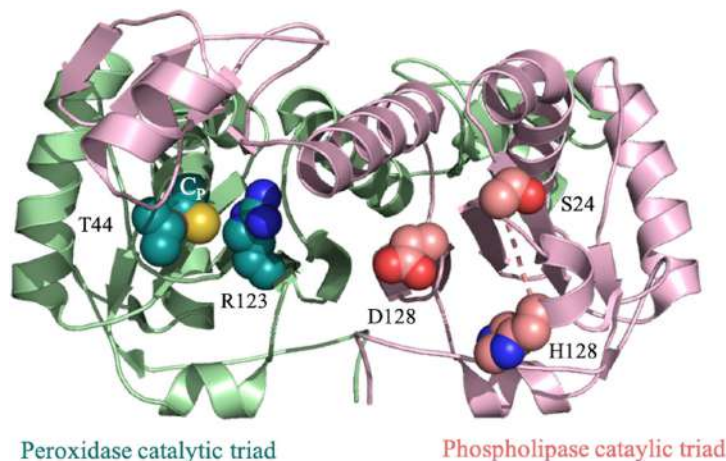
(Tairum et al. 2016). The Thr/Ser substitution may also provide a different microenvironment to ligands targeted to Prx from pathogens (Fig. 4a). For some pathogenic bacteria, such as *Haemophilus influenzae*, *Neisseria meningitidis*, and *Vibrio cholerae*, the differences among the Prx5 isoforms are accentuated as a consequence of a glutaredoxin domain fused to the C-terminal region (Fig. 4c), a feature that is not observed in animal and plant isoforms (Kim et al. 2003).

Besides the peroxidase activity, some peroxiredoxins present additional functions. At high hydroperoxide concentrations, C_p is hyperoxidized to sulfinic acid (C_p -SO₂H), resulting in loss of peroxidase activity and concomitant acquisition of chaperone (holdase) function. In parallel, these 2-Cys Prxs undergo structural rearrangements to form high molecular weight species (HMW), presenting a spherical or filamentous organization (Fig. 3 d and e) (Hall et al. 2009; Jang et al. 2004; Saccoccia et al. 2012; Tairum et al. 2016). A C-terminal α -helix, which is restricted to eukaryotic isoforms with rare exceptions (e.g., *Helicobacter pylori* AhpC), facilitates C_p hyperoxidation by impairing disulfide formation (Hall et al. 2009). Molecular chaperone activity has also been described in Prx from some microorganisms previously

exposed to heat shock without the requirement of C_p hyperoxidation (Kamariah et al. 2018; Morais et al. 2017; Saccoccia et al. 2012; Teixeira et al. 2015). In addition, a BCP-PrxQ from *Deinococcus radiodurans* and a Prx6 from *Anabaena* sp. also present molecular chaperone activity (Cho et al. 2019; Mishra et al. 2017).

Another additional function displayed by some members of the Prx6 class is a calcium-independent phospholipase A₂ activity (PLA₂), which is unrelated to the peroxidase function (Bannitz-Fernandes et al. 2019). The PLA₂ activity of Prx6 depends on a second active site separated from the peroxidase center and composed of a catalytic Ser, a His and an Asp (Fig. 5). In pathogens, PLA₂ activity was described in Prx6 enzymes from *Pseudomonas aeruginosa* and from *Aspergillus fumigatus*, two respiratory airway pathogens. These phospholipase activities might be related to the metabolism of surfactants in the lungs during colonization by damaging phospholipids from the host cell membrane or nutrient acquisition (Bannitz-Fernandes et al. 2019). Besides the peroxidase function, these additional activities of some Prx groups may support the ideal condition for organism survival, especially microorganisms.

Fig. 5 Catalytic triads of peroxidase and PLA₂ activities of a representative Prx6 enzyme. The active sites of the two functions performed by members of the Prx6 class are independent. The LsfA structure (PDB = 6P0W) is represented as cartoon and each monomer present a different color (pale green and light pink). Corresponding residues for each catalytic triad are represented as spheres and colored as follows: C = green or pink (peroxidase or phospholipase catalytic triad, respectively), N = blue, O = red, and S = light orange



Peroxioredoxin reductants

To perform catalysis efficiently, Prx requires appropriate reductants for fast turnover and the Trx system is the most frequently employed. The Trx system comprises thioredoxin (Trx), thioredoxin reductase (TrxR), and NADPH as electron source (Oliveira et al. 2010). Although Trx enzymes are highly conserved, TrxR enzymes are distinct between microorganisms/plants and mammals and have been explored as drug targets (Koch et al. 2013). In both cases, TrxR are obligate dimers, but the enzymes present low molecular weight (molecular mass of monomers ~ 33 kDa) in bacteria, fungi, and plants. In mammals, TrxR possesses ~ 50 kDa per monomer and contains a selenocysteine residue (Oliveira et al. 2010). Below, we describe variations found in the reduction of Prx enzymes from the canonical Trx system.

AhpC-Prx1

A specialized Trx system in bacteria, named AhpF, is dedicated to the reduction of AhpC-Prx1 enzymes. AhpF is a multidomain enzyme, containing a TrxR portion fused to two Trx domains in a single polypeptide. Although AhpF can use either NADH or NADPH as the electron donor, NADH is the preferred reductant (~ 100×), in contrast to the canonical TrxR that preferentially uses NADPH (Poole and Ellis 1996).

Mycobacterium tuberculosis presents another specialized system for the reduction of some AhpC-Prx1 isoforms. This system comprises AhpD, a bacterial protein with thioredoxin-like activity, but without significant structural homology to Trx. It acts together with dihydrolipoamide succinyltransferase (SucB) and dihydrolipoamide dehydrogenase (Lpd), also using electrons from NADH (Bryk et al. 2002).

Protozoans from the *Trypanosoma* and *Leishmania* genera present a unique thiol-disulfide network that is able to reduce AhpC-Prx1 members (so-called in trypanosomatids as trypanedoxin peroxidase, TxnPx) (Netto et al. 2016; Piacenza et al. 2008). The system uses NADH as an electron donor and includes the enzymes trypanedoxin (Txn), an oxidoreductase homologue of thioredoxin, and trypanedoxin reductase (TR), a flavoenzyme structurally similar to the high molecular weight TrxR. The system also comprises trypanothione (TSH), an abundant low molecular weight thiol that is only present in trypanosomatids (Jaeger and Flohe 2006). In this case, peroxiredoxin disulfide is directly reduced by Txn, which is restored to the reduced state by trypanothione. The oxidized trypanothione molecules are reduced by TR using NADH.

Prx5

Prx5 enzymes can be reduced by the glutathione (GSH) system, which is composed of glutaredoxin (Grx), GSH, glutathione reductase (GR), and NADPH. GSH forms a mixed disulfide with C_P-SOH, which is then reduced by Grx, regenerating reduced Prx. A second GSH molecule recovers reduced Grx, generating oxidized glutathione (GSSG), which is then reduced by glutathione reductase using electrons from NADPH. It is worth to highlight that Prx domain is fused to a Grx domain (Fig. 5c) in some bacterial Prx5 enzymes, which appears to confer higher reducing efficiency to the system (Kim et al. 2003).

Prx6

For some Prx6, the GSH system was proposed to turnover the enzyme (Pedrajas et al. 2016). The Prx6 class is mostly composed of 1-Cys Prx enzymes and so distinct agents directly

reduce C_p-SOH intermediate. Although several biological reductants have been tested, most of them failed to reduce microbial Prx6, including Trx system (Longo et al. 2020; Rocha et al. 2018). Prx1 from *S. cerevisiae* is an exception, being reduced by the mitochondrial Trx system (Pedrajas et al. 2000). In contrast, ascorbate is a general reductant for Prx6 enzymes (Anschau et al. 2020). We demonstrated that the sulfenic acids in yeast Prx1 and mammalian Prx6 are reduced by ascorbate, the only biological and non-thiolic reductant described for peroxiredoxins so far (Monteiro et al. 2007). Later, we determined that Prx6 isoforms from pathogenic microorganisms, such as bacteria (LsfA, *P. aeruginosa*) are reduced by ascorbate at moderate rates (Anschau et al. 2020).

AhpE

Like Prx6 class, most AhpE enzymes are 1-Cys Prx and the identities of the biological reductants are still controversial. We have previously demonstrated that AhpE from *M. tuberculosis* can be reduced by ascorbate (Monteiro et al. 2007). Additionally, *M. tuberculosis* presents a thiolic enzymatic system that is involved in AhpE reduction. This system was only described in the actinobacteria group and is represented by mycoredoxin (Mrx1), mycothiol disulfide reductase (MR), and a low molecular weight thiolic compound named mycothiol (MSH) (Hugo et al. 2014).

The unique aspects of some of the reductive systems of various Prx enzymes from microorganisms, without counterparts in the hosts, are frequently considered as important chemotherapeutic targets (Budde and Flohe 2003; Jaeger and Flohe 2006). In fact, inhibitors for peroxiredoxin reducing systems in bacteria and fungi have already been identified (Koshkin et al. 2004; Marshall et al. 2019) and inhibitors for the Prx trypanosomatid reducing system have recently shown promising results in preclinical studies (Tunes et al. 2020).

Substrate diversity and protective roles of peroxiredoxins

Prx enzymes display lower specificity towards hydroperoxides, decomposing them with high efficiency. For instance, Prx enzymes from several microbial pathogens decompose hydrogen peroxide and peroxynitrite very efficiently, at rate constants from 10^4 to $10^8 \text{ M}^{-1} \text{ s}^{-1}$ (Pineyro et al. 2011; Reyes et al. 2016; Tairum et al. 2016). The reduction of OHPs by Prx is also efficient (up to $10^8 \text{ M}^{-1} \text{ s}^{-1}$) and important for pathogen survival (Estelle et al. 2020; Jacobson et al. 1989; Reyes et al. 2011). The vast majority of studies used synthetic organic molecules such as *tert*-butyl (*t*-BOOH) and cumene (CHP) hydroperoxides to mimic biological OHPs and members of all Prx classes efficiently decompose these molecules (Akerman and Muller 2005; Baker and Poole 2003;

Parsonage et al. 2008; Tairum et al. 2016). Table 1 summarizes different kinds hydroperoxides used to challenge distinct Prx classes.

It is important to note that OHPs are bulkier than hydrogen peroxide or peroxynitrite, so Prx active sites are large and hydrophobic to accommodate such distinct molecules. In fact, some studies demonstrate that even bulky substrates are efficiently decomposed by Prx. For instance, *M. tuberculosis* AhpE achieves rate constants of approximately $10^8 \text{ M}^{-1} \text{ s}^{-1}$ over arachidonic acid hydroperoxide (15-HpETE), a very bulky hydroperoxide produced by reticulocytes, eosinophils, and T-lymphocytes (Reyes et al. 2011). Interestingly, arachidonic acid is an integrant of cell membranes and AhpE was detected in the membrane fraction of *M. tuberculosis* (Gu et al. 2003). In *H. pylori*, AhpC and BCP efficiently decompose OHP, making the pathogen tolerant to oxidative stress (Baker et al. 2001). In the fungus *Paracoccidioides brasiliensis*, a Prx6 isoform can decompose linoleic hydroperoxide more efficiently than *t*-BOOH and CHP (3–8-fold higher, respectively) (Longo et al. 2020). Accordingly, linoleic and linolenic acid hydroperoxides are also of high importance in infection by pathogens due to their pronounced microbicidal effect (Estelle et al. 2020). Together, the information presented in this section demonstrates the substrate versatility of Prx, indicating the role of these enzymes in cell protection, including against oxidative stress generated by OHPs.

Effects of Prx inactivation in pathogenic microorganism cells

Silencing of Prx usually renders cells more sensitive to hydroperoxides insults (Castro et al. 2020; Comtois et al. 2003; Cosgrove et al. 2007; Hillmann et al. 2016; Missall et al. 2004; Rocha et al. 2018; Teixeira et al. 2015). Their importance in hydroperoxide decomposition can be associated with DNA protection. In fact, the first bacterial peroxiredoxins described (AhpC from *Escherichia coli* and *S. typhimurium*) were identified by observing that strains lacking *ahpC* genes presented high mutation rates, while strains overexpressing AhpC suppressed DNA alterations (Storz et al. 1987). This protective role was later related to the ability of AhpC to decompose OHPs (Jacobson et al. 1989), a phenomenon also observed in other bacterial species and eukaryotic microorganisms. For instance, Δ *ahpC* from *Bacteroides fragilis* presents five times more mutations than the wild-type strain (Rocha and Smith 1999). Although the deletion of one or more Prx isoforms in the yeast *S. cerevisiae* is not lethal, it compromises genome stability (Ogusucu et al. 2009). In *Candida albicans*, the Δ *tsa1* strains also present increased mutation rates, indicating that Prx contributes to genome stability (Urban et al. 2005). The protective role of Prx in DNA

Table 1 List of different hydroperoxides that are decomposed by Prx of each class in bacteria, fungi, and protozoans

Prx class	Group	Hydroperoxide	Enzyme/organism	Reference(s)
AhpC-Prx1	Bacteria	H ₂ O ₂ ; <i>t</i> -BOOH; CHP; peroxynitrite; ethyl hydroperoxide; linoleic acid hydroperoxide; 5'-hydroperoxymethyluracil (thymine hydroperoxide); 5-hydroperoxy-6-hydroxy-dihydrothymine; 5-hydroperoxy-6-methyl-6-hepten-2-one and 6-hydroperoxy-6-methyl-4-hepten-2-one; 7 α -hydroperoxy-3 β -hydroxycholest-6-ene; trans-9-hydroperoxyoctadec-10-enoic acid and trans-10-hydroperoxyoctadec-8-enoic acid; trans-pinocarveyl hydroperoxide (3)	<i>EcAhpC</i> , <i>HpAhpC</i> , <i>MtAhpC</i> , <i>StAhpC</i>	Baker et al. 2001; Bryk et al. 2000; Hillas et al. 2000; Jacobson et al. 1989; Jaeger et al. 2004; Parsonage et al. 2005
AhpC-Prx1	Fungi	H ₂ O ₂ ; <i>t</i> -BOOH; CHP; peroxynitrite	<i>ScTsa1</i> , <i>ScTsa2</i>	Munhoz and Netto 2004; Ogunseun et al. 2007; Tatum et al. 2016
AhpC-Prx1	Protozoa	H ₂ O ₂ ; <i>t</i> -BOOH; CHP; peroxynitrite; linoleic acid hydroperoxide; phosphatidylcholine hydroperoxide	<i>CjTXNPrx</i> , <i>GiPrx1a</i> , <i>GiPrx1b</i> , <i>LmTryP1</i> , <i>TbcTXNPrx</i> , <i>TccTXNPrx</i> , <i>TcmTXNPrx</i> , <i>TgcTXNPrx</i>	Körmann and Müller 2005; Flohé et al. 2002; König and Fairlamb 2007; Mastromicola et al. 2014; Nogueira et al. 1997; Pineyro et al. 2011; Trujillo et al. 2004
BCP-PrxQ	Bacteria	H ₂ O ₂ ; <i>t</i> -BOOH; CHP; peroxynitrite; linoleic acid hydroperoxide; fatty acids hydroperoxides	<i>EcBCP</i> , <i>HpBCP</i> , <i>MbPrxQB</i> , <i>XPrxQ</i>	Horta et al. 2010; Jeong et al. 2000; Reyes et al. 2016; Wang et al. 2005
Prx	Bacteria	H ₂ O ₂ ; <i>t</i> -BOOH; CHP; peroxynitrite	<i>EcTpx</i> , <i>MtTpx</i>	Baker and Poole 2003; Jaeger et al. 2004
Prx5	Bacteria	H ₂ O ₂	<i>VvPrx3</i>	Ahn et al. 2018
Prx5	Fungi	H ₂ O ₂ ; <i>t</i> -BOOH	<i>AAsp f3</i>	Hillmann et al. 2016
Prx5	Protozoa	<i>t</i> -BOOH	<i>PfAOP</i>	Staudacher et al. 2015
Prx6	Fungi	H ₂ O ₂ ; <i>t</i> -BOOH; CHP; linoleic acid hydroperoxide	<i>AfPrx1</i> , <i>AfPrxC</i> , <i>PbPrx1</i>	Longo et al. 2020; Rocha et al. 2018
AhpE	Bacteria	H ₂ O ₂ ; <i>t</i> -BOOH; CHP; peroxynitrite; 15-HpETE (arachidonic hydroperoxide); 3-CPBA (3-chloroperoxybenzoic acid); HCO ₄ (peroxymonocarbonate); linoleic acid hydroperoxide	<i>MtAhpE</i>	Hugo et al. 2009; Reyes et al. 2011

Af = *Aspergillus fumigatus*; *Cj* = *Critidia fasciculata*; *Ec* = *Escherichia coli*; *Gi* = *Giardia intestinalis*; *Hp* = *Helicobacter pylori*; *Lm* = *Leishmania major*; *Mb* = *Mycobacterium bovis*; *Mt* = *Mycobacterium tuberculosis*; *Pb* = *Paracoccidioides brasiliensis*; *Pf* = *Plasmodium falciparum*; *Sc* = *Saccharomyces cerevisiae*; *St* = *Salmonella typhimurium*; *Tb* = *Trypanosoma brucei*; *Tc* = *Trypanosoma cruzi*; *Tg* = *Toxoplasma gondii*; *Vv* = *Vibrio vulnificus*

integrity was also observed under nitrosative stress in *Trypanosoma brucei*. Defective strains in the enzyme uracil-DNA glycosylase (UNG), a component of the base excision DNA repair system, exhibit reduced infectivity and increased levels of DNA damage (Yague-Capilla et al. 2019). Notably, surviving cells of the *T. brucei* Δ ung strain present increased expression of AhpC-Prx1 peroxiredoxin (Tryp1), which is correlated with peroxynitrite detoxification (Yague-Capilla et al. 2019).

Prx also provide protection through a mechanism related to their chaperone/holdase activity. As explained before, the acquisition of chaperone activity can occur by C_P hyperoxidation (Jang et al. 2004). Since almost all bacterial AhpCs are resistant to C_P hyperoxidation (Wood et al. 2003), the acquisition of chaperone activity to these microorganisms probably involves a redox independent mechanism, which is dependent on thermal insults (Jang et al. 2004), but still poorly understood. Only few descriptions of chaperone activities of bacterial Prx isoforms have been performed to date (Buranajitpakorn et al. 2011; Kamariah et al. 2018). The chaperone function of *E. coli* AhpC was mostly investigated in cell-free systems. In one case, no evidence of heat shock protection by AhpC in *Xanthomonas campestris* was obtained (Buranajitpakorn et al. 2011).

In fungi, the acquisition of chaperone function was described for the two cytosolic isoforms of the AhpC-Prx1 class. Tsa1 and Tsa2 undergo transitions from peroxidase to chaperone in response to oxidative stress and thermal insults. Additionally, null individual mutants or the double mutant Δ ttsa1/ Δ ttsa2 present a significant reduction in cell viability compared with the wild-type strain when subjected to heat shock (Jang et al. 2004). A decrease in cell survival as a consequence of thermal insult has also been reported in *Cryptococcus neoformans* Δ ttsa1 mutant (Missall et al. 2004).

In protozoans, the AhpC-Prx1 enzymes from *Schistosoma mansoni* and *Leishmania infantum* were described to switch from peroxidase to chaperone in response to oxidative stress or heat shock (Saccoccia et al. 2012; Teixeira et al. 2015). Furthermore, genetic inactivation of the *L. infantum* mitochondrial AhpC-Prx1 isoform (Δ mtcnp1) rendered cells a thermosensitive phenotype. High levels of protein aggregation were detected at 37°C (Teixeira et al. 2015), which is compatible with the physiological temperature of mammalian hosts.

Besides inactivating Prx by genetic approaches, pharmacological inhibitors represent an alternative to investigate the roles of these proteins in pathogens. However, only a few microbial peroxiredoxin inhibitors have been identified to date. For instance, showdomycin is a potent C-glycosyl nucleoside antibiotic isolated from *Streptomyces showdoensis* that was detected as a ligand of AhpC catalytic cysteines from *Staphylococcus aureus* (Bottcher and Sieber 2010). In protozoans, conoidin A was initially described as an inhibitor of host cell invasion by the pathogenic protozoan *Toxoplasma*

gondii; this compound was later shown to bind covalently to cysteine residues of TgPrxII, a peroxiredoxin from the AhpC-Prx1 subclass. Conoidin A also prevents the growth of *Plasmodium falciparum* and increases the sensitivity of the parasite in host cells to chloroquine, an antimalarial drug (Haraldsen et al. 2009). Both conoidin A and showdomycin are bulky molecules with large hydrophobic backbones, which may mimic large biological substrates, as OHPs. Therefore, inactivating Prx isoforms may be a promising approach to enhance the death of pathogens resistant to multiple drugs.

Prx as virulence factors

Since Prx play important roles in pathogen survival, they may represent virulence factors. In fact, enzymes from five of the six Prx classes have been described as virulence factors in pathogens (Table 2). No studies have yet been performed to examine the involvement of AhpE enzymes in virulence. The role of different Prx classes in the virulence of the pathogenic microorganisms is summarized below.

AhpC-Prx1

AhpC-Prx1 isoforms were associated with *Helicobacter cinaedi* and *H. pylori* virulence, since *ahpC* mutants (Δ *ahpC*) could not to colonize mice stomach and were more susceptible to killing by macrophages (Charoenlap et al. 2012; Olczak et al. 2003). Moreover, *H. cinaedi* lacking the *ahpC* gene presented a reduced ability to colonize the intestine of both wild-type and defective in interleukin-10 (IL-10) mice (Charoenlap et al. 2012).

In the pathogenic marine bacterium *Vibrio vulnificus*, AhpC is related to both the bacterial capacity to grow during infection of human intestinal cells and the ability to infect mice (Baek et al. 2009). Infecting wild-type mice with a Δ *ahpC* strain of *Listeria monocytogenes* diminishes bacterial virulence. This Δ *ahpC* strain was virulent when infecting mice with iNOS deficiency, indicating that AhpC protects bacteria from nitric oxide-derived oxidants, such as peroxynitrite (Dons et al. 2014).

AhpC-Prx1 members from the *Mycobacterium* genus are indirectly related to the actions of catalase and isoniazid (INH), a drug largely employed in the treatment of tuberculosis. *Mycobacterium tuberculosis* is able to grow inside host macrophages using diverse strategies to detoxify distinct ROS and RNS. In addition, some strains of *M. tuberculosis* present INH resistance that is related to catalase (KatG) mutations (Rintiswati et al. 2011). INH is a prodrug that requires “activation” by KatG to inhibit enoyl-acyl carrier protein reductase from *M. tuberculosis* by the formation of an isonicotinoyl-NAD adduct (Yu

Table 2 Prx enzymes and virulence

Prx class	Virulence factor	Protein(s)	Organism	Reference
AhpC-Prx1	Yes	AhpC	<i>Helicobacter cinaedi</i>	Charoenlap et al. 2012
	Yes	AhpC	<i>Helicobacter pylori</i>	Olczak et al. 2003
	Yes	Prx	<i>Listeria monocytogenes</i>	Dons et al. 2014
	Yes	AhpC	<i>Mycobacterium bovis</i>	Wilson et al. 1998
	No	AhpC	<i>Mycobacterium tuberculosis</i>	Heym et al. 1997
	No	AhpC	<i>Mycobacterium tuberculosis</i>	Kaufmann et al. 2001
	No	AhpC	<i>Porphyromonas gingivalis</i>	Johnson et al. 2004
	No	AhpC	<i>Salmonella typhimurium</i>	Taylor et al. 1998
	No	AhpC	<i>Staphylococcus aureus</i>	Cosgrove et al. 2007
	Yes	AhpC	<i>Streptococcus pyogenes</i>	Brenot et al. 2005
	No	AhpC	<i>Streptococcus pyogenes</i>	Brenot et al. 2005
	Yes	AhpC1	<i>Vibrio vulnificus</i>	Baek et al. 2009
	No	Tsa1	<i>Candida albicans</i>	Urban et al. 2005
	Yes	Tsa1/Tsa2	<i>Candida glabrata</i>	Gutierrez-Escobedo et al. 2020
	Yes	Tsa1	<i>Cryptococcus neoformans</i>	Missall et al. 2004
	Yes	Prx	<i>Entamoeba histolytica</i>	Davis et al. 2006
	Yes	mTXPx	<i>Leishmania infantum</i>	Castro et al. 2011
	No	GPrx	<i>Leishmania infantum</i>	Castro et al. 2020
	Yes	Prx	<i>Plasmodium berghei</i>	Usui et al. 2015
	Yes	TXNPx	<i>Trypanosoma cruzi</i>	Piñeyro et al. 2008
BCP-PrxQ	Yes	BCP	<i>Brucella melitensis</i>	Zygmunt et al. 2006
	No	Prx1	<i>Helicobacter pullorum</i>	Parente et al. 2017
	Yes	BCP	<i>Helicobacter pylori</i>	Wang et al. 2005
Tpx	Yes	Tpx	<i>Enterococcus faecalis</i>	La Carbona et al. 2007
	Yes	Tpx	<i>Helicobacter pylori</i>	Olczak et al. 2003
	Yes	Tpx	<i>Mycobacterium tuberculosis</i>	Hu and Coates 2009
	No	Tpx	<i>Salmonella enterica</i>	Horst et al. 2010
	Yes	TpxD	<i>Streptococcus pneumoniae</i>	Hajaj et al. 2012
Prx5	Yes	Prx5	<i>Brucella abortus</i>	Hu et al. 2019
	Yes	Prx5-Grx	<i>Neisseria meningitidis</i>	Aljannat et al. 2020
	Yes	Prx3	<i>Vibrio vulnificus</i>	Lim et al. 2014
	Yes	Aps f3	<i>Aspergillus fumigatus</i>	Hillmann et al. 2016
Prx6	Yes	LsfA	<i>Pseudomonas aeruginosa</i>	Kaihami et al. 2014
	Yes	Prx1	<i>Aspergillus fumigatus</i>	Rocha et al. 2018
	No	PrxB	<i>Aspergillus fumigatus</i>	Rocha et al. 2018
	No	PrxC	<i>Aspergillus fumigatus</i>	Rocha et al. 2018
	No	Tsa3	<i>Cryptococcus neoformans</i>	Missall et al. 2004
	Yes	MoPrx1	<i>Magnaporthe oryzae</i>	Mir et al. 2015

et al. 2003). Mutants of KatG are defective in the activation of INH and in the elimination of hydrogen peroxide. Therefore, increased levels of AhpC compensate the loss

in KatG activity and are relevant in *M. tuberculosis* resistance to INH (Sherman et al. 1996).

Catalase (KatA) and AhpC also have compensatory roles in *S. aureus*, especially during aerobic growth, even though

these enzymes are not required for bacterial virulence in mice or for survival of the bacteria in human neutrophils (Cosgrove et al. 2007). AhpC deletion did not affect the virulence of *Porphyromonas gingivalis* and *S. typhimurium* in murine models (Johnson et al. 2004; Taylor et al. 1998). Curiously, *Streptococcus pyogenes* Δ ahpC was less virulent in one of two murine models tested, suggesting a differential immune response according to the host's genetic background or related to infection technique (Brenot et al. 2005).

In the fungus *C. glabrata*, deletion of *tsa2* gene has a profound impact on virulence compared with the Δ tsa1 strain, while Tsa1 and Tsa2 are highly induced when challenged by neutrophils and are important for both the survival and growth of this pathogen in the immune cells (Gutierrez-Escobedo et al. 2020). Similarly, mice infected with *C. neoformans* Δ tsa1 strains are able to survive for a longer period than animals infected with wild-type pathogenic yeast. The levels of the pathogen in the lung and in the brain of animals infected with the mutant strain were significantly lower (Missall et al. 2004). In contrast, *C. albicans* Tsa1 is an important component against oxidative stress, but it has no impact on virulence (Urban et al. 2005).

In *T. cruzi*, overexpression of two AhpC-Prx1 isoforms made the pathogen more efficient against the oxidative defenses of macrophages, indicating virulence role of both enzymes (Leitsch et al. 2018). In *Plasmodium berghei*, Δ prx1 cells were not able to survive in a murine model (Usui et al. 2015). For *Entamoeba histolytica*, nonvirulent strains had low expression levels of AhpC-Prx1 and overexpression of this enzyme rendered higher resistance to oxidative stress and partially restored pathogenicity to this strain, while gene deletion in virulent strains lead to avirulence (Davis et al. 2006). Deletion of the mitochondrial isoform of *L. infantum* decreased the long-term persistence of the pathogen in the liver and spleen of guinea pigs compared to the wild-type strain (Angelucci et al. 2016; Castro et al. 2011). Conversely, experiments using the *L. infantum* strain with a deletion of the glycosomal isoform Prx showed that this enzyme is dispensable for virulence in mice (Castro et al. 2020).

BCP-PrxQ

The *H. pylori* Δ bcp mutant has a significantly lower ability to colonize and survive in mouse stomach than the wild-type strain, mainly after 3 weeks of infection (Wang et al. 2005). In contrast, *Helicobacter pullorum* Δ prx1 and wild-type strains show no differences when challenged with hydrogen peroxide and peroxynitrite, or during macrophage infection (Parente et al. 2017). *Brucella melitensis* expresses a BCP-PrxQ required for bacterial virulence in the caprine host, which is probably related to its capacity to survive inside host macrophages (Zygmunt et al. 2006). The BCP-PrxQ from the phytopathogen *Candidatus Liberibacter asiaticus* is able to

repress the recognition of some pathogen-associated molecular patterns (PAMPs) in tobacco. This BCP-PrxQ can also protect bacteria from lipid peroxidation, preventing the formation of oxylipin (Jain et al. 2018), a class of oxygenated derivatives of polyunsaturated fatty acids that contribute to plant defense.

Tpx

Concerning the Tpx class, the *H. pylori* Δ tpx strain has a reduced capacity to colonize mouse stomach, revealing its importance during infection (Olczak et al. 2003). It is worth mentioning that isoforms from three different Prx classes are related to the virulence of *H. pylori*. Deletion of the *tpx* gene of *Enterococcus faecalis* affects survival during macrophage infection and dependence on virulence in a mouse peritonitis model (La Carbona et al. 2007). Mice infected with the Δ tpxD strain of *Streptococcus pneumoniae* showed significantly longer survival. However, this difference is not observed during blood infection, which is probably related to the high oxygen levels in the respiratory system (Hajaj et al. 2012). The inactivation of the *tpx* gene of *Salmonella enterica* makes bacteria more sensitive to hydrogen peroxide than the single mutants of the other Prx. During macrophage infection, the Δ tpx strain mutant was more sensitive, while no difference was detected in a mice infection model (Horst et al. 2010). *Mycobacterium tuberculosis* mutants lacking *tpx* cannot grow in organs of infected mice, fail to induce an inflammatory response, and show low resistance to macrophage infections. Additionally, Tpx is able to protect *M. tuberculosis* against the RNS generated by macrophages, as demonstrated by infecting iNOS knockout host cells (Hu and Coates 2009). This finding is important since *M. tuberculosis* AhpC-Prx1 isoforms are unable to exert virulence effects.

Prx5

Studies on the virulence of members from the Prx5 class are scarce. *Vibrio vulnificus* strain lacking the *prx5* isoform causes a delay in mice death by intragastric infection (Lim et al. 2014). Remarkably, a Prx5-Grx fusion of *N. meningitidis* has been related to virulence, as verified using an ex vivo human whole blood model of meningococcal bacteremia (Aljannat et al. 2020). As mentioned before, the bacterial Prx5-Grx enzymes have no counterparts in the host and may represent an important target for drugs. In fungi, an isoform from Prx5 subfamily has also been characterized as a virulence factor in *A. fumigatus* (Hillmann et al. 2016).

Prx6

Only one bacterial Prx6 has been related to virulence so far. LsfA from *P. aeruginosa* is able to specifically protect

bacteria against the oxidative burst promoted by the macrophages. The authors used an acute pneumonia model to infect mice with the mutant lacking the *lsfa* gene strain and observed an improved survival rate compared with those infected with wild-type strain (Kaihami et al. 2014).

Virulence studies were also performed on members of Prx6 subfamily in fungi. *Aspergillus fumigatus* presents three Prx6 enzymes. Experiments using the individual mutant strains for each isoform revealed that the absence of only one of them (Prx1) caused lower mortality rates compared with the wild-type. Additionally, the organs of animals infected with the $\Delta prx1$ strain have an elevated rate of pathogenic cell death and high levels of immunological defense cells (Rocha et al. 2018). For *Magnaporthe oryzae*, the lack of a Prx6 class enzyme impacts virulence in a rice seedling root model (Mir et al. 2015).

Although no obvious general rule emerges from these studies, several Prx from five subfamilies are involved in virulence of distinct pathogens. The obvious role of Prx in virulence is related to pathogen protection from the host's oxidative responses. However, the demonstration of the role of Prxs enzymes in virulence may be hampered by the fact that some pathogens have a large repertoire of antioxidant enzymes. On the other hand, Prx have additional activities such as phospholipase and chaperone that can also be involved in pathogen virulence. Therefore, two key points are probably relevant to understand the complex mechanisms underlying virulence: (i) investigating the cooperation among antioxidant enzymes to fight oxidative insults in different developmental stages and in different cell compartments and (ii) the role of each Prx activity during microbial infection and colonization.

Concluding remarks

The relevance of Prx in microorganism's response to oxidative stress has been demonstrated since their discovery in *S. typhimurium* and *E. coli* in the 1980's. Over the years, Prx have been biochemically and structurally characterized in diverse microorganisms, along with the identification of reducing and oxidizing substrates, and structural peculiarities. It has become clear that Prx enzymes display unique and key roles in the establishment and progression of infectious diseases. In fact, a number of Prx enzymes among bacteria, fungi, and protozoans present virulence properties, which may be related to any peroxiredoxin function (e.g., peroxidase, phospholipase, chaperone, or cell signaling to eukaryotes). Despite these enzymes being important for pathogen survival, few studies have applied knowledge on Prx to fight pathogenic microorganisms. An obstacle to applications like inhibitors or vaccines is that only a small number of molecules capable of efficiently and selectively inhibiting Prx enzymes is known. Overall, Prx should be regarded as an important alternative for

the development of new therapeutical approaches to overcome the globally relevant growing resistance of pathogenic microorganisms to antimicrobials.

Author contributions All authors wrote, revised, edited, and approved the final version of the paper.

Funding This work was supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). The authors M.A.O., C.A.T., L.E.S.N., R.L.A.S., A.L.P.O., V.I.M.C., and M.C.S. are members of the CEPID REDOXOMA from FAPESP (grant number 2013/07937-8). This work was also supported by grants from FAPESP to M.A.O. (2017/19942-7), C.A.T., A.L.P.O., R.L.A.S., V.I.M.C., M.C.S., and C.A.B. are recipients of fellowships from FAPESP (16/15849-0; 19/04054-4; 20/00845-4; 20/02868-1; 17/06263-4; and 11/13500-6, respectively).

Declarations

Ethics approval and consent to participate This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

References

- Ahn J, Jang KK, Jo I, Nurhasni H, Lim JG, Yoo J-W, Choi SH, Ha N-C, Ahn J, Jang KK, Jo I, Nurhasni H, Lim JG, Yoo J-W, Choi SH, Ha N-C (2018) Crystal structure of peroxiredoxin 3 from and its implications for scavenging peroxides and nitric oxide. *IUCr* 5(1):82–92
- Akerman SE, Muller S (2005) Peroxiredoxin-linked detoxification of hydroperoxides in *Toxoplasma gondii*. *J Biol Chem* 280(1):564–570. <https://doi.org/10.1074/jbc.M406367200>
- Aljannat MAK, Oldfield NJ, Albasri HM, Dorrington LKG, Ohri RL, Wooldridge KG, Turner DPJ (2020) The moonlighting peroxiredoxin-glutaredoxin in *Neisseria meningitidis* binds plasminogen via a C-terminal lysine residue and contributes to survival in a whole blood model. *Microb Pathog* 139:103890. <https://doi.org/10.1016/j.micpath.2019.103890>
- Andrade HM, Murta SM, Chapeaurouge A, Perales J, Nirde P, Romanha AJ (2008) Proteomic analysis of *Trypanosoma cruzi* resistance to Benznidazole. *J Proteome Res* 7(6):2357–2367. <https://doi.org/10.1021/pr700659m>
- Angelucci F, Miele AE, Ardini M, Boumis G, Saccoccia F, Bellelli A (2016) Typical 2-Cys peroxiredoxins in human parasites: several physiological roles for a potential chemotherapy target. *Mol Biochem Parasitol* 206(1–2):2–12. <https://doi.org/10.1016/j.molbiopara.2016.03.005>
- Anschau V, Ferrer-Sueta G, Aleixo-Silva RL, Bannitz-Fernandes R, Tairum CA, Tonoli CCC, Murakami MT, de Oliveira MA, Netto LES (2020) Reduction of sulfenic acids by ascorbate in proteins, connecting thiol-dependent to alternative redox pathways. *Free Radic Biol Med* 156:207–216. <https://doi.org/10.1016/j.freeradbiomed.2020.06.015>
- Baek WK, Lee HS, Oh MH, Koh MJ, Kim KS, Choi SH (2009) Identification of the *Vibrio vulnificus* ahpCl gene and its influence on survival under oxidative stress and virulence. *J Microbiol* 47(5): 624–632. <https://doi.org/10.1007/s12275-009-0130-x>

- Baker LM, Poole LB (2003) Catalytic mechanism of thiol peroxidase from *Escherichia coli*. Sulfenic acid formation and overoxidation of essential CYS61. *J Biol Chem* 278(11):9203–9211. <https://doi.org/10.1074/jbc.M209888200>
- Baker LM, Raudonikienė A, Hoffman PS, Poole LB (2001) Essential thioredoxin-dependent peroxiredoxin system from *Helicobacter pylori*: genetic and kinetic characterization. *J Bacteriol* 183(6):1961–1973. <https://doi.org/10.1128/JB.183.6.1961-1973.2001>
- Bannitz-Fernandes R, Aleixo-Silva R, Silva JP, Dodia C, Vazquez-Medina JP, Tao JQ, Fisher A, Netto L (2019) Non-mammalian Prdx6 enzymes (proteins with 1-Cys Prdx mechanism) display PLA(2) activity similar to the human orthologue. *Antioxidants* (Basel) 8(3). <https://doi.org/10.3390/antiox8030052>
- Bottcher T, Sieber SA (2010) Showdomycin as a versatile chemical tool for the detection of pathogenesis-associated enzymes in bacteria. *J Am Chem Soc* 132(20):6964–6972. <https://doi.org/10.1021/ja909150y>
- Brenot A, King KY, Caparon MG (2005) The PerR regulon in peroxide resistance and virulence of *Streptococcus pyogenes*. *Mol Microbiol* 55(1):221–234. <https://doi.org/10.1111/j.1365-2958.2004.04370.x>
- Bryk R, Griffin P, Nathan C (2000) Peroxynitrite reductase activity of bacterial peroxiredoxins. *Nature* 407(6801):211–215
- Bryk R, Lima CD, Erdjument-Bromage H, Tempst P, Nathan C (2002) Metabolic enzymes of mycobacteria linked to antioxidant defense by a thioredoxin-like protein. *Science* 295(5557):1073–1077. <https://doi.org/10.1126/science.1067798>
- Budde H, Flohe L (2003) Enzymes of the thiol-dependent hydroperoxide metabolism in pathogens as potential drug targets. *Biofactors* 17(1–4):83–92. <https://doi.org/10.1002/biof.5520170109>
- Buranajitpakorn S, Piwkam A, Charoenlap N, Vattanaviboon P, Mongkolsuk S (2011) Genes for hydrogen peroxide detoxification and adaptation contribute to protection against heat shock in *Xanthomonas campestris* pv. *campestris*. *FEMS Microbiol Lett* 317(1):60–66. <https://doi.org/10.1111/j.1574-6968.2011.02211.x>
- Castro H, Teixeira F, Romao S, Santos M, Cruz T, Florido M, Appelberg R, Oliveira P, Ferreira-da-Silva F, Tomas AM (2011) Leishmania mitochondrial peroxiredoxin plays a crucial peroxidase-unrelated role during infection: insight into its novel chaperone activity. *PLoS Pathog* 7(10):e1002325. <https://doi.org/10.1371/journal.ppat.1002325>
- Castro H, Rocha MI, Silva R, Oliveira F, Gomes-Alves AG, Cruz T, Duarte M, Tomas AM (2020) Functional insight into the glycosomal peroxiredoxin of Leishmania. *Acta Trop* 201:105217. <https://doi.org/10.1016/j.actatropica.2019.105217>
- Cha MK, Kim HK, Kim IH (1995) Thioredoxin-linked “thiol peroxidase” from periplasmic space of *Escherichia coli*. *J Biol Chem* 270(48):28635–28641. <https://doi.org/10.1074/jbc.270.48.28635>
- Charoenlap N, Shen Z, McBee ME, Muthupalani S, Wogan GN, Fox JG, Schauer DB (2012) Alkyl hydroperoxide reductase is required for *Helicobacter cinaedi* intestinal colonization and survival under oxidative stress in BALB/c and BALB/c interleukin-10^{-/-} mice. *Infect Immun* 80(3):921–928. <https://doi.org/10.1128/IAI.05477-11>
- Chen H, Liu Y, Zhao C, Xiao D, Zhang J, Zhang F, Chen M, Wang H (2013) Comparative proteomics-based identification of genes associated with glycopeptide resistance in clinically derived heterogeneous vancomycin-intermediate *Staphylococcus aureus* strains. *PLoS One* 8(6):e66880. <https://doi.org/10.1371/journal.pone.0066880>
- Cho C, Lee GW, Hong SH, Kaur S, Jung KW, Jung JH, Lim S, Chung BY, Lee SS (2019) Novel functions of peroxiredoxin Q from *Deinococcus radiodurans* R1 as a peroxidase and a molecular chaperone. *FEBS Lett* 593(2):219–229. <https://doi.org/10.1002/1873-3468.13302>
- Choi W, Yoo YJ, Kim M, Shin D, Jeon HB, Choi W (2003) Identification of proteins highly expressed in the hyphae of *Candida albicans* by two-dimensional electrophoresis. *Yeast* 20(12):1053–1060. <https://doi.org/10.1002/yea.1022>
- Comtois SL, Gidley MD, Kelly DJ (2003) Role of the thioredoxin system and the thiol-peroxidases Tpx and Bcp in mediating resistance to oxidative and nitrosative stress in *Helicobacter pylori*. *Microbiology (Reading)* 149(Pt 1):121–129. <https://doi.org/10.1099/mic.0.25896-0>
- Condeles AL, Gomes F, de Oliveira MA, Soares Netto LE, Toledo Junior JC (2020) Thiol peroxidases as major regulators of intracellular levels of peroxynitrite in live *Saccharomyces cerevisiae* cells. *Antioxidants* (Basel) 9(5). <https://doi.org/10.3390/antiox9050434>
- Copley SD, Novak WR, Babbitt PC (2004) Divergence of function in the thioredoxin fold suprafamily: evidence for evolution of peroxiredoxins from a thioredoxin-like ancestor. *Biochemistry* 43(44):13981–13995. <https://doi.org/10.1021/bi048947r>
- Cosgrove K, Coutts G, Jonsson IM, Tarkowski A, Kokai-Kun JF, Mond JJ, Foster SJ (2007) Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) have compensatory roles in peroxide stress resistance and are required for survival, persistence, and nasal colonization in *Staphylococcus aureus*. *J Bacteriol* 189(3):1025–1035. <https://doi.org/10.1128/JB.01524-06>
- Das UN (2018) Arachidonic acid and other unsaturated fatty acids and some of their metabolites function as endogenous antimicrobial molecules: a review. *J Adv Res* 11:57–66. <https://doi.org/10.1016/j.jare.2018.01.001>
- Davis PH, Zhang X, Guo J, Townsend RR, Stanley SL Jr (2006) Comparative proteomic analysis of two *Entamoeba histolytica* strains with different virulence phenotypes identifies peroxiredoxin as an important component of amoebic virulence. *Mol Microbiol* 61(6):1523–1532. <https://doi.org/10.1111/j.1365-2958.2006.05344.x>
- de Godoy LM, Marchini FK, Pavoni DP, Rampazzo Rde C, Probst CM, Goldenberg S, Krieger MA (2012) Quantitative proteomics of *Trypanosoma cruzi* during metacyclogenesis. *Proteomics* 12(17):2694–2703. <https://doi.org/10.1002/pmic.201200078>
- Dons LE, Mosa A, Rottenberg ME, Rosenkrantz JT, Kristensson K, Olsen JE (2014) Role of the *Listeria monocytogenes* 2-Cys peroxiredoxin homologue in protection against oxidative and nitrosative stress and in virulence. *Pathog Dis* 70(1):70–74. <https://doi.org/10.1111/2049-632X.12081>
- Dwyer DJ, Belenky PA, Yang JH, MacDonald IC, Martell JD, Takahashi N, Chan CT, Lobritz MA, Braff D, Schwarz EG, Ye JD, Pati M, Vercurryse M, Ralifo PS, Allison KR, Khalil AS, Ting AY, Walker GC, Collins JJ (2014) Antibiotics induce redox-related physiological alterations as part of their lethality. *Proc Natl Acad Sci U S A* 111(20):E2100–E2109. <https://doi.org/10.1073/pnas.1401876111>
- Enany S, Yoshida Y, Yamamoto T (2014) Exploring extra-cellular proteins in methicillin susceptible and methicillin resistant *Staphylococcus aureus* by liquid chromatography-tandem mass spectrometry. *World J Microbiol Biotechnol* 30(4):1269–1283. <https://doi.org/10.1007/s11274-013-1550-7>
- Estelle D, Laurence L, Marc O, Caroline C, Magali D, Marie-Laure F (2020) Linolenic fatty acid hydroperoxide acts as biocide on plant pathogenic bacteria: biophysical investigation of the mode of action. *Bioorg Chem* 100:103877. <https://doi.org/10.1016/j.bioorg.2020.103877>
- Fereig RM, Kuroda Y, Terkawi MA, Mahmoud ME, Nishikawa Y (2017) Immunization with *Toxoplasma gondii* peroxiredoxin 1 induces protective immunity against toxoplasmosis in mice. *PLoS One* 12(4):e0176324. <https://doi.org/10.1371/journal.pone.0176324>
- Fisher RA, Gollan B, Helaine S (2017) Persistent bacterial infections and persister cells. *Nat Rev Microbiol* 15(8):453–464. <https://doi.org/10.1038/nrmicro.2017.42>
- Flohé L, Budde H, Bruns K, Castro H, Clos J, Hofmann B, Kansal-Kalavar S, Krumme D, Menge U, Plank-Schumacher K, Sztajer H, Wissing J, Wylegalla C, Hecht H-J (2002) Tryparedoxin

- peroxidase of *Leishmania donovani*: molecular cloning, heterologous expression, specificity, and catalytic mechanism. *Arch Biochem Biophys* 397(2):324–335
- Francis KP, Taylor PD, Inchley CJ, Gallagher MP (1997) Identification of the *ahp* operon of *Salmonella typhimurium* as a macrophage-induced locus. *J Bacteriol* 179(12):4046–4048. <https://doi.org/10.1128/jb.179.12.4046-4048.1997>
- Gautam P, Shankar J, Madan T, Sirdeshmukh R, Sundaram CS, Gade WN, Basir SF, Sarma PU (2008) Proteomic and transcriptomic analysis of *Aspergillus fumigatus* on exposure to amphotericin B. *Antimicrob Agents Chemother* 52(12):4220–4227. <https://doi.org/10.1128/AAC.01431-07>
- Gautam P, Mushahary D, Hassan W, Upadhyay SK, Madan T, Sirdeshmukh R, Sundaram CS, Sarma PU (2016) In-depth 2-DE reference map of *Aspergillus fumigatus* and its proteomic profiling on exposure to itraconazole. *Med Mycol* 54(5):524–536. <https://doi.org/10.1093/mmy/myv122>
- Ghaemmaghami S, Huh WK, Bower K, Howson RW, Belle A, Dephoure N, O'Shea EK, Weissman JS (2003) Global analysis of protein expression in yeast. *Nature* 425(6959):737–741. <https://doi.org/10.1038/nature02046>
- Gu S, Chen J, Dobos KM, Bradbury EM, Belisle JT, Chen X (2003) Comprehensive proteomic profiling of the membrane constituents of a *Mycobacterium tuberculosis* strain. *Mol Cell Proteomics* 2(12):1284–1296. <https://doi.org/10.1074/mcp.M300060-MCP200>
- Gutierrez-Escobedo G, Hernandez-Carreón O, Morales-Rojano B, Revuelta-Rodríguez B, Vazquez-Franco N, Castano I, De Las PA (2020) *Candida glabrata* peroxiredoxins, Tsa1 and Tsa2, and sulfiredoxin, Srx1, protect against oxidative damage and are necessary for virulence. *Fungal Genet Biol* 135:103287. <https://doi.org/10.1016/j.fgb.2019.103287>
- Hajaj B, Yesilkaya H, Benisty R, David M, Andrew PW, Porat N (2012) Thiol peroxidase is an important component of *Streptococcus pneumoniae* in oxygenated environments. *Infect Immun* 80(12):4333–4343. <https://doi.org/10.1128/IAI.00126-12>
- Hall A, Karplus PA, Poole LB (2009) Typical 2-Cys peroxiredoxins—structures, mechanisms and functions. *FEBS J* 276(9):2469–2477. <https://doi.org/10.1111/j.1742-4658.2009.06985.x>
- Hall A, Parsonage D, Poole LB, Karplus PA (2010) Structural evidence that peroxiredoxin catalytic power is based on transition-state stabilization. *J Mol Biol* 402(1):194–209. <https://doi.org/10.1016/j.jmb.2010.07.022>
- Hall A, Nelson K, Poole LB, Karplus PA (2011) Structure-based insights into the catalytic power and conformational dexterity of peroxiredoxins. *Antioxid Redox Signal* 15(3):795–815. <https://doi.org/10.1089/ars.2010.3624>
- Halliwell B, Gutteridge JMC (2015) Free radicals in biology and medicine, 5th edn. Oxford University Press, Oxford
- Haraldsen JD, Liu G, Botting CH, Walton JG, Storm J, Phalen TJ, Kwok LY, Soldati-Favre D, Heintz NH, Muller S, Westwood NJ, Ward GE (2009) Identification of conoidin A as a covalent inhibitor of peroxiredoxin II. *Org Biomol Chem* 7:3040–3048. <https://doi.org/10.1039/B901735F>
- Heym B, Stavropoulos E, Honoré N, Domenech P, Saint-Joanis B, Wilson TM, Collins DM, Colston MJ, Cole ST (1997) Effects of overexpression of the alkyl hydroperoxide reductase AhpC on the virulence and isoniazid resistance of *Mycobacterium tuberculosis*. *Infect Immun* 65(4):1395–1401
- Hicks LD, Raghavan R, Battisti JM, Minnick MF (2010) A DNA-binding peroxiredoxin of *Coxiella burnetii* is involved in countering oxidative stress during exponential-phase growth. *J Bacteriol* 192(8):2077–2084. <https://doi.org/10.1128/JB.01324-09>
- Hillas PJ, del Alba FS, Oyarzabal J, Wilks A, Ortiz de Montellano PR (2000) The AhpC and AhpD antioxidant defense system of *Mycobacterium tuberculosis*. *J Biol Chem* 275(25):18801–18809
- Hillmann F, Bagramyan K, Strassburger M, Heinekamp T, Hong TB, Bzymek KP, Williams JC, Brakhage AA, Kalkum M (2016) The crystal structure of peroxiredoxin Asp f3 provides mechanistic insight into oxidative stress resistance and virulence of *Aspergillus fumigatus*. *Sci Rep* 6:33396. <https://doi.org/10.1038/srep33396>
- Horst SA, Jaeger T, Denkel LA, Rouf SF, Rhen M, Bange FC (2010) Thiol peroxidase protects *Salmonella enterica* from hydrogen peroxide stress *in vitro* and facilitates intracellular growth. *J Bacteriol* 192(11):2929–2932. <https://doi.org/10.1128/JB.01652-09>
- Horta BB, de Oliveira MA, Discola KF, Cussiol JRR, Netto LES (2010) Structural and biochemical characterization of peroxiredoxin Qβ from *Xylella fastidiosa*. *J Biol Chem* 285(21):16051–16065
- Hu Y, Coates AR (2009) Acute and persistent *Mycobacterium tuberculosis* infections depend on the thiol peroxidase TrpX. *PLoS One* 4(4):e5150. <https://doi.org/10.1371/journal.pone.0005150>
- Hu H, Tian M, Li P, Bao Y, Guan X, Lian Z, Yin Y, Ding C, Yu S (2019) Brucella infection regulates peroxiredoxin-5 protein expression to facilitate intracellular survival by reducing the production of nitric oxide and reactive oxygen species. *Biochem Biophys Res Commun* 516(1):82–88
- Hugo M, Turell L, Manta B, Botti H, Monteiro G, Netto LES, Alvarez B, Radi R, Trujillo M (2009) Thiol and Sulfenic acid oxidation of AhpE, the one-cysteine Peroxiredoxin from : kinetics, acidity constants, and conformational dynamics. *Biochemistry* 48(40):9416–9426
- Hugo M, Van Laer K, Reyes AM, Vertommen D, Messens J, Radi R, Trujillo M (2014) Mycothiol/mycoredoxin 1-dependent reduction of the peroxiredoxin AhpE from *Mycobacterium tuberculosis*. *J Biol Chem* 289(8):5228–5239. <https://doi.org/10.1074/jbc.M113.510248>
- Jacobson FS, Morgan RW, Christman MF, Ames BN (1989) An alkyl hydroperoxide reductase from *Salmonella typhimurium* involved in the defense of DNA against oxidative damage. Purification and properties. *J Biol Chem* 264(3):1488–1496
- Jaeger T, Flohe L (2006) The thiol-based redox networks of pathogens: unexploited targets in the search for new drugs. *Biofactors* 27(1–4):109–120. <https://doi.org/10.1002/biof.5520270110>
- Jaeger T, Budde H, Flohé L, Menge U, Singh M, Trujillo M, Radi R (2004) Multiple thioredoxin-mediated routes to detoxify hydroperoxides in *Mycobacterium tuberculosis*. *Arch Biochem Biophys* 423(1):182–191
- Jain M, Munoz-Bodnar A, Zhang S, Gabriel DW (2018) A secreted ‘*Candidatus Liberibacter asiaticus*’ peroxiredoxin simultaneously suppresses both localized and systemic innate immune responses in planta. *Mol Plant-Microbe Interact* 31(12):1312–1322. <https://doi.org/10.1094/MPMI-03-18-0068-R>
- Jang HH, Lee KO, Chi YH, Jung BG, Park SK, Park JH, Lee JR, Lee SS, Moon JC, Yun JW, Choi YO, Kim WY, Kang JS, Cheong GW, Yun DJ, Rhee SG, Cho MJ, Lee SY (2004) Two enzymes in one; two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function. *Cell* 117(5):625–635. <https://doi.org/10.1016/j.cell.2004.05.002>
- Jeong W, Cha M-K, Kim I-H (2000) Thioredoxin-dependent Hydroperoxide peroxidase activity of Bacterioferritin Comigratory protein (BCP) as a new member of the Thiol-specific antioxidant protein (TSA)/alkyl Hydroperoxide peroxidase C (AhpC) family. *J Biol Chem* 275(4):2924–2930
- Johnson NA, Liu Y, Fletcher HM (2004) Alkyl hydroperoxide peroxidase subunit C (ahpC) protects against organic peroxides but does not affect the virulence of *Porphyromonas gingivalis* W83. *Oral Microbiol Immunol* 19(4):233–239. <https://doi.org/10.1111/j.1399-302X.2004.00145.x>
- Kaihami GH, Almeida JR, Santos SS, Netto LE, Almeida SR, Baldini RL (2014) Involvement of a 1-Cys peroxiredoxin in bacterial virulence. *PLoS Pathog* 10(10):e1004442. <https://doi.org/10.1371/journal.ppat.1004442>

- Kamariah N, Eisenhaber B, Eisenhaber F, Gruber G (2018) Molecular mechanism of the *Escherichia coli* AhpC in the function of a chaperone under heat-shock conditions. *Sci Rep* 8(1):14151. <https://doi.org/10.1038/s41598-018-32527-7>
- Kaufmann SHE, Springer B, Master S, Sander P, Zahrt T, McFalone M, Song J, Papavinasundaram KG, Colston MJ, Boettger E, Deretic V (2001) Silencing of oxidative stress response in : expression patterns of in virulent and avirulent strains and effect of inactivation. *Infect Immun* 69(10):5967–5973
- Kaneva IN, Longworth J, Sudbery PE, Dickman MJ (2018) Quantitative proteomic analysis in *Candida albicans* using SILAC-based mass spectrometry. *Proteomics* 18(5–6):e1700278. <https://doi.org/10.1002/pmic.201700278>
- Kim SJ, Woo JR, Hwang YS, Jeong DG, Shin DH, Kim K, Ryu SE (2003) The tetrameric structure of *Haemophilus influenzae* hybrid Prx5 reveals interactions between electron donor and acceptor proteins. *J Biol Chem* 278(12):10790–10798. <https://doi.org/10.1074/jbc.M209553200>
- Koch O, Jäger T, Heller K, Khandavalli PC, Pretzel J, Becker K, Flohé L, Selzer PM (2013) Identification of *M. tuberculosis* thioredoxin reductase inhibitors based on high-throughput docking using constraints. *J Med Chem* 56(12):4849–4859. <https://doi.org/10.1021/jm3015734>
- König J, Fairlamb AH (2007) A comparative study of type I and type II trypanothione peroxidases. *FEBS J* 274(21):5643–5658
- Koshkin A, Zhou XT, Kraus CN, Brenner JM, Bandyopadhyay P, Kuntz ID, Barry CE 3rd, Ortiz de Montellano PR (2004) Inhibition of *Mycobacterium tuberculosis* AhpD, an element of the peroxiredoxin defense against oxidative stress. *Antimicrob Agents Chemother* 48(7):2424–2430. <https://doi.org/10.1128/AAC.48.7.2424-2430.2004>
- Kumar B, Sharma D, Sharma P, Katoch VM, Venkatesan K, Bisht D (2013) Proteomic analysis of *Mycobacterium tuberculosis* isolates resistant to kanamycin and amikacin. *J Proteome* 94:68–77. <https://doi.org/10.1016/j.jprot.2013.08.025>
- La Carbona S, Sauvageot N, Giard JC, Benachour A, Posteraro B, Auffray Y, Sanguinetti M, Hartke A (2007) Comparative study of the physiological roles of three peroxidases (NADH peroxidase, Alkyl hydroperoxide reductase and Thiol peroxidase) in oxidative stress response, survival inside macrophages and virulence of *Enterococcus faecalis*. *Mol Microbiol* 66(5):1148–1163. <https://doi.org/10.1111/j.1365-2958.2007.05987.x>
- Lam PL, Wong RS, Lam KH, Hung LK, Wong MM, Yung LH, Ho YW, Wong WY, Hau DK, Gambari R, Chui CH (2020) The role of reactive oxygen species in the biological activity of antimicrobial agents: an updated mini review. *Chem Biol Interact* 320:109023. <https://doi.org/10.1016/j.cbi.2020.109023>
- Leitsch D, Williams CF, Hrdy I (2018) Redox pathways as drug targets in microaerophilic parasites. *Trends Parasitol* 34(7):576–589. <https://doi.org/10.1016/j.pt.2018.04.007>
- Li W, Zhang S, Wang X, Yu J, Li Z, Lin W, Lin X (2018) Systematically integrated metabolomic-proteomic studies of *Escherichia coli* under ciprofloxacin stress. *J Proteome* 179:61–70. <https://doi.org/10.1016/j.jprot.2018.03.002>
- Lim JG, Bang YJ, Choi SH (2014) Characterization of the *Vibrio vulnificus* 1-Cys peroxiredoxin Prx3 and regulation of its expression by the Fe-S cluster regulator IscR in response to oxidative stress and iron starvation. *J Biol Chem* 289(52):36263–36274. <https://doi.org/10.1074/jbc.M114.611020>
- Longo LVG, Breyer CA, Novaes GM, Gegembauer G, Leitao NP Jr, Octaviano CE, Toyama MH, de Oliveira MA, Puccia R (2020) The human pathogen *Paracoccidioides brasiliensis* has a unique 1-Cys peroxiredoxin that localizes both intracellularly and at the cell surface. *Front Cell Infect Microbiol* 10:394. <https://doi.org/10.3389/fcimb.2020.00394>
- Lynch AS, Robertson GT (2008) Bacterial and fungal biofilm infections. *Annu Rev Med* 59:415–428. <https://doi.org/10.1146/annurev.med.59.110106.132000>
- Marshall AC, Kidd SE, Lamont-Friedrich SJ, Arentz G, Hoffmann P, Coad BR, Bruning JB (2019) Structure, mechanism, and inhibition of Thioredoxin Reductase. *Antimicrob Agents Chemother* 63(3)
- Mastronicola D, Falabella M, Testa F, Pucillo LP, Teixeira M, Sarti P, Saraiva LM, Giuffrè A (2014) Functional characterization of peroxiredoxins from the human protozoan parasite *Giardia intestinalis*. *PLoS Negl Trop Dis* 8(1):e2631. <https://doi.org/10.1371/journal.pntd.0002631>
- Medzhitov R (2007) Recognition of microorganisms and activation of the immune response. *Nature* 449(7164):819–826. <https://doi.org/10.1038/nature06246>
- Mir AA, Park SY, Abu Sadat M, Kim S, Choi J, Jeon J, Lee YH (2015) Systematic characterization of the peroxidase gene family provides new insights into fungal pathogenicity in *Magnaporthe oryzae*. *Sci Rep* 5:11831. <https://doi.org/10.1038/srep11831>
- Mishra Y, Hall M, Locmelis R, Nam K, Soderberg CAG, Storm P, Chaurasia N, Rai LC, Jansson S, Schroder WP, Sauer UH (2017) Active-site plasticity revealed in the asymmetric dimer of AnPrx6 the 1-Cys peroxiredoxin and molecular chaperone from *Anabaena* sp. PCC 7210. *Sci Rep* 7(1):17151. <https://doi.org/10.1038/s41598-017-17044-3>
- Missall TA, Pusateri ME, Lodge JK (2004) Thiol peroxidase is critical for virulence and resistance to nitric oxide and peroxide in the fungal pathogen, *Cryptococcus neoformans*. *Mol Microbiol* 51(5):1447–1458. <https://doi.org/10.1111/j.1365-2958.2004.03921.x>
- Monteiro G, Horta BB, Pimenta DC, Augusto O, Netto LE (2007) Reduction of 1-Cys peroxiredoxins by ascorbate changes the thiol-specific antioxidant paradigm, revealing another function of vitamin C. *Proc Natl Acad Sci U S A* 104(12):4886–4891. <https://doi.org/10.1073/pnas.0700481104>
- Morais MAB, Giuseppe PO, Souza T, Castro H, Honorato RV, Oliveira PSL, Netto LES, Tomas AM, Murakami MT (2017) Calcium and magnesium ions modulate the oligomeric state and function of mitochondrial 2-Cys peroxiredoxins in *Leishmania* parasites. *J Biol Chem* 292(17):7023–7039. <https://doi.org/10.1074/jbc.M116.762039>
- Munhoz DC, Netto LES (2004) Cytosolic Thioredoxin peroxidase I and II are important defenses of yeast against organic Hydroperoxide insult. *J Biol Chem* 279(34):35219–35227
- Murphy TF, Kirkham C, Sethi S, Lesse AJ (2005) Expression of a peroxiredoxin-glutaredoxin by *Haemophilus influenzae* in biofilms and during human respiratory tract infection. *FEMS Immunol Med Microbiol* 44(1):81–89. <https://doi.org/10.1016/j.femsim.2004.12.008>
- Nelson KJ, Knutson ST, Soito L, Klomsiri C, Poole LB, Fetrow JS (2011) Analysis of the peroxiredoxin family: using active-site structure and sequence information for global classification and residue analysis. *Proteins* 79(3):947–964. <https://doi.org/10.1002/prot.22936>
- Netto LE, de Oliveira MA, Tairum CA, da Silva Neto JF (2016) Conferring specificity in redox pathways by enzymatic thiol/disulfide exchange reactions. *Free Radic Res* 50(2):206–245. <https://doi.org/10.3109/10715762.2015.1120864>
- Nogoceke E, Gommel DU, Kiess M, Kalisz HM, Flohe L (1997) A unique cascade of oxidoreductases catalyses trypanothione-mediated peroxide metabolism in *Crithidia fasciculata*. *Biol Chem* 378(8):827–836. <https://doi.org/10.1515/bchm.1997.378.8.827>
- Numberger T, Brunner F, Kemmerling B, Piater L (2004) Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol Rev* 198:249–266. <https://doi.org/10.1111/j.0105-2896.2004.0119.x>
- Ogusucu R, Rettori D, Munhoz DC, Netto LES, Augusto O (2007) Reactions of yeast thioredoxin peroxidases I and II with hydrogen

- peroxide and peroxynitrite: rate constants by competitive kinetics. *Free Radic Biol Med* 42(3):326–334
- Ogusucu R, Rettori D, Netto LE, Augusto O (2009) Superoxide dismutase 1-mediated production of ethanol- and DNA-derived radicals in yeasts challenged with hydrogen peroxide: molecular insights into the genome instability of peroxiredoxin-null strains. *J Biol Chem* 284(9):5546–5556. <https://doi.org/10.1074/jbc.M805526200>
- Olczak AA, Seyler RW Jr, Olson JW, Maier RJ (2003) Association of *Helicobacter pylori* antioxidant activities with host colonization proficiency. *Infect Immun* 71(1):580–583. <https://doi.org/10.1128/iai.71.1.580-583.2003>
- Oliveira MA, Discola KF, Alves SV, Medrano FJ, Guimaraes BG, Netto LE (2010) Insights into the specificity of thioredoxin reductase-thioredoxin interactions. A structural and functional investigation of the yeast thioredoxin system. *Biochemistry* 49(15):3317–3326. <https://doi.org/10.1021/bi901962p>
- O'Riordan AA, Morales VA, Mulligan L, Faheem N, Windle HJ, Kelleher DP (2012) Alkyl hydroperoxide reductase: a candidate *Helicobacter pylori* vaccine. *Vaccine* 30(26):3876–3884. <https://doi.org/10.1016/j.vaccine.2012.04.002>
- Parente MR, Forte E, Falabella M, Boneca IG, Teixeira M, Giuffrè A, Saraiva LM (2017) Nitrosative stress defences of the enterohepatic pathogenic bacterium *Helicobacter pullorum*. *Sci Rep* 7(1):9909. <https://doi.org/10.1038/s41598-017-10375-1>
- Park SG, Cha MK, Jeong W, Kim IH (2000) Distinct physiological functions of thiol peroxidase isoenzymes in *Saccharomyces cerevisiae*. *J Biol Chem* 275(8):5723–5732. <https://doi.org/10.1074/jbc.275.8.5723>
- Parsonage D, Youngblood DS, Sarma GN, Wood ZA, Andrew Karplus P, Poole LB (2005) Analysis of the link between enzymatic activity and oligomeric state in AhpC, a bacterial Peroxiredoxin. *Biochemistry* 44(31):10583–10592
- Parsonage D, Karplus PA, Poole LB (2008) Substrate specificity and redox potential of AhpC, a bacterial peroxiredoxin. *Proc Natl Acad Sci U S A* 105(24):8209–8214. <https://doi.org/10.1073/pnas.0708308105>
- Pedrajas JR, Miranda-Vizuete A, Javanmardy N, Gustafsson JA, Spyrou G (2000) Mitochondria of *Saccharomyces cerevisiae* contain one-conserved cysteine type peroxiredoxin with thioredoxin peroxidase activity. *J Biol Chem* 275(21):16296–16301. <https://doi.org/10.1074/jbc.275.21.16296>
- Pedrajas JR, McDonagh B, Hernandez-Torres F, Miranda-Vizuete A, Gonzalez-Ojeda R, Martinez-Galisteo E, Padilla CA, Barcena JA (2016) Glutathione is the resolving thiol for thioredoxin peroxidase activity of 1-Cys peroxiredoxin without being consumed during the catalytic cycle. *Antioxid Redox Signal* 24(3):115–128. <https://doi.org/10.1089/ars.2015.6366>
- Perkins A, Nelson KJ, Parsonage D, Poole LB, Karplus PA (2015) Peroxiredoxins: guardians against oxidative stress and modulators of peroxide signaling. *Trends Biochem Sci* 40(8):435–445. <https://doi.org/10.1016/j.tibs.2015.05.001>
- Piacenza L, Peluffo G, Alvarez MN, Kelly JM, Wilkinson SR, Radi R (2008) Peroxiredoxins play a major role in protecting *Trypanosoma cruzi* against macrophage- and endogenously-derived peroxynitrite. *Biochem J* 410(2):359–368. <https://doi.org/10.1042/BJ20071138>
- Piñeyro MD, Parodi-Talice A, Arcari T, Robello C (2008) Peroxiredoxins from *Trypanosoma cruzi*: virulence factors and drug targets for treatment of Chagas disease? *Gene* 408(1–2):45–50
- Pineyro MD, Arcari T, Robello C, Radi R, Trujillo M (2011) Tryparedoxin peroxidases from *Trypanosoma cruzi*: high efficiency in the catalytic elimination of hydrogen peroxide and peroxynitrite. *Arch Biochem Biophys* 507(2):287–295. <https://doi.org/10.1016/j.abb.2010.12.014>
- Poole LB, Ellis HR (1996) Flavin-dependent alkyl hydroperoxide reductase from *Salmonella typhimurium*. 1. Purification and enzymatic activities of overexpressed AhpF and AhpC proteins. *Biochemistry* 35(1):56–64. <https://doi.org/10.1021/bi951887s>
- Poole LB, Nelson KJ (2016) Distribution and features of the six classes of peroxiredoxins. *Mol Cell* 39(1):53–59. <https://doi.org/10.14348/molcells.2016.2330>
- Poole LB, Hall A, Nelson KJ (2011) Overview of peroxiredoxins in oxidant defense and redox regulation. *Curr Protoc Toxicol* Chapter 7:Unit7 9. <https://doi.org/10.1002/0471140856.tx0709s49>
- Prolo C, Alvarez MN, Radi R (2014) Peroxynitrite, a potent macrophage-derived oxidizing cytotoxin to combat invading pathogens. *Biofactors* 40(2):215–225. <https://doi.org/10.1002/biof.1150>
- Radi R (2018) Oxygen radicals, nitric oxide, and peroxynitrite: redox pathways in molecular medicine. *Proc Natl Acad Sci U S A* 115(23):5839–5848. <https://doi.org/10.1073/pnas.1804932115>
- Repolés BM, Machado CR, Florentino PT (2020) DNA lesions and repair in trypanosomatids infection. *Genet Mol Biol* 43(1):e20190163
- Reyes AM, Hugo M, Trostchansky A, Capece L, Radi R, Trujillo M (2011) Oxidizing substrate specificity of *Mycobacterium tuberculosis* alkyl hydroperoxide reductase E: kinetics and mechanisms of oxidation and overoxidation. *Free Radic Biol Med* 51(2):464–473. <https://doi.org/10.1016/j.freeradbiomed.2011.04.023>
- Reyes AM, Vazquez DS, Zeida A, Hugo M, Pineyro MD, De Armas MI, Estrin D, Radi R, Santos J, Trujillo M (2016) PrxQ B from *Mycobacterium tuberculosis* is a monomeric, thioredoxin-dependent and highly efficient fatty acid hydroperoxide reductase. *Free Radic Biol Med* 101:249–260. <https://doi.org/10.1016/j.freeradbiomed.2016.10.005>
- Rhee SG, Kil IS (2017) Multiple functions and regulation of mammalian peroxiredoxins. *Annu Rev Biochem* 86:749–775. <https://doi.org/10.1146/annurev-biochem-060815-014431>
- Richard D, Bartfai R, Volz J, Ralph SA, Muller S, Stunnenberg HG, Cowman AF (2011) A genome-wide chromatin-associated nuclear peroxiredoxin from the malaria parasite *Plasmodium falciparum*. *J Biol Chem* 286(13):11746–11755. <https://doi.org/10.1074/jbc.M110.198499>
- Rintiswati N, Wibawa T, Asmara W, Soebono H (2011) Effect of oxidative stress on AhpC activity and virulence in katG Ser315 Thr *Mycobacterium tuberculosis* mutant. *Indonesian J Biotechnol* 16(2):100–110
- Rocha ER, Smith CJ (1999) Role of the alkyl hydroperoxide reductase (ahpCF) gene in oxidative stress defense of the obligate Anaerobe *Bacteroides fragilis*. *J Bacteriol* 181(18):5701–5710. <https://doi.org/10.1128/JB.181.18.5701-5710.1999>
- Rocha MC, de Godoy KF, Bannitz-Fernandes R, Fabri J, Barbosa MMF, de Castro PA, Almeida F, Goldman GH, da Cunha AF, Netto LES, de Oliveira MA, Malavazi I (2018) Analyses of the three 1-Cys peroxiredoxins from *Aspergillus fumigatus* reveal that cytosolic Prx1 is central to H₂O₂ metabolism and virulence. *Sci Rep* 8(1):12314. <https://doi.org/10.1038/s41598-018-30108-2>
- Rodrigues AM, Kubitschek-Barreira PH, Pinheiro BG, Teixeira-Ferreira A, Hahn RC, de Camargo ZP (2020) Immunoproteomic analysis reveals novel candidate antigens for the diagnosis of paracoccidioidomycosis due to *Paracoccidioides lutzii*. *J Fungi* (Basel) 6(4). <https://doi.org/10.3390/jof6040357>
- Saccoccia F, Di Micco P, Boumis G, Brunori M, Kouris I, Miele AE, Morea V, Sriratanana P, Williams DL, Bellelli A, Angelucci F (2012) Moonlighting by different stressors: crystal structure of the chaperone species of a 2-Cys peroxiredoxin. *Structure* 20(3):429–439. <https://doi.org/10.1016/j.str.2012.01.004>
- Seaver LC, Imlay JA (2001) Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. *J Bacteriol* 183(24):7173–7181. <https://doi.org/10.1128/JB.183.24.7173-7181.2001>
- Sherman DR, Mdululi K, Hickey MJ, Arain TM, Morris SL, Barry CE 3rd, Stover CK (1996) Compensatory ahpC gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. *Science*

- 272(5268):1641–1643. <https://doi.org/10.1126/science.272.5268.1641>
- Shishodia SK, Tiwari S, Shankar J (2019) Resistance mechanism and proteins in *Aspergillus* species against antifungal agents. *Mycology* 10(3):151–165. <https://doi.org/10.1080/21501203.2019.1574927>
- Silva KSE, Neto BRS, Zambuzzi-Carvalho PF, de Oliveira CM, Pires LB, Kato L, Bailao AM, Parente-Rocha JA, Hernandez O, Ochoa JG, Soares MC, Pereira M (2018) Response of *Paracoccidioides lutzii* to the antifungal camphene thiosemicarbazide determined by proteomic analysis. *Future Microbiol* 13:1473–1496. <https://doi.org/10.2217/fmb-2018-0176>
- Skrzypek MS, Binkley J, Binkley G, Miyasato SR, Simison M, Sherlock G (2017) The Candida Genome Database (CGD): incorporation of Assembly 22, systematic identifiers and visualization of high throughput sequencing data. *Nucleic Acids Res* 45(D1):D592–D596. <https://doi.org/10.1093/nar/gkw924>
- Souza Goebel C, de Mattos Oliveira F, Severo LC (2013) *Saccharomyces cerevisiae* infections. *Rev Iberoam Micol* 30(3):205–208. <https://doi.org/10.1016/j.riam.2013.03.001>
- Staerck C, Gastebois A, Vandeputte P, Calenda A, Larcher G, Gillmann L, Papon N, Bouchara JP, Fleury MJJ (2017) Microbial antioxidant defense enzymes. *Microb Pathog* 110:56–65. <https://doi.org/10.1016/j.micpath.2017.06.015>
- Staudacher V, Djuika CF, Koduka J, Schlossarek S, Kopp J, Büchler M, Lanzer M, Deponte M (2015) Plasmodium falciparum antioxidant protein reveals a novel mechanism for balancing turnover and inactivation of peroxiredoxins. *Free Radic Biol Med* 85:228–236
- Storz G, Christman MF, Sies H, Ames BN (1987) Spontaneous mutagenesis and oxidative damage to DNA in *Salmonella typhimurium*. *Proc Natl Acad Sci U S A* 84(24):8917–8921. <https://doi.org/10.1073/pnas.84.24.8917>
- Tairum CA, Santos MC, Breyer CA, de Oliveira ALP, Cabrera VIM, Toledo-Silva G, Mori GM, Toyama MH, Netto LES, de Oliveira MA (2021) Effects of Serine or Threonine in the active site of typical 2-Cys Prx on hyperoxidation susceptibility and on chaperone activity. *Antioxidants (Basel)*. <https://doi.org/10.3390/antiox10071032>
- Tairum CA, Santos MC, Breyer CA, Geyer RR, Nieves CJ, Portillo-Ledesma S, Ferrer-Sueta G, Toledo JC Jr, Toyama MH, Augusto O, Netto LE, de Oliveira MA (2016) Catalytic Thr or Ser residue modulates structural switches in 2-Cys peroxiredoxin by distinct mechanisms. *Sci Rep* 6:33133. <https://doi.org/10.1038/srep33133>
- Taylor PD, Inchley CJ, Gallagher MP (1998) The *Salmonella typhimurium* AhpC polypeptide is not essential for virulence in BALB/c mice but is recognized as an antigen during infection. *Infect Immun* 66(7):3208–3217. <https://doi.org/10.1128/IAI.66.7.3208-3217.1998>
- Teixeira F, Castro H, Cruz T, Tse E, Koldewey P, Southworth DR, Tomas AM, Jakob U (2015) Mitochondrial peroxiredoxin functions as crucial chaperone reservoir in *Leishmania infantum*. *Proc Natl Acad Sci U S A* 112(7):E616–E624. <https://doi.org/10.1073/pnas.1419682112>
- Trujillo M, Budde H, Piñeyro MD, Stehr M, Robello C, Flohé L, Radi R (2004) Trypanosoma brucei and Trypanosoma cruzi Tryparedoxin peroxidases catalytically detoxify Peroxynitrite via oxidation of fast reacting Thiols. *J Biol Chem* 279(33):34175–34182
- Tunes LG, Morato RE, Garcia A, Schmitz V, Steindel M, Corrêa-Junior JD, Dos Santos HF, Frézar F, de Almeida MV, Silva H, Moretti NS, de Barros ALB, do Monte-Neto RL (2020) Preclinical gold complexes as oral drug candidates to treat leishmaniasis are potent trypanothione reductase inhibitors. *ACS Infect Dis* 6(5):1121–1139. <https://doi.org/10.1021/acinfed.9b00505>
- Urban C, Xiong X, Sohn K, Schroppel K, Brunner H, Rupp S (2005) The moonlighting protein Tsa1p is implicated in oxidative stress response and in cell wall biogenesis in *Candida albicans*. *Mol Microbiol* 57(5):1318–1341. <https://doi.org/10.1111/j.1365-2958.2005.04771.x>
- Usui M, Masuda-Suganuma H, Fukumoto S, Angeles JM, Hakimi H, Inoue N, Kawazu S (2015) Effect of thioredoxin peroxidase-1 gene disruption on the liver stages of the rodent malaria parasite *Plasmodium berghei*. *Parasitol Int* 64(3):290–294. <https://doi.org/10.1016/j.parint.2014.09.013>
- Vallejo MC, Nakayasu ES, Matsuo AL, Sobreira TJ, Longo LV, Ganiko L, Almeida IC, Puccia R (2012) Vesicle and vesicle-free extracellular proteome of *Paracoccidioides brasiliensis*: comparative analysis with other pathogenic fungi. *J Proteome Res* 11(3):1676–1685. <https://doi.org/10.1021/pr200872s>
- Vediyappan G, Rossignol T, d'Enfert C (2010) Interaction of *Candida albicans* biofilms with antifungals: transcriptional response and binding of antifungals to beta-glucans. *Antimicrob Agents Chemother* 54(5):2096–2111. <https://doi.org/10.1128/AAC.01638-09>
- Vranakis I, De Bock PJ, Papadioti A, Tselentis Y, Gevaert K, Tsiotis G, Psaroulaki A (2012) Quantitative proteome profiling of *C. burnetii* under tetracycline stress conditions. *PLoS One* 7(3):e33599. <https://doi.org/10.1371/journal.pone.0033599>
- Wang G, Olczak AA, Walton JP, Maier RJ (2005) Contribution of the *Helicobacter pylori* thiol peroxidase bacterioferritin comigratory protein to oxidative stress resistance and host colonization. *Infect Immun* 73(1):378–384. <https://doi.org/10.1128/IAI.73.1.378-384.2005>
- Wilson T, de Lisle GW, Marcinkeviciene JA, Blanchardand JS, Collins DM (1998) Antisense RNA to ahpC, an oxidative stress defence gene involved in isoniazid resistance, indicates that AhpC of *Mycobacterium bovis* has virulence properties. *Microbiology* 144(10):2687–2695
- Winterbourn CC, Hampton MB (2008) Thiol chemistry and specificity in redox signaling. *Free Radic Biol Med* 45(5):549–561. <https://doi.org/10.1016/j.freeradbiomed.2008.05.004>
- Winterbourn CC, Kettle AJ, Hampton MB (2016) Reactive oxygen species and neutrophil function. *Annu Rev Biochem* 85:765–792. <https://doi.org/10.1146/annurev-biochem-060815-014442>
- Wood ZA, Poole LB, Karplus PA (2003) Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* 300(5619):650–653. <https://doi.org/10.1126/science.1080405>
- Yague-Capilla M, Garcia-Caballero D, Aguilar-Pereyra F, Castillo-Acosta VM, Ruiz-Perez LM, Vidal AE, Gonzalez-Pacanowska D (2019) Base excision repair plays an important role in the protection against nitric oxide- and in vivo-induced DNA damage in *Trypanosoma brucei*. *Free Radic Biol Med* 131:59–71. <https://doi.org/10.1016/j.freeradbiomed.2018.11.025>
- Yu S, Girotto S, Lee C, Magliozzo RS (2003) Reduced affinity for isoniazid in the S315T mutant of *Mycobacterium tuberculosis* KatG is a key factor in antibiotic resistance. *J Biol Chem* 278(17):14769–14775. <https://doi.org/10.1074/jbc.M300326200>
- Zhu X, Tu ZJ, Coussens PM, Kapur V, Janagama H, Naser S, Sreevatsan S (2008) Transcriptional analysis of diverse strains *Mycobacterium avium* subspecies *paratuberculosis* in primary bovine monocyte derived macrophages. *Microbes Infect* 10(12-13):1274–1282. <https://doi.org/10.1016/j.micinf.2008.07.025>
- Zygmunt MS, Hagius SD, Walker JV, Elzer PH (2006) Identification of *Brucella melitensis* 16M genes required for bacterial survival in the caprine host. *Microbes Infect* 8(14-15):2849–2854. <https://doi.org/10.1016/j.micinf.2006.09.002>