FACULDADE DE MEDICINA DE BOTUCATU
DEPARTAMENTO DE PATOLOGIA

ANDRÉ LUIZ VENTURA SÁVIO

EFEITOS TOXICOGENÉTICOS E TOXICOGENÔMICOS DO ISOTIOCIANATO DE ALILA (ÓLEO DE MOSTARDA) EM LINHAGENS CELULARES DE CARCINOMA DE BEXIGA

BOTUCATU
2014

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ANDRÉ LUIZ VENTURA SÁVIO

Dissertação apresentada à Faculdade de Medicina de Botucatu - FMB/UNESP, para obtenção do título de Mestre, pelo curso de Pós-Graduação em Patologia.

Orientadora: Profa. Dra. Daisy Maria Fávero Salvadori
Co-orientadora: Profa. Dra. Glenda Nicioli da Silva

BOTUCATU
2014

"Não diga e nem faça nada antes de pensar. Seja amistoso sem ser vulgar. Prenda os amigos de verdade à sua alma com ganchos de aço, mas não faça calos na mão cumprimentando e abraçando qualquer um que apareça. Nunca entre em brigas, mas se entrar, faça o adversário ficar com medo de você. Escute todo mundo, mas fale com poucos. Leve sempre em consideração as críticas dos outros, mas não julgue ninguém. E acima de tudo, o seguinte, a saber: seja verdadeiro com você mesmo; a consequência disso (assim como a noite e o dia vêm um depois do outro) é que você não será falso com ninguém" Hamlet, William Shakespeare

DEDICATÓRIA

À Deus.

Aos meus pais ***Genivaldo Sávio*** *e* ***Vera Lúcia Ventura Sávio*** *pelo exemplo de honestidade, caráter, por todo amor, orações, incentivo e por acreditarem e me fazerem acreditar na minha capacidade, pela compreensão pelos momentos de ausência e por não medirem esforços para que esse sonho fosse concretizado. À minha noiva* ***Brenda Anfilo****, por todo companheirismo durante essa etapa de minha vida, por me fazer acreditar nos meus sonhos e me ajudar a superar todos os obstáculos que surgiram no caminho, pela motivação incondicional e também por, além de compreender, me auxiliar nos experimentos em finais de semanas e feriados. Sem vocês esta trajetória seria muito mais difícil, vocês são a base de tudo. Amo vocês.*

"Os sonhos não determinam o lugar em que você vai estar, mas produzem a força necessária para tirá-lo do lugar em que está"
Augusto Cury

AGRADECIMENTOS ESPECIAIS

*À **Dra. Daisy Maria Fávero Salvadori:** pela orientação, por acreditar neste trabalho, mesmo quando os resultados não surgiam, e me proporcionar às condições necessárias para desenvolver esta pesquisa. Obrigado pelos incentivos, conselhos, pela paciência, dedicação e principalmente pelos puxões de orelha, pois foram com eles que aprendi e busquei me tornar cada dia melhor.*

*À **Dra. Glenda Nicioli da Silva:** pela co-orientação, pelo incentivo incondicional durante todo o desenvolvimento deste trabalho, por me mostrar a "luz" quando eu já não enxergava as respostas para as minhas próprias perguntas, por não medir esforços para me ajudar mesmo estando ausente, além disso, muito obrigado por acreditar em minha capacidade mesmo quando fui alvo de críticas e me estimular a buscar novos desafios.*

Vocês são referências de competência, profissionalismo e dedicação. Vocês foram e são fundamentais para minha formação e amadurecimento profissional. Muito obrigado pela oportunidade de aprender e trabalhar com vocês.

"Se enxerguei mais longe, foi porque me apoiei em ombros de gigantes" Isaac Newton

AGRADECIMENTOS

Às minhas irmãs ***Mariele Ventura Sávio Cesarin, Aline Fernanda Ventura Sávio Leite*** *e aos meus cunhados "irmãos"* ***Julio Cesar Cesarin e Caio Fernando Gianini Leite****, obrigado pelas palavras de incentivo, por me fazerem sentir presente mesmo estando longe e pelas inúmeras formas (cada um do seu jeito) de me ajudaram durante esta fase, muito obrigado. Família é a base de tudo.*

Aos meus pequenos sobrinhos ***Gabriel Sávio Cesarin, Rafaela Sávio Cesarin e Elis Sávio Leite****, embora tão pequenos, por compreender a ausência do tio e pelo imenso amor e alegria que me proporcionam quando nos reencontramos.*

À minha sogra ***Maria Aparecida Prieto Anfilo****, ao meu sogro* ***Wilson Luiz Anfilo*** *e a* ***Helenice Anfilo****, não tenho palavras para agradecer a maneira com que vocês me acolheram, me apoiaram e me ajudaram, muito obrigado por tudo.*

Aos meus queridos tios ***José Ventura e Odete Ventura****, pelo carinho e incentivo.*

Aos amigos do ***Laboratório de Citopatologia do Departamento de Patologia****, por desde minha chegada a Botucatu me incentivarem incondicionalmente.*

Aos meus amigos ***Igor Minatel*** *e* ***Renata Vilasboas****, sem vocês este sonho talvez não tivesse se concretizado.*

Ao ***Samuel Thomaz da Silva****, pelo companheirismo e pelas palavras de incentivo e apoio.*

Aos colegas do ***Laboratório de Toxicogenômica e Nutrigenômica: Luciana Maria Feliciano, Maruhen Silveira, Vanessa Peressi, Renato Prato, Bruno Luperini, Babatound Romuald, Bruna Jerônimo, Helenice Marcello, Victória Fiere, Mario Botasso, Pablo Bertoni, Raphael Toledo,*** *obrigado por todo incentivo e apoio. Em especial a* ***João Paulo de Castro Marcondes*** *pelo auxílio nas análises estatísticas e orientações; à* ***Elaine Aparecida de Camargo,*** *pelo incentivo e orientação na padronização dos testes de cometa e micronúcleo.*

Aos amigos ***Carla Munari**, Juliana Lara Padovani** e Fábio Henrique Fernandes*****, *pela amizade, incentivo, momentos de descontração e companheirismo durante todo esse período e, principalmente, pela paciência no momento mais difícil na elaboração desta dissertação, muito obrigado. Vocês foram os resultados mais significativos dessa dissertação (**P<0.0001).*

Aos meus amigos ***Luiz Marcelo Sarti (Penteio), Danilo Correa (Vando), Matheus Camili (Chico Rey)****, com quem eu convivi desde minha chegada a botucatu, muito obrigado pelos momentos descontraídos e conselhos.*

Ao amigo ***Carlos Roberto Padovani*** *e aos* ***Mestres da Bola,*** *pelos clássicos aos sábados na arena* ***UNIFAC****, que serviram como uma válvula de escape em frente às dificuldades.*

À senhora ***Valquíria Montanha,*** *pelo auxílio na limpeza do laboratório e amizade.*

Ao Professor ***José Eduardo Corrente****, pela disponibilidade em realizar prontamente todos os testes estatísticos.*

À ***Vânia Soler****, da Seção de Pós-Graduação, por sempre estar disposta a ajudar.*

À ***Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP)*** *e ao* ***Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq)****, pela bolsa de estudo e apoio financeiro.*

"O êxito da vida não se mede pelo caminho que conquistou, mas sim pelas dificuldades que superou no percurso" Abraham Lincoln

Sumário

RESUMO

1 – RESUMO

Nos últimos anos, tem crescido o interesse pela identificação de compostos naturais com potencial medicinal. O isotiocianato de alila (AITC), encontrado em plantas da família *Cruciferae* e composto majoritário da semente de mostarda, vem sendo avaliado quanto a sua atividade antineoplásica em bexiga devido à sua alta biodisponibilidade na urina a após ingestão. Neste estudo foram investigadas as atividades toxicogenética e toxicogenômica do AITC em linhagens celulares de carcinoma de bexiga com diferentes *status* do gene *TP53* (RT4 – *TP53* selvagem / T24 – *TP53* mutado). As concentrações de AITC testadas nos ensaios de citotoxicidade, sobrevivência celular e clonogênica foram 0,005, 0,0625, 0,0725, 0,0825, 0,0925, 0,125 e 0,25 µM; nos testes do cometa, micronúcleo, ciclo celular, apoptose, morfologia e expressão gênica (*ANLN, BAX, BCL-2, CDK-1, SMAD4, S100P e TP53*) foram utilizadas as concentrações de 0,005, 0,0625, 0,0725, 0,0825 e 0,0925 µM. Os resultados mostraram aumento de danos primários no DNA (teste do cometa) para todas as concentrações testadas do AITC na linhagem T24 e para as concentrações mais altas (0,0725, 0,0825 e 0,0925 µM) na linhagem RT4; não foram observados aumentos nas frequências de células micronucleadas em ambas as linhagens. Nas análises de ciclo celular e apoptose foram observadas a parada do ciclo na fase G2/M para a linhagem T24 e altas taxas de apoptose precoce e necrose na linhagem RT4. Com relação à expressão gênica, houve aumento da expressão de *S100P* e *BAX* e diminuição da expressão de *BCL-2* na linhagem RT4 e aumento da expressão de *BCL-2, BAX* e *ANLN* e diminuição da expressão de *S100P* na T24. Nenhuma alteração foi identificada para os genes *SMAD4* e *CDK1* nas duas linhagens tratadas com o AITC. Concluindo, o AITC foi capaz de induzir lesões primárias no DNA de células de ambas as linhagens de carcinoma de bexiga, mas que não se refletiram em alterações cromossômicas detectadas pelo teste do micronúcleo. Por outro lado, os dados mostraram que o *status* do gene *TP53* interferiu nas respostas celulares ao AITC: enquanto os principais efeitos observados nas células *TP53* selvagens (RT4) estavam relacionados à morte celular por apoptose, com modulação da expressão de genes da via *BAX/BCL-2*, na linhagem *TP53* mutada (T24) estavam relacionados ao bloqueio do ciclo celular, com indução da expressão do gene *ANLN*, relacionado à citocinese.

ABSTRACT

2 – *ABSTRACT*

Compounds obtained from fruits, vegetables and essential oils have been widely used to treat many diseases. The allyl isothiocyanate (AITC), also known as mustard essential oil, is found in plants of the cruciferous family and is abundant in the mustard seeds. Due to its high bioavailability in urine, AITC has been considered as a promising antineoplastic agent against bladder cancer. Therefore, the aims of this study were to investigate the toxicogenetic and toxicogenomic effects of AITC in the wild-type (RT4) and in a mutant (T24) *TP53* bladder transitional carcinoma cell lines. AITC was tested at concentrations of 0.005, 0.0625, 0.0725, 0.0825, 0.0925, 0.125 and 0.25 µM in the cytotoxicity and cell and clonogenic survival assays; in the comet and micronucleus assays, flow cytometry, apoptosis and gene expression (*ANLN, BAX, BCL-2, CDK-1, SMAD4, S100P e TP53*) evaluations the concentrations of 0.005; 0.0625; 0.0725; 0.0825 e 0.0925 µM were used. The results showed increased primary DNA damage in T24 (0.005, 0.0625, 0.0725, 0.0825 and 0.0925 µM) and RT4 (0.0725, 0.0825 and 0.0925 µM) cells. However, no significant difference was detected in the frequency of micronucleated cells. A significant increase of cells at sub-G1 phase (0.0625, 0.0725 and 0.0825 µM) and significant decrease at S phase (0.005, 0.0625, 0.0725 and 0.0825 µM) were observed in RT4 cells after AITC treatments. In T24 cells, an increased sub-G1 rate (0.0725 and 0.0825 µM) and cell cycle arrest at G2 phase (0.0625, 0.0725 and 0.0825 µM) were detected. Furthermore, it was observed increased apoptosis and necrosis rates and increase of *S100P* and *BAX* and decrease of *BCL-2* expression in the wild type *TP53* cells. For the mutated *TP53* cell line, increased expression of *BCL-2*, *BAX* and *ANLN* and decreased expression *S100P* were observed. No change was detected for the *SMAD4* and *CDK-1* genes. In conclusion, AITC was able to reach DNA and induce primary damage in both bladder transitional carcinoma cell lines, but those damages were not converted into chromosome aberrations detected as micronuclei. Data also showed that the *TP53* status interfered with cell responses to AITC: while the major effects in the wild *TP53* cells were related to apoptosis and to the modulation of *BAX/BCL-2* via, in mutated *TP53* cells were associated to cell cycle arrest and induction of *ANLN* expression, which is related to cytokinesis.

REVISÃO DA LITERATURA

3. REVISÃO DA LITERATURA

3.1 CÂNCER DE BEXIGA E ISOTIOCIANATO DE ALILA

No mundo, o câncer de bexiga é a quarta neoplasia mais comum em homens e a nona em mulheres. A mortalidade por este tumor é três vezes maior em homens atingindo 113.000 óbitos em 2011 (Jemal, 2011). No Brasil, o câncer de bexiga representa, em homens, o 6º mais frequente nas regiões sudeste (10/100 mil), o 7º na região centro-oeste (5,86/100 mil), o 8º na região sul (9,10/100 mil) e o 11º nas regiões norte (1,98/100 mil) e nordeste (2,82/100 mil). Em mulheres, é o 13º mais frequente nas regiões centro oeste (2,72/ 100 mil) e norte (0,70/100 mil) e o 14º nas regiões sul (3,29/100 mil), sudeste (2,55/100 mil) e nordeste (1,24/100 mil) (Figura 1). Na última estimativa realizada pelo Instituto Nacional do Câncer, cerca de 8.940 novos casos estão previstos para 2014 (INCA, 2014).

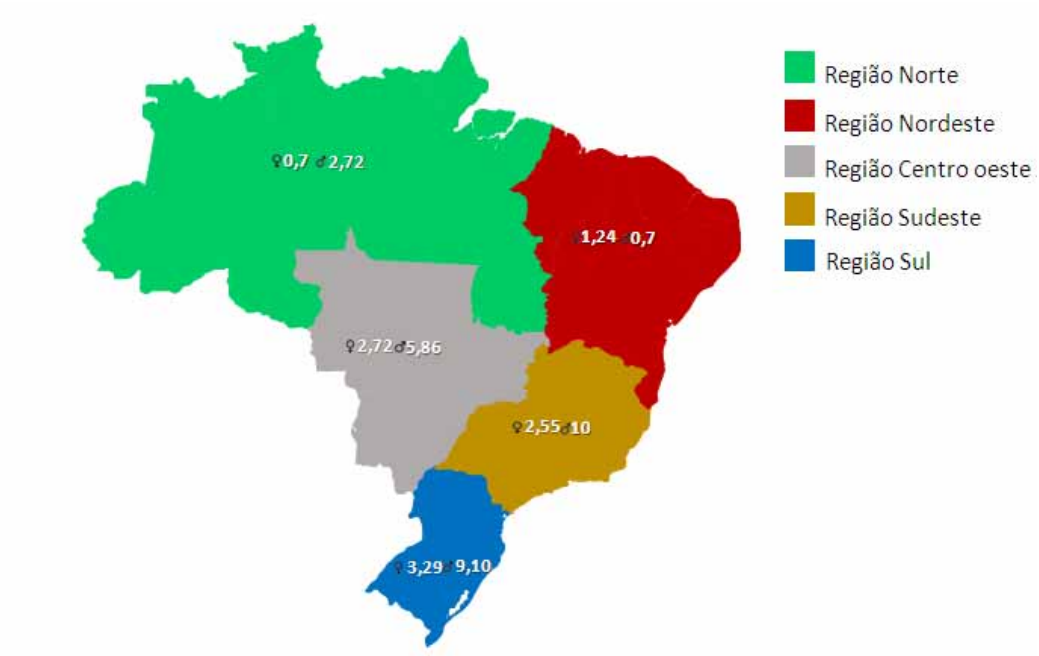

Figura 1: Incidência de câncer de bexiga nas cinco regiões do país. Os valores representam número de casos/100.000 homens ou mulheres (Adaptado de INCA, 2014).

Os Carcinomas de Células Uroteliais (UCC) de bexiga correspondem a 90% dos tumores malignos nesse órgão, sendo os demais representados pelos carcinomas de células escamosas

(7%), pelos adenocarcinomas (2%) e pelos sarcomas (<1%) (Castillo-Martin et al., 2010). Estruturalmente, a bexiga caracteriza-se por ser um órgão oco e elástico, composto por quatro túnicas: a mucosa, a sub-mucosa, a muscular e a adventícia. A túnica mucosa apresenta uma camada de células epiteliais transicionais, o urotélio, que recobre a superfície interna do órgão (Lewis, 2000; Junqueira et al., 2004). O urotélio é composto por três camadas celulares morfologicamente distintas: as células basais, as intermediárias e as células superficiais, também chamadas de "umbrela" (Castillo-Martin et al., 2010). Patologicamente, o UCC de bexiga é classificado pela extensão do tumor primário (estadiamento) e pelo grau de diferenciação histológica, podendo apresentar-se como tumor papilar, carcinoma *in situ* (CIS), tumor músculo-invasivo, doença avançada e doença metastática (Chester et al., 2004). Os UCCs são superficiais quando atingem, no máximo, a lâmina própria do epitélio vesical (TA e T1), e profundos, quando invadem a camada muscular superficial e profunda (T2) e tecido perivesical (T3) (Figura 2) (Stenzl et al., 2011). A principal característica clínica dos tumores vesicais não invasivos é o seu alto índice de recorrência, com indiferenciação progressiva e aumento da agressividade (Kaufman, 2006).

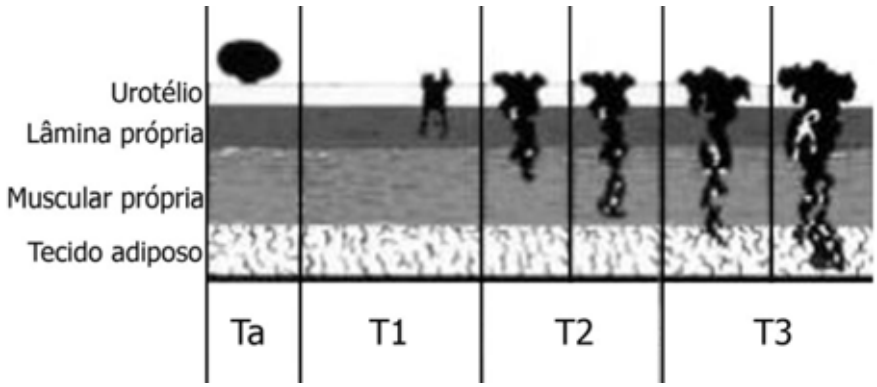

Figura 2 – Estadiamento dos tumores de bexiga – (adaptado de Pompeo et al., 2008).

os primeiros fatores relacionados à indução do UCC foram as exposições ocupacionais que ocorreiam em indústrias têxteis e fábricas de pneus. Posteriormente, os casos foram também associados à exposição prolongada ao analgésico fenacetina, à ciclofosfamida e às

aminas aromáticas (Messing et al., 2002; Wallerand et al., 2005). O tabagismo, atualmente considerado como a principal causa deste tipo de tumor, parece estar mais diretamente relacionado ao tumor invasivo do que aos tumores superficiais (Jiang, 2012).

Dentre as diferentes alterações genéticas descritas no câncer de bexiga, mutações no gene *TP53* representam as mais frequentes. O *TP53* codifica a proteína p53, fosfoproteína nuclear que normalmente ativa a parada do ciclo celular na fase G1 em resposta a danos no DNA. A proteína p53, responsável por estender o tempo disponível para o reparo do DNA antes da célula entrar na fase S do ciclo, tem papel importante na apoptose, estabilidade genômica, senescência e diferenciação celular (Cavalcanti et al., 2002; Levine et al., 2009; Cheng et al., 2011). Mutações no gene *TP53* já foram associadas a UCCs de alto risco em indivíduos fumantes, enquanto nenhuma relação foi encontrada para o gene *FGFR3*, que está associado à regulação do crescimento e divisão celular em tumores de baixo grau (Wallerand et al., 2005).Além de mutações no *TP53*, mutações nos oncogenes *FGFR3* e *HRAS* foram descritas em, respectivamente, 75% e 30% dos tumores papilares de baixo grau (Wu, 2005; Jebar et al., 2005; Cheng et al., 2011). A inativação dos genes supressores tumorais *TP53*, *PTEN* e *RB1* é frequentemente observada em doença avançada e no carcinoma *in situ* (CIS) (Bakkar et al., 2003). Segundo Goebell et al. (2010), essas alterações seriam responsáveis pela instabilidade genética e pela ativação de mecanismos anti-apoptóticos.

Devido a alta taxa de recorrência, progressão para doença invasiva e aumento da agressividade, o câncer de bexiga é considerado uma doença altamente mórbida (Kaufman, 2006). Os pacientes submetidos a procedimentos cirúrgicos periódicos são considerados grupo de risco para o desenvolvimento neoplásico de *novo* e são mais susceptíveis à recorrência tumoral (Nilsson et al., 2001; Latini et al., 2010). Além da cirurgia, a radio e a quimioterapia são também utilizadas para este tipo de câncer. Kotwal et al. (2008) observaram que o índice de sobrevivência entre os pacientes com tumores invasivos submetidos a

radioterapia é equivalente ao daqueles submetidos a cistectomia, porém com a vantagem da preservação do órgão. No caso da quimioterapia, são vários os protocolos utilizados e envolvem combinações de drogas como metrotrexato, vinblastina, doxorrubicina e cisplatina (protocolo conhecido como MVAC) e também a combinação de gencitabina e cisplatina. A terapia MVAC produz taxas de resposta em torno de 72%, mas apresenta como desvantagens a alta toxicidade, vômitos e neutropenia febril (Herr, 2001). Por outro lado, a combinação de cisplatina e gencitabina, que possui eficácia similar ao MVAC, apresenta níveis de toxicidade muito menores (Mcaninch, et al 2010). Além desses, o tratamento com BCG (Bacilo de Calmette-Guérin) tem se mostrado eficaz para os casos de carcinoma *in situ* e como adjuvante em terapias de tumores com alto risco de recidiva e progressão (Machado et. al., 2009).

Na maioria dos casos, os protocolos antineoplásicos atuam induzindo alterações no DNA e eventos moleculares que resultam em alterações no ciclo celular e apoptose. Os compostos quimioterápicos convencionais causam efeitos colaterais intensos, às vezes com taxa de sucesso limitada devido à concentração insuficiente da droga, alta toxicidade sistêmica, ausência de seletividade, além de contribuirem para resistência dos tumores após tratamento prolongado (Arkin, 2005). Portanto, algumas alternativas aos tratamentos químicos convencionais têm sido avaliadas. Uma delas é o uso de compostos naturais com potencial anticancerígeno e mais seletivo (Dumont et al., 2007). Os produtos provenientes de "plantas medicinais" apresentam melhor compatibilidade com o organismo humano, com menores taxas de efeitos colaterais e com a vantagem de serem mais acessíveis para a população, embora não possa ser descartada a possibilidade desses compostos também serem tóxicos e causarem efeitos colaterais ao paciente (Arora et al., 2010).

Nas últimas décadas, o uso de substâncias presentes em frutas, vegetais e óleos essenciais herbáceos têm merecido atenção especial, uma vez que foi demonstrado que diversas delas apresentam importante atividade antiproliferativa, induzindo apoptose e

alterações no ciclo celular (Son et. al., 2003; Chen et al., 2011; Bhattacharya et al., 2012). É crescente, portanto, o número de estudos que avaliam o potencial antineoplásico de óleos essenciais em diversos tipos celulares. Recentemente, Celia et al. (2013) demonstraram que o óleo essencial da tangerina diminuiu a taxa de proliferação de células de neuroblastoma humano *in vitro,* enquanto Jeong et al. (2013) demonstraram que o óleo essencial de *Pogostemon cablin* diminuiu a capacidade de proliferação celular e aumentou a taxa de apoptose em células de carcinoma colorretal também *in vitro.* Anteriormente, havia sido descrito que o eugenol, extraído do cravo-da-índia, também apresentava propriedades antiproliferativas e atividades pró-apoptóticas em células de melanoma *in vitro* (Pisano et al., 2007).

Grande destaque tem sido dado, também, aos vegetais crucíferos (Brassicaceae), cujos compostos fitoquímicos glicozinados auxiliam o organismo na produção enzimas que atuam na desintoxicação de carcinógenos (Bergerot et al., 2006). Estudos epidemiológicos mostram a associação inversa entre o consumo de vegetais crucíferos e o risco para o câncer de bexiga (Michaud et al., 1999; Tang et al., 2008). Parte dos efeitos desses vegetais tem sido atribuída a compostos da família dos isotiocianatos. Alguns pesquisadores descreveram, por exemplo, que o sulforano (SFN), isotiocianato extraído do brócolis, e o fenetil isotiocianato (FEITC) extraído do agrião, possuem atividades quimiprotetora em linhagens de adenocarcinoma de cólon, câncer de próstata e em células Jurkat (leucemia) (Fimogari et al., 2007; Cheung et al., 2009). Estudos conduzidos por Lin et al. (2011) mostraram que o tratamento com o benzil isotiocianato (BEITC), com o FEITC e com o SFN em linhagens celulares de leucemia mieloide aguda e leucemia promielocítica aumentou a taxa de apoptose, devido ao efeito antiproliferativo dos compostos. Ma et al. (2006) demonstraram que o fenilhexil isotiocianato (PHITC) inibiu a atividade do gene *HDAC1* em linhagem celular de leucemia (HL-60), enquanto Shan et al. (2009) observaram que o sulforano aumentou a expressão do gene *p38*

MAPK, inibiu a ligação do fator NF-κB e diminuiu a expressão da ciclo-oxigenase-2 (COX-2) em linhagem T24 de tumor de bexiga. Mais recentemente, Korenori et al. (2013) demonstraram que o 6-metiltiohexil isotiocinato (6-MTITC) ativou a expressão da quinona oxidoredutase 1 (NQO1), que modula o sistema Nrf2/Keap1, mecanismo de defesa contra estresse oxidativo (Kensler et al., 2007; Niture et al., 2010; Antelmannet al., 2011).

Em 2008, Munday et al. relataram que o isotiocianato de alila (AITC), presente em vegetais da família das crucíferas e abundante em semente de mostarda, rábano e *wasabi* (raiz forte), possuía atividade promissora para o tratamento e prevenção do câncer de bexiga. Os autores relataram que a principal razão para tal suposição era a alta biodisponibilidade do composto na urina, ou seja, a quase totalidade administrada por via oral era eliminada passando por esse órgão. No período em que o composto permanece na bexiga até ser eliminado as células neoplásicas são expostas à substância, a qual é rapidamente absorvida, induzindo bloqueio do ciclo celular e apoptose (Geng et al., 2011). Havia sido previamente descrito que o AITC poderia ser seletivo para células tumorais, uma vez que foram encontrados baixos níveis do composto em células de epitélio normal (Musk, 1993). Mais tarde, esses achados foram confirmados por Bhattacharya (2010), que demonstrou que a administração do AITC por via oral inibiu o desenvolvimento e invasão muscular do câncer de bexiga em ratos e que os níveis do composto na urina eram duas a três vezes maiores do que os encontrados no plasma.

Na tentativa de explicar os possíveis mecanismos de ação do AITC, Geng et al. (2011) utilizaram diferentes linhagens celulares de câncer de bexiga (UM-UC-3, UM-UC-6 e T24). Os autores mostraram que o AITC leva à parada do ciclo celular em mitose, que ocorre devido à degradação da α e β-tubulinas, além de induzir apoptose mediada por *Bcl-2*, citocromo C e ativação das caspases 9 e 3. Outros pesquisadores descreveram o envolvimento de proteínas de fase 2, por meio da ativação do Nrf2 (*nuclear factor erythroid 2-related factor*

2), um ativador transcricional (Ye et al., 2001). Embora vários estudos tenham demonstrado a ação do AITC sobre o ciclo celular e apoptose, seu mecanismo de ação não está ainda completamente elucidado.

3.2 CICLO CELULAR, APOPTOSE E NECROSE

O ciclo celular é dividido em quatro fases distintas - G1, S, G2 e M - e, para assegurar a correta execução dos eventos em cada uma delas, as células possuem mecanismos de checagem que garantem a manutenção da estabilidade genética (Lopes, 2003; Weaver, 2006). A progressão do ciclo celular é dependente de enzimas do tipo quinase, que possuem uma subunidade catalítica conhecida por quinase dependente de ciclinas (CDK1, CDK2, CDK4 e CDK6) e uma subunidade conhecida como ciclina (tipos A, B, D e E). Os sinais mitóticos são detectados pela expressão de ciclinas do tipo D, que se ligam e ativam as ciclinas CDK4 e CDK6 durante a fase G1, quando as células se preparam para iniciar a síntese de DNA; no final da replicação do DNA ocorre a ativação de CDK2. A degração das ciclinas do tipo A facilita a formação do complexo CDK1-ciclina B que é responsável pelo início da mitose. Além dessas enzimas, o gene *ANLN*, que codifica a proteína anilina, é também de fundamental importância no ciclo celular, participando no processo de citocinese (Oegema et al, 2000; Malumbres, 2001, Campo et al, 2005; Malumbres, 2005; Malumbres et al. 2009).

Além do ciclo, a morte celular por apoptose é também um evento regulado por mecanismos moleculares e bioquímicos, e que se caracteriza por alterações morfológicas como encolhimento celular, condensação e fragmentação nuclear e perda de adesão ou da matriz extracelular (Nishida, 2008). Esse tipo de morte celular ocorre, principalmente, quando os danos no DNA são irreparáveis. A apoptose pode ser ativada por suas vias: a extrínseca (via receptor) e a intrínseca (via mitocondrial) (Eum, 2011). A via extrínseca ocorre por meio da ativação de receptores de TNF (R-TNF) que são capazes de induzir a ativação das

caspases. Classicamente, os ligantes desta família são proteínas triméricas ou multiméricas da membrana celular. Seus receptores compartilham um domínio intracelular conhecido como "domínio de morte" (*Death domain*) e, por isso, são classificados como "receptores de morte celular" (Daniel, 2001). Na via intrínseca, em um dos mecanismos, as membranas mitocondriais tornam-se permeáveis ao citocromo C, que é liberado para o citosol ligando-se a uma molécula conhecida como APAF-1, que tem a capacidade de se ligar à caspase 9. Esse agregado, que recebe o nome de apoptossoma, desencadeia uma cascata de sinalização que culmina na apoptose (Ghobrial, 2005). Em outro mecanismo, a proteína BCL-2, inibi a interação do citocromo C e a ativação da caspase 9 pela molécula APAF-1. Por outro lado, a proteina BAX apresenta atividade pró-apoptótica, auxiliando na liberação do citocromo C e na formação dos apoptossomas (Shamas-Din, 2011; Wen, 2012). Outra importante molécula envolvida no processo de apoptose é a proteína p53, que ativa uma série de reguladores positivos de apoptose, como o DR-5 e a BAX (Benchimol, 2001; Yu, 2003). Outros genes, como por exemplo, o *S100P* e o *SMAD4*, também podem desempenhar papel importante na indução de apoptose (Aitchison et a., 2008; Ding et al., 2011; Jiang, 2012).

A necrose é outro tipo de morte celular e que se caracteriza pela desestabilização da integridade da membrana e extravasamento de componentes intracelulares para o meio extracelular, gerando respostas inflamatórias (Zong, 2004; Agostinis, 2004). Moléculas como a PIR (proteínas de interação do receptor), quinases, PARP1 (poli ADPribose polimerase-1), oxidases e NADPH já foram identificadas e relacionadas a este tipo de morte celular (Golstein, 2007; Galluzi, 2008). A diferenciação entre células necróticas e apoptóticas pode ser realizada com base na morfologia e em características moleculares. Morfologicamente, as células apoptóticas apresentam retração celular, incluindo a condensação da cromatina e a preservação da membrana plasmática, enquanto a célula necrótica apresenta-se mais

volumosa e com ruptura precoce de membrana. Em relação a características moleculares, a ativação das caspases é um pré-requisito para o início da apoptose, enquanto a proteína necrótica RIP1 é necessária para a necrose (Krysko et al., 2006).

OBJETIVOS

4 – OBJETIVOS

O objetivo deste estudo foi avaliar o potencial antineoplásico e possíveis mecanismos de ação do AITC em células tumorais de bexiga com diferentes *status* do gene *TP53* (RT4 - tumor grau 1, com gene *TP53* selvagem; e T24 – tumor grau 3 e *TP53* mutado). Mais especificamente, o AITC foi avaliado quanto a seus efeitos:

- citotóxico e antiproliferativo;
- genotóxico (indução de danos primários no DNA, como quebras de fita simples e duplas e sítios álcali lábeis) e mutagênico (indução de danos clastogênicos e aneugênicos);
- sobre o ciclo celular e na possível indução de apoptose;
- sobre a morfologia celular;
- na modulação da expressão dos genes *TP53, ANLN, SMAD4, S100P, BCL-2, CDK-1* e *BAX*.

NOTA DO AUTOR: o delineamento experimental, assim como os resultados do estudo e a discussão dos dados estão apresentados no formato manuscritos para publicação.

ARTIGO I

5 – CELL CYCLE KINETICS, APOPTOSIS RATES, DNA DAMAGE AND *TP53* GENE EXPRESSION IN BLADDER CANCER CELLS TREATED WITH ALLYL ISOTHIOCYANATE (MUSTARD ESSENTIAL OIL)

André Luiz Ventura Sávio[*1], Glenda Nicioli da Silva[2], Elaine Aparecida de Camargo[1], Daisy Maria Fávero Salvadori[1]

[1] UNESP – Universidade Estadual Paulista, Faculdade de Medicina de Botucatu, 18618-970, Botucatu, SP, Brazil.

[2]UFOP – Universidade Federal de Ouro Preto, Escola de Farmácia, Departamento de Análises Clínicas, 35400-000, Ouro Preto, MG, Brazil.

*Corresponding author: André Luiz Ventura Sávio, Faculdade de Medicina de Botucatu – UNESP, Departamento de Patologia, Rubião Junior, 18618-970, Botucatu, SP, Brazil (e-mail: andresavio8@hotmail.com). Phone: +55-14 38807263. Fax: +55-14 38807263.

Artigo publicado na revista Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis, 762: 40-46, 2014 (http://dx.doi.org/10.1016/j.mrfmmm.2014.02.006).

ABSTRACT

Allyl isothiocyanate (AITC) is present in plants of the cruciferous family and is abundant in mustard seed. Due to its high bioavailability in urine after ingestion, AITC has been considered a promising antineoplastic agent against bladder cancer. Because *TP53* mutations are the most common alterations in bladder cancer cells and are frequently detected in *in situ* carcinomas, in this study, we investigated whether the AITC effects in bladder cancer cells are dependent on the *TP53* status. Two bladder transitional carcinoma cell lines were used: RT4, with wild-type *TP53*; and T24, with a mutated *TP53*. AITC was tested at concentrations of 0.005, 0.0625, 0.0725, 0.0825, 0.0925, 0.125 and 0.25 µM in cytotoxicity, cell and clonogenic survival assays, comet and micronucleus assays and for its effects on cell cycle and apoptosis by flow cytometry and on *TP53* gene expression. The data showed increased primary DNA damage in both cell lines; however, lower concentrations of AITC were able to induce genotoxicity in the mutated *TP53* cells. Furthermore, the results demonstrated increased apoptosis and necrosis rates in the wild-type cells, but not in mutated *TP53* cells, and cell cycle arrest in the G2 phase for mutated cells after AITC treatment. No significant differences were detected in *TP53* gene expression in the two cell lines. In conclusion, AITC caused cell cycle arrest, increased apoptosis rates and varying genotoxicity dependent on the *TP53* status. However, we cannot rule out the possibility that those differences could reflect other intrinsic genetic alterations in the examined cell lines, which may also carry mutations in genes other than *TP53*. Therefore, further studies using other molecular targets need to be performed to better understand the mechanisms by which AITC may exert its antineoplastic properties against tumor cells.

Key words: allyl isothiocyanate, apoptosis, bladder cancer, cell cycle, genotoxicity.

1. INTRODUCTION

Bladder cancer is the fourth most common neoplasm diagnosed in men and the ninth most common in women in the Western world [1]. Approximately 90% of malignant bladder tumors are represented by urothelial cell carcinomas (UCC), which present as small papillae or invasive lesions [2]. Due to the high recurrence rates, progression to muscle invasive disease and increased aggressiveness, bladder cancer is considered a highly morbid disease [3]. The majority of cases are associated with cigarette smoking and occupational exposure to aromatic amines [4]. It has been reported that tobacco metabolites excreted into the urine of smokers are responsible for approximately 50% of bladder tumors. Furthermore, smokers have a four-fold higher incidence of bladder cancer than non-smokers [5].

Therapies for UCC include surgical procedures (partial or radical cystectomy), radio and chemotherapy. However, currently, natural substances found in fruits, vegetables and essential oils have been investigated as alternative approaches for treating diseases such as cancer [6, 7, 8]. Allyl isothiocyanate (AITC), known as mustard essential oil, is abundant in mustard seed and wasabi (horseradish) and is a natural compound with chemopreventive potential. An epidemiological study has demonstrated that raw cruciferous vegetables containing AITC may reduce the risk of bladder cancer [9]. In fact, it was shown that approximately 80% of AITC oral doses are selectively delivered to the bladder tissue through urinary excretion and can potently inhibit cancer development and muscle invasion [10, 11]. Furthermore, it has been suggested that this compound has promising activity for treating and preventing bladder cancer because of its capacity for interfering with mitosis, increasing ubiquitination and tubulin degradation and inducing apoptosis [12].

TP53 mutations are the most common alterations in bladder cancer cells and are frequently detected in *in situ* carcinomas (CIS) and in advanced disease states [13, 14, 15]. These mutations are related to cellular transformation, malignancy and the high recurrence

rate of urinary bladder cancers [13, 16]. *TP53* is a critical gene in the G1 checkpoint and is evolved in the tumor response to several anticancer drugs [17, 18, 19]. Its encoded protein, p53, can activate G1 cell cycle arrest in response to DNA damage, extending the time available for DNA repair before entry into the S phase, and can play an important role in the apoptosis pathways [20, 21]. Therefore, *TP53* mutations can affect the p53DNA-binding activity, abolishing the transcriptional activation of *TP53* target genes and apoptosis [22].

Numerous studies have investigated the relationship between p53 and/or *TP53* mutations and the response to antineoplastic drugs, once both protein and gene are related to the DNA damage response pathway [17, 18, 23]. Recently, it was demonstrated that multiple myeloma cell death caused by the exposure to gemcitabine and clofarabine is p53-dependent [19]. Therefore, to investigate the antineoplastic potential of AITC for bladder carcinoma, we used two cell lines with different *TP53* status: one carrying the wild-type gene (RT4) and the other carrying a mutated *TP53* (T24). The cytotoxicity, mutagenicity, apoptosis rates, cell cycle alterations and toxicogenomic activities of AITC were evaluated in the two cell lines.

2. MATERIALS AND METHODS

2.1 CELL LINES AND TEST COMPOUND

The human urothelial carcinoma cell lines RT4 (from a low grade tumor with the wild type *TP53* gene) and T24 (from an invasive tumor with the *TP53* allele encoding an in-frame deletion of tyrosine 126) were purchased from the Cell Bank of the Federal University of Rio de Janeiro, Brazil, and maintained as previously described by da Silva et al. [24]. Allyl isothiocyanate (AITC) was purchased from Sigma-Aldrich (USA) and was diluted into 2% Tween 20 prior to use. The test compound concentrations were selected based on the data

obtained in the clonogenic survival assay. All treatments with AITC were performed for three hours, as described by Zhang et al. [25].

2.2 CYTOTOXICITY AND CELL PROLIFERATION

Cytotoxicity and cell proliferation rates were assessed using the Cell Proliferation Kit II (XTT) (ROCHE Diagnostics; Mannheim, Germany). Briefly, cells were seeded into 12-well culture plates (12×10^4 and 8×10^4 cells/well for cytotoxicity and cell survival, respectively). After 24 hours, the cells were treated with AITC at concentrations of 0.005, 0.0625, 0.0725, 0.0825, 0.0925, 0.125 and 0.25 µM for three hours. Untreated cells and cells treated only with Tween 20 were cultured as controls. Three hours after incubation, the cells were washed with Hank's solution (0.4 g KCl, 0.06 g KH_2PO_4, 0.04 g Na_2HPO_4, 0.35 g $NaHCO_3$, 1 g glucose and 8 g NaCl in 1 L H_2O). After washing, 50 µL of XTT test solution (1 mL XTT labeling solution/20 µL of electron-coupling reagent) was added to each well, and the absorbance was measured at 492 and 690 nm after 90 min (absorbance results are proportional to the percentage of viable cells). For evaluating cytotoxicity and cell proliferation, complete fresh medium was added, and the cells were incubated at 37°C for 21 and 69 hours, respectively. Then, a 50-µL aliquot of XTT solution was added to each well, and the absorbance was measured after 90 min. Both tests were conducted in triplicate.

2.3 CLONOGENIC SURVIVAL

A clonogenic assay was used for evaluating the long-term effects of AITC. For clonogenic ability, cells were plated at a density of 1×10^6 cells/25 cm^3 culture flask; 24 hours later, they were treated with AITC at concentrations of 0.005, 0.0625, 0.0725, 0.0825 and 0.0925 µM for three hours. Cultures were rinsed with Hank's solution, trypsinized, and approximately 1000 cells were plated into 25-cm^3 culture flasks and allowed to grow for 15

days to form colonies. The cells were Giemsa stained, and the number of colonies with 50 or more cells was counted. The experiments were performed in triplicate.

2.4 COMET ASSAY

Initially, 8 x 10^4 cells were seeded into 12-well plates for 24 hours. Then, cells were treated with AITC at concentrations of 0.005, 0.0625, 0.0725, 0.0825 and 0.0925 µM for three hours. Methyl methanesulfonate (0.006 M, 5 min, 37°C; Sigma-Aldrich, Inc.; St. Louis, MO, USA) was used as positive control. The comet assay was conducted based on the methods described in Singh et al. [26] and Tice et al. [27]. Briefly, 10 µl of cells was added to 100 µl of 0.5% low-melting-point agarose at 37°C. This mixture was layered onto pre-coated slides with 1.5% standard agarose and covered with a coverslip. After agarose solidification at 4°C, the coverslip was gently removed. Then, the slides were immersed into lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl at pH 10, 1% sodium sarcosinate, 1% Triton X-100 and 10% DMSO) overnight, at 4°C, which was followed by an incubation step in alkaline buffer (0.3 mM NaOH and 1 mM EDTA; pH > 13) for 20 minutes, to allow DNA unwind and alkali-labile site expression. Electrophoresis was conducted in the same alkaline buffer at 4°C, for 20 minutes, at 25 V (0.86 V cm^{-1}) and 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris-HCl (pH 7.5) for 15 min, fixed with absolute ethanol and stored at room temperature until analysis. All steps were conducted in the dark to prevent additional DNA damage. The slides were stained with SYBR Gold (1:10,000; Invitrogen; Grand Island, NY, USA), immediately before analysis. Cell viability was assessed using the trypan blue exclusion test (0.4% trypan blue, Sigma-Aldrich, Inc.; St. Louis, MO, USA) exclusion test (viability was never lower than 90%). A total of 150 randomly selected nucleoids per treatment was analyzed under 400x magnification with a fluorescence microscope connected to an image analysis system (Comet Assay IV, Perceptive Instruments; Suffolk, Haverhill,

UK). Tail intensity (% DNA in tail) was used to estimate DNA damage. The slides were prepared in duplicate from three independent experiments.

2.5 CYTOKINESIS-BLOCK MICRONUCLEUS ASSAY

The MN assay was performed based on the technique described by Fenech [28]. Briefly, 1 x 10^6 cells were seeded into a dish (100 mm x 20 mm). Twenty-four hours later, cells were treated with AITC at concentrations of 0.005, 0.0625, 0.0725, 0.0825 and 0.0925 µM, for three hours. Then, cytochalasin B (3 µg/mL) was added and the cells were incubated at 37°C and 5% CO_2 for 28 (T24) or 44 (RT4) hours. At the end of the incubation steps, cells were collected and centrifuged at 800 rpm for five minutes. The supernatant was discarded, and 5 mL of ice-cold hypotonic solution (0.075 M KCL) was added. After cell fixation, the slides were stained with 5% Giemsa solution and analyzed under a light microscope at 1000x magnification. One thousand binucleated cells were analyzed in each slide. Doxorubicin was used as a positive control at a concentration of 0.4 µg/mL for two hours, and all treatments were performed in triplicate.

2.6 CELL CYCLE KINETICS AND APOPTOSIS DETECTION

For cell cycle kinetics, 2 x 10^5 cells were seeded into 12-well plates; 24 hours later, the cells were treated with AITC at concentrations of 0.005, 0.0625, 0.0725 and 0.0825 µM for three hours and then washed with Hank's solution. Fresh medium was added, and the cells were incubated again at 37°C for 21 hours. Afterwards, cells were detached using trypsin-EDTA, resuspended into fresh medium and centrifuged at 1000 rpm for 10 min. The pellet was resuspended in 200 µL of HFS (50 µg/ml propidium iodide, 0.1% sodium citrate and 0.1% Triton X-100), placed on ice and protected from the light for at least 30 min. The percentage of cells in the G0/G1, S and G2/M phases were measured using GUAVA Cytosoft version 4.2.1 software. The cell cycle analyses were performed in triplicate.

A quantitative assessment for apoptosis was performed using a Guava Annexin reagent (Merck Millipore). Annexin V was used for detecting the externalization of phosphatidylserine to the cell surface and 7-AAD as an indicator of cell membrane structural integrity. Briefly, $2x10^5$ cells were seeded into 12-well culture plates. After 24 hours, the cells were treated with AITC at concentrations of 0.005, 0.0625, 0.0725 and 0.0825 µM for three hours. Afterwards, cells were washed with Hank's solution, and fresh medium was added. Untreated cells and cells treated only with Tween 20 were cultured as controls. Cells were collected 21 and 45 hours after incubation, resuspended into 100 µL Guava Nexin reagent for 20 minutes in the dark and immediately analyzed in the Guava easyCyte flow cytometer (Millipore) using the Guava System software. Data from 5000 cells were collected in each data file. Cellular status was defined as follows: unstained cells were classified as 'alive'; cells stained only by annexin V were classified as 'early apoptotic'; cells stained by both annexin V and 7-AAD were classified as 'late apoptotic'; and cells stained only by 7-AAD were classified as 'dead'.

2.7 *TP53* EXPRESSION

Total RNA from the cultured T24 and RT4 cells was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed using 6 µL of random hexamer primers (10X), 6 µL of reaction buffer (10X), 2.5 µL of dNTPs (25X) and 3 µL of MultiScribe (50 U/mL, High Capacity; Applied Biosystems). After incubation at 25°C for 10 min and 37°C for two hours, the cDNA was stored at 4°C and at –20°C. Differential expression of *TP53* in the two cell lines was assayed using the TaqMan system (Applied Biosystems; Foster City, CA, USA). Each tube contained 2 µL of cDNA template, 5 µL of Master Mix TaqMan 2X (Applied Biosystems) and 0.5 µL of 20X primers/probe (Assays-on-Demand gene expression products; Applied Biosystems). β-actin

was used as a housekeeping gene. The reaction was performed using the following thermal cycler conditions: 94°C for 10 min followed by 40 cycles at 94°C for 30 s and 60°C for 1 min. Fluorescence data were collected during each annealing/extension step. The reactions were performed using an Applied Biosystems 7500 FAST Real-Time PCR System and SDS software, version 1.2.3 (Sequence Detection Systems 1.2.3, 7500 Real-Time PCR Systems, Applied Biosystems). For every PCR sample, a negative (no template) control was processed as a routine control. Assays were performed in triplicate. Relative gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ method [29].

2.8 STATISTICAL ANALYSIS

Statistical analyses were performed using SAS software, v.9.2 (Statistical Analysis System, SAS Institute; Cary, NC, USA). For the cytotoxicity, comet and cell proliferation assays, data were analyzed using ANOVA and Tukey's test; for the cell proliferation assay (nonparametric distribution – T24 cells), the analysis was performed using a gamma distribution followed by a multiple comparisons test using the DIFF option of the GENMOD procedure; for clonogenic survival and apoptosis and cell cycle analyses, a Poisson distribution and a factorial analysis based on a binomial distribution were performed, respectively; for the micronucleus test, data were analyzed using the Poisson (RT4 cells) and binomial (T24 cells) distributions. Gene expression values were analyzed by one-way ANOVA followed by Tukey's test. The results were considered statistically significant when $p < 0.05$.

3. RESULTS

3.1 CYTOTOXICITY, CELL PROLIFERATION AND CLONOGENIC SURVIVAL ASSAYS

The data revealed a significant decrease of cell proliferation only in RT4 cells (wild type *TP53*) three hours after treatment with 0.25 µM AITC (Figure 1A). In contrast, significant decreases were detected for both RT4 (0.125 and 0.25 µM AITC) and T24 (0.25 µM) cells three days after treatments (Figure 1B). No cytotoxicity was visualized 24 after treatments in either cell line (Figure 2). The clonogenic survival assay revealed significant decreases in cell colonies (RT4 and T24) after AITC treatment at concentrations of 0.0625, 0.0725, 0.0825 and 0.0925 µM (Figure 3).

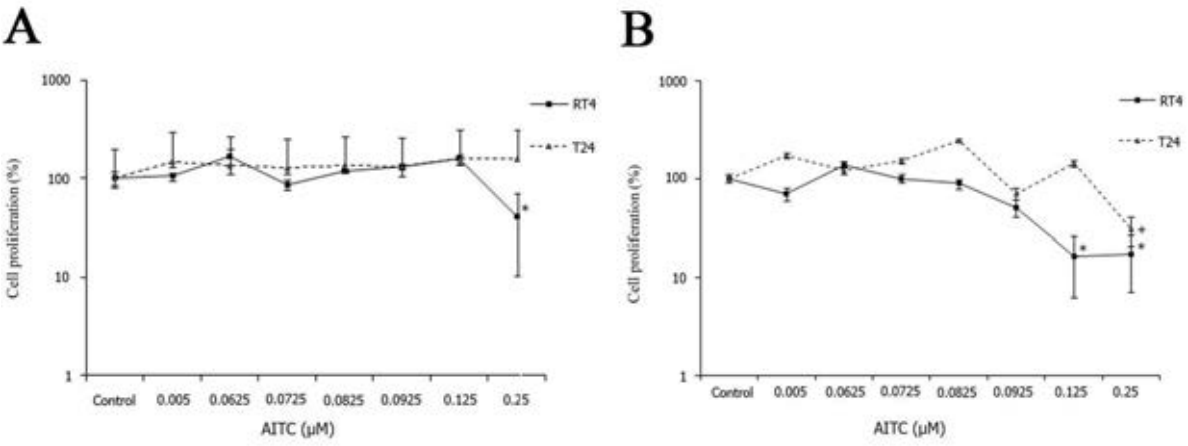

Figure 1 – Percentages of proliferation (logarithmic scale) in RT4 and T24 cell lines immediately (A) and three days (B) after treatment with allyl isothiocyanate (AITC). * $p < 0.05$ in relation to the AITC vehicle control (2% Tween 20%). Each point represents the mean value obtained from three independent experiments.

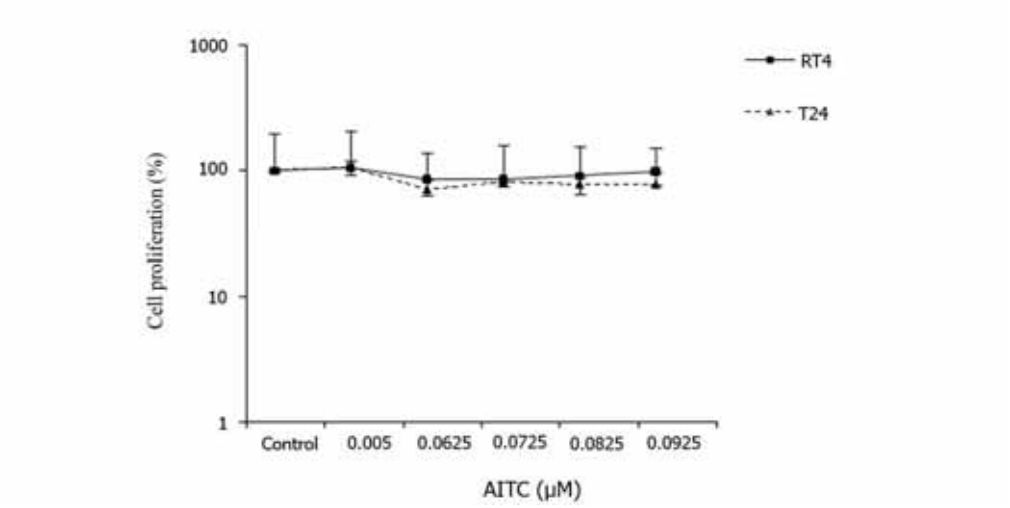

Figure 2 - Percentages of proliferation (logarithmic scale) in RT4 and T24 cell lines 24 hours after treatment with allyl isothiocyanate (AITC). * $p < 0.05$ in relation to the AITC vehicle control (Tween 20%). Each point represents the mean value obtained from three independent experiments.

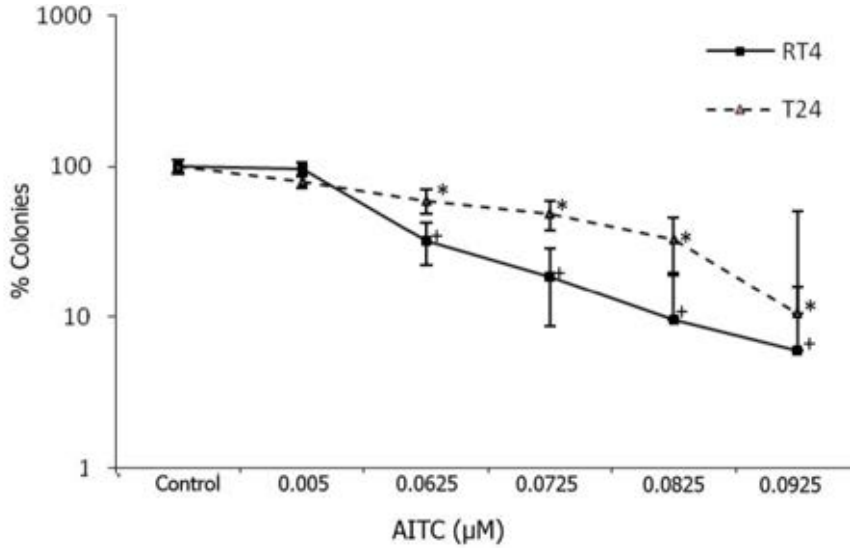

Figure 3 – Percentages of cell colonies (logarithmic scale) 10 (T24 cells) and 15 (RT4 cells) days after treatment with allyl isothiocyanate (AITC). $p < 0.05$ (*RT4; +T24). Each point represents the mean value obtained from three independent experiments.

3.2 COMET AND MICRONUCLEUS ASSAYS

All concentrations of AITC increased primary DNA damage in T24 cells; in RT4 cells, increases were observed for the three highest concentrations (0.0725, 0.0825 and 0.0925 µM) (Table I). No significant differences were detected in the frequency of micronucleus between negative control and treated cells (Table II).

Table I. DNA damage (tail intensity) in bladder carcinoma cell-lines (RT4 and T24) treated with allyl isothiocyanate (AITC)

Cell line	Negative control[1]	Control Tween[2]	Positive control[3]	AITC (µM)				
				0.005	0.0625	0.0725	0.0825	0.0925
RT4	14.462± 1.024	12.055 ± 1.229	80.361± 6.127*	18.085 ± 3.118	19.871 ± 2.018	24.923 ± 6.330*	27.400 ± 3.264*	30.472 ± 3.820*
T24	5.807 ± 1.800	5.063 ± 0.218	89.430b ± 3.906*	14.807 ± 1.900*	13.903 ± 1.790*	15.767 ± 1.825*	13.933 ± 2.268*	15.557 ± 0.659*

[1]No treatment; [2]cells treated with 2% Tween 20 (vehicle control); [3]cells treated with Methyl Methane Sulfonate (0.006 M); * $p < 0.05$ compared to the Tween control.

Table II: Frequencies of micronuclei (‰ MNC) in cells RT4 and T24 after treatment with allyl isothiocyanate (AITC)

Cell line	Negative control[1]	Control Tween[2]	Positive control[3]	AITC (µM)				
				0.005	0.0625	0.0725	0.0825	0.0925
T24	0.08	0.05	0.38	0.16	0.10	0.16	0.10	0.10
RT4	0	0	0.30	0	0	0	0	0.03

[1]No treatment; [2]cells treated with 2% Tween 20 (vehicle control); [3] cells treated with doxorubicin (0.4 µg/mL); frequency of micronucleated cells in 1000 cells analyzed (‰ MNC); * $p < 0.05$ compared to the Tween control.

3.3 CELL CYCLE ANALYSIS

Significant decreases in the numbers of RT4 cells in the S phase were detected after AITC treatment at 0.005, 0.0625, 0.0725 and 0.0825 µM. For T24 cells, significant decreases ($p<0.05$) in the numbers of cells in the G1 phase (0.0725 and 0.0825 µM) and the S phase (0.0625 and 0.0825 µM) accompanied by an increased number of cells in the G2 phase were detected, suggesting G2/M cell cycle arrest (Table III).

Table III – Cell cycle kinetics in bladder carcinoma cell lines (RT4 and T24) treated with allyl isothiocyanate (AITC)

		RT4	T24
G1 (%)	Control	40.90 ± 8.84	48.07±1.25
	Tween 20	51.41 ±12.30	49.38±0.68
	0.005 µM	58.58 ± 6.91	42.29±3.89
	0.0625 µM	59.00 ± 3.02	42.34±5.95
	0.0725 µM	40.57 ± 3.09	30.47±4.47*
	0.0825 µM	39.10 ± 2.37	14.94±2.82*
S (%)	Control	22.75±17.80	18.22±1.23
	Tween 20	26.14±25.75	22.70±1.30
	0.005 µM	8.75±1.72*	23.05±1.73
	0.0625 µM	5.03±0.82*	13.80±1.44*
	0.0725 µM	8.05±2.12*	16.89±3.58
	0.0825 µM	6.24±1.50*	5.72±2.16*
G2 (%)	Control	24.94±1.06	24.89±1.97
	Tween 20	20.88±7.48	21.14±4.60
	0.005 µM	21.87±2.09	27.96±4.88
	0.0625 µM	17.28±1.83	34.48±5.81*
	0.0725 µM	21.89±7.54	39.44±13.49*
	0.0825 µM	18.64±3.97	64.38±0.70*

Percentage of cells in the G1, S and G2/M mitotic phases are expressed as the mean ± standard deviation from triplicate**. *** $p < 0.05$ compared to the Tween control.

3.4 APOPTOSIS

Increased necrosis (0.0625, 0.0725, 0.0825 and 0.0925 µM) and early apoptosis (0.0625, 0.0725, 0.0825 and 0.0925 µM) rates were observed in RT4 cells 48 hours after treatment with AITC. For T24 cells, an increased necrosis rate was detected only 24 hours after treatment with AITC at 0.0625 µM (Table IV).

Table IV – Cell cycle analysis of bladder carcinoma cell lines RT4 and T24 treated with allyl isothiocyanate (mustard essential oil).

		RT4		T24	
		24 hours	**48 hours**	**24 hours**	**48 hours**
Necrosis (%)	Control	11	14	2	2
	Tween	9	24	1	3
	0.005µM	5	24	4	2
	0.0625µM	11	11*	5*	2
	0.0725µM	11	6*	4	2
	0.0825µM	10	8*	4	2
	0.0925µM	7	6*	4	2
Late apoptosis (%)	Control	22	17	8	12
	Tween	19	9	14	12
	0.005µM	7*	7	15	15
	0.0625µM	18	18*	29*	18
	0.0725µM	34*	35*	20*	26*
	0.0825µM	20	15*	19	21*
	0.0925µM	18	43*	23*	19*
Early apoptosis (%)	Control	1	5	0	5
	Tween	6	6	1	7
	0.005µM	3	7	1	8
	0.0625µM	3	10*	1	9
	0.0725µM	4	13*	1	11
	0.0825µM	2*	14*	1	10
	0.0925 µM	3	10*	1	12

Percentage of cells in the necrosis, late apoptosis, viable cells, early apoptosis. ***** $p < 0.05$ compared to the Tween control.

3.5 *TP53* EXPRESSION

The data showed no significant differences for *TP53* mRNA expression after treatment with AITC in either cell line (Figure 4).

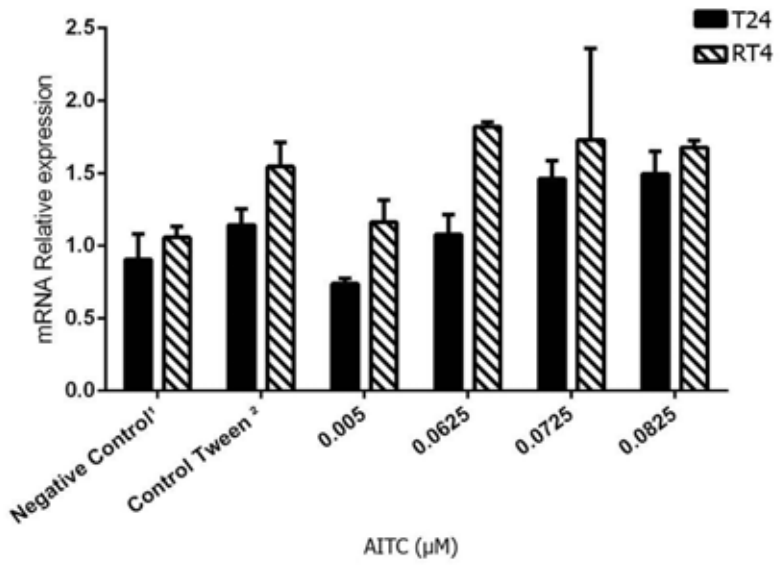

Figure 4- Relative *TP53* mRNA levels in RT4 and T24 cell lines after treatment with allyl isothiocyanate (AITC). The relative expression values correspond to fold-change values. [1]No treatment; [2]cells treated with 2% Tween 20 (vehicle control); β-actin (endogenous RNA control).

4. DISCUSSION

Epidemiological studies have shown that cruciferous vegetables may act on lung, breast, prostate, pancreas and bladder cancers, mainly because of their high concentrations of isothiocyanates (ITCs) [30]. Each vegetable may have one different ITC: for instance, phenethyl isothiocyanate (PEITC), derived from gluconasturtiin hydrolysis, is found in wasabi and watercress; benzyl isothiocyanate (BITC), derived from glucotropaeolin hydrolysis, is found in cabbage; and the allyl isothiocyanate (AITC), which is derived from sigrina hydrolysis, is found in mustard and horseradish [31]. ITCs may act by inhibiting cytochrome P450 isoforms, which modulate phase II enzymes and prevent DNA adducts caused by some carcinogens [30]. Mi [32] has observed significant inhibition of the proteasome activity in several cell lines, including cervical (HeLa), lung (A549), colon (HT29) and breast cancer (MCF-7) cells treated with BITC and PEITC. Additionally, this

same author demonstrated that these compounds cause rapid accumulation of p53 and NF-kB (IkB) inhibition, apoptosis and G2/M phase arrest [32]. *In vitro* growth inhibition of UM-UC3 human bladder carcinoma cells was detected after treatment with AITC [33]. Furthermore, AITC is markedly less toxic to normal cells than to cancer cells, suggesting its selective activity [6, 34]. The high bioavailability of AITC in urine has been suggested as an important feature for bladder cancer therapy [11]. In fact, some authors have described higher levels of AITC in urinary bladder tissues than in other organs after administration [33,35].

Therefore, based on the chemotherapeutic potential of AITC, and considering the fact that chemotherapy exposure induces widespread p53-dependent DNA damage, we tested the effect of this compound in wild-type and mutated *TP53* cell lines. The cellular response due to *TP53* mutations after different treatments is poorly understood, as the response depends on a complex signaling cascade. Hofseth et al. [36] reported that a mutant p53 impaired the DNA damage response and rendered the tumor cells more resistant to drug-induced apoptosis. In this way, it has been already shown that a functional *TP53*/p53 pathway improves gemcitabine cytotoxicity [37]. Moreover, a previous study from our group demonstrated higher apoptosis rates in wild-type cells compared to mutated *TP53* cells after cisplatin and gemcitabine treatments [24].

Herein, we observed that AITC induced decreased cell proliferation three days after treatment at the highest concentrations and no cytotoxicity 24 hours after treatment in both cell lines. These results provide evidence that the AITC concentrations used led to the loss of reproductive integrity, i.e., decreased cell proliferation capacity, most likely because of sustained lethal damage. In contrast, some authors have reported cytotoxic effects of AITC (percentages of viable cells) in human brain malignant glioma cells (GBM 8401), though at concentrations higher that 1 μM [38]. It is important to remember that the clonogenic cell

survival assay determines the ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large colony. This assay is widely used to examine the effects of agents with potential application in the clinic [39]. Therefore, the low survival observed in this assay could merely be the control of the lysis rates for cells that had already lost their reproductive potential at 24 hours and/or three days [40]). Our present study also revealed increased amounts of primary DNA damage (comet assay) in both wild-type and mutated *TP53* cells treated with AITC. Interestingly, in mutated cells, even the two lowest concentrations of AITC were able to induce significant damage. Therefore, it seems that the *TP53* background must be considered with regard to the genotoxic potential of AITC. Nevertheless, although AITC was able to reach DNA, no mutagenic effect was detected by the micronucleus assay. These findings suggest that AITC, although genotoxic, is not aneugenic or clastogenic in the two cell lines. Similar results were previously reported by Lamy et al. [41] testing other ITCs. MTPITC, MTBITC and MTPeITC were genotoxic, but not mutagenic, in HepG2 cells. Additionally, it has been reported that AITC is unable to induce either chromosome aberrations or sister chromatid exchanges, even at highly cytotoxic doses [42]. The authors suggested that either the DNA was efficiently repaired or factors that trigger apoptosis might be active. Actually, several molecular events are involved in DNA repair.

Regarding the toxicogenomic effect of AITC, our results revealed no change in *TP53* gene expression in either cell line. Recently, it was demonstrated that PEITC might deplete mutant p53 without causing changes in p53 mRNA expression [43]. One explanation for this finding was a possible mechanism involving protein modification via covalent binding to the ITC functional group, making the mutant p53 cells significantly more sensitive to PEITC-induced apoptosis than the wild-type cells. Similarly, we suggest that AITC can act at

translational and/or post-translational levels, as we observed increased early apoptosis rates in the wild-type cells but not in *TP5*3 mutated cells, suggesting that AITC-induced DNA damage triggers apoptosis, most likely through p53 pathways. Kumar et al. [44] have discussed that decreased *BCL-2* and increased *BAX* expression, as well as CAD (caspase-activated DNase) activation by caspase-3, might also explain AITC induced-apoptosis.

When the effect of AITC on the cell cycle was analyzed, we detected activities dependent on the *TP53* status. While a decreased number of cells in S phase was observed for the wild-type *TP53* cells, significantly decreased numbers of cells in G1 and S phases and an increased number in the G2/M phase (suggestive of cell arrest) were detected in mutated *TP53* cells after treatment with AITC. G2/M cell cycle arrest was also observed in human glioma cells after treatment with AITC [39]. Moreover, AITC is able to bind to cysteine residues and α- and β-tubulins in a bladder cancer cell line, promoting their degradation and ubiquitination and inducing cell cycle arrest in mitosis [12]. Studies using colon and prostate cancer cell lines treated with sulforaphane (which also belongs to the isothiocyanate family) demonstrated a G2/M cell cycle arrest, loss of *Bcl-2* gene expression and increased caspase activity [45, 46]

In conclusion, AITC caused cell cycle arrest and increased apoptosis rates and genotoxicity dependent on the *TP53* status. However, we cannot rule out the possibility that these differences could reflect other intrinsic genetic alterations in the examined cell lines, which may also carry mutations in genes other than *TP53*. Therefore, further studies using other molecular targets need to be performed to better understand the mechanisms by which AITC may exert its antineoplastic properties against tumor cells.

5. AUTHOR CONTRIBUTIONS

All of the authors reviewed the manuscript. ALVS conducted all of the experiments, interpreted the data and wrote the manuscript. GNS suggested the experimental design, conducted the flow cytometry experiments and interpreted the data. EAC performed the flow cytometry experiments, micronucleus test and comet assay. DMFS was the advisor, contributed to the experimental design and data interpretation as well as critically read the manuscript.

6. ACKNOWLEDGMENTS

The authors are grateful to Dr. José Eduardo Corrente and Dr. João Paulo de Castro Marcondes for the statistical analyses.

7. FUNDING

This study was supported by FAPESP (scholarship) and CNPq.

8. COMPETING INTERESTS

The authors declare that they have no competing interests.

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MANUSCRITO I

6. INHIBITION OF BLADDER CANCER CELL PROLIFERATION BY ALLYL ISOTHIOCYANATE (MUSTARD ESSENTIAL OIL) IS DEPENDENT ON THE *TP53* GENOTYPE

André Luiz Ventura Sávio[*1], Glenda Nicioli da Silva[2], Daisy Maria Fávero Salvadori[1]

[1] UNESP – Universidade Estadual Paulista, Faculdade de Medicina de Botucatu, Departamento de Patologia, Botucatu, SP, Brazil.

[2] UFOP – Universidade Federal de Ouro Preto, Escola de Farmácia, Departamento de Análises Clínicas, Ouro Preto, MG, Brazil.

* Corresponding author: André Luiz Ventura Sávio, Faculdade de Medicina de Botucatu – UNESP, Departamento de Patologia, Rubião Junior, 18618-970, Botucatu, SP, Brazil (e-mail: savio.alv@gmail.com). Phone: +55-14 38807553.

Keywords: Allyl isothiocyanate, bladder cancer, cell proliferation, gene expression, *TP53* gene

Manuscrito submetido à publicação na revista Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis.

1. ABSTRACT

Natural compounds hold great promise for combating antibiotic resistance, the failure to control some diseases, the emergence of new diseases and the toxicity of some contemporary medical products. Allyl isothiocyanate (AITC), which is abundant in cruciferous vegetables and mustard seeds and is commonly referred to as mustard essential oil, exhibits promising antineoplastic activity against bladder cancer, although its mechanism of action is not fully understood. Therefore, the aim of this study was to investigate the effects of AITC activity on bladder cancer cell lines carrying a wild type (*wt*; RT4) or mutated (T24) *TP53* gene. Morphological changes, cell cycle kinetics and *CDK1, SMAD4, BAX, BCL2, ANLN* and *S100P* gene expression were evaluated. In both cell lines, treatment with AITC inhibited cell proliferation (at 0.0625, 0.0725, 0.0825 and 0.0925 μM AITC) and induced morphological changes, including scattered and elongated cells and other aberrations. Gene expression profiles revealed increased *S100P* and *BAX* and decreased *BCL2* expression in RT4 cells following AITC treatment. T24 cells displayed increased *BCL2*, *BAX* and *ANLN* and decreased *S100P* expression. No changes in *SMAD4* and *CDK1* expression were observed in either cell line. In conclusion, AITC inhibits cell proliferation independent of *TP53* status. However, the mechanism of action of AITC differed in the two cell lines; in RT4 cells, it mainly acted via the classical *BAX/BCL2* pathway, while in T24 cells, AITC modulated the activities of *ANLN* (related to cytokinesis) and *S100P*. These data confirm the role of AITC as a potential antiproliferative compound that modulates gene expression according to the tumor cell *TP53* genotype.

2. INTRODUCTION

It has been estimated that up to 33% of cancer cases are preventable by changes in diet and associated factors [1]. Nutritional interventions may not only prevent disease in healthy populations but may also confer therapeutic effects in affected individuals [2, 3]. For example, *Ficus racemosa* bark, a medicinal herb, enhances the effects of hypoglycemic drugs, leading to decreased glucose levels in diabetic patients without side effects [4]. Anti-atherosclerotic effects of garlic-based drugs were observed in 196 asymptomatic men aged 40-74 [5]. Recently, Loganathan et al. [6] demonstrated that the *Ganoderma lucidum* mushroom inhibits breast-to-lung cancer metastasis in mice by downregulating pro-invasive genes such as *HRAS, VIL2, S100A4, MCAM, I2PP2A* and *FN1*. Furthermore, decreased *BCL2* and increased *BAX* gene expression have been previously reported to occur in association with increased apoptosis rates in human pharyngeal squamous carcinoma cells treated with capsaicin derived from hot peppers [7]. Substances derived from fruits and vegetables and essential oils have been extensively investigated, and some possess the ability to induce apoptosis in addition to being associated with significant antiproliferative activities [8, 9]. Furthermore, these natural compounds can potentially reduce or eliminate undesirable effects of typical therapies [10]. For example, an epidemiological study revealed an inverse relationship between the ingestion of crude broccoli and the risk of bladder cancer [11]. Because of its high bioavailability in urine, allyl isothiocyanate (AITC) is considered a promising agent for the treatment and prevention of bladder cancer [12, 13]. Cell cycle arrest at G2/M phase due to decreased activity of the CDK1/cyclin B complex has been observed in human glioma cells (GBM 8401) treated with AITC [14].

AITC is an aliphatic isothiocyanate that is derived from sinigrin and is abundant in cruciferous vegetables and mustard seeds [15]. It has been proposed that AITC would be selective for tumor cells because low levels of this compound have been detected in normal

epithelial cells [16]. However, its mechanism of action has not yet been fully elucidated. Kumar et al. [17] reported that AITC induces apoptosis in Ehrlich ascites tumors (EAT) by modulating the expression of the *BCL-2* and *BAX* genes. Recently, Wang et al. [18] demonstrated the therapeutic benefits of AITC in lung diseases via its promotion of increased expression of *MRP1*, a gene involved in protection against oxidative stress and xenobiotics.

Globally, bladder cancer is the seventh most common neoplasia in men and the seventeenth in women, and specifically, urothelial cell carcinoma (UCC) is responsible for approximately 90% of these malignant tumors [19, 20]. Urothelial cancer frequently involves mutations in the *TP53* gene [21], and high rates of recurrence and progression are observed [22]. Cyclophosphamide, arylamines and cigarette smoking are the main risk factors associated with the etiology of UCC [23]. The most used and successful chemotherapies for UCC include combinations of methotrexate, vinblastine, doxorubicin and cisplatin (MVAC) or a combination of gemcitabine and cisplatin [24, 25]. However, these protocols are associated with adverse effects, such as high systemic toxicity, a lack of selectivity and tumor resistance following prolonged treatment [26]. Furthermore, genes related to the sensitivity of tumors to chemotherapy may play critical roles in the selection of preferential treatments [27].

Therefore, the aim of this study was to investigate the effects of AITC on cellular responses and gene (*ANLN*, *S100P*, *SMAD4*, *BCL2*, *BAX* and *CDK1*) expression in UCC cell lines with a wild type (*wt*) or mutated *TP53* gene. Our results will contribute to the successful identification of alternative antineoplastic drug therapies for urothelial cancer in addition to the elucidation of their possible mechanisms of action.

3. MATERIALS AND METHODS

3.1 CELL LINES AND TEST COMPOUND

The human urothelial carcinoma cell lines RT4 (from a low grade tumor with a *wt TP53* gene) and T24 (from an invasive tumor with the *TP53* allele, encoding an in-frame deletion of tyrosine 126) were obtained from the Cell Bank of the Federal University of Rio de Janeiro, Brazil, and maintained as previously described by da Silva et al. [28]. AITC was purchased from Sigma-Aldrich (USA) and diluted into 2% Tween-20 prior to use. All AITC treatments were performed for three hours, as suggested by Zhang et al. [29].

3.2 CELL MORPHOLOGY

Initially, 8 x 10^4 cells were seeded into 12-well culture plates and incubated at 37°C with 5% CO_2. Twenty-four hours later, the cells were treated with 0.005, 0.0625, 0.0725 or 0.0825 µM AITC for 3 hours. Next, the cells were washed with Hank's solution (0.4 g KCl, 0.06 g KH_2PO_4, 0.04 g Na_2HPO_4, 0.35 g $NaHCO_3$, 1 g glucose and 8 g NaCl in 1 L H_2O), and fresh medium was added for an additional incubation period of 21 hours. Untreated cells and cells treated with 2% Tween-20 were cultured as controls. Morphological changes were evaluated by phase-contrast microscopy before and after AITC treatment. Cultures and treatments were performed in triplicate.

3.3 CELL CYCLE KINETICS AND CELL VIABILITY

Cells were seeded into 12-well plates at a density of 2 x 10^5 cells/well to evaluate the cell cycle kinetics. Twenty-four hours later, the cells were treated with AITC (0.005, 0.0625, 0.0725, 0.0825 or 0.0925 µM) for three hours. The cells were then washed with Hank´s solution, and fresh medium was added, followed by incubation at 37°C for 21, 45 or 69 h. The cells were detached with trypsin-EDTA and counted using an automated cell counter. Cell

viability was evaluated by the trypan blue exclusion test at 21 hours following treatment initiation (0.005, 0.0625, 0.0725 or 0.0825 µM AITC). Assays were performed in triplicate.

3.4 QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (RT-PCR) AND EVALUATION OF *ANLN, S100P, SMAD4, BCL2, BAX* AND *CDK1* GENE EXPRESSION

Total RNA was isolated using the RNeasy Mini Kit® (Qiagen) according to the manufacturer's protocol. RNA concentrations and purities were determined using a NanoVue spectrophotometer (GE Healthcare). Complementary DNA was synthesized using the High Capacity Kit (Applied Biosystems, USA) according to the manufacturer's instructions. *ANLN, S100P, BCL2, SMAD4, BAX* and *CDK1* gene expression levels were assayed using the TaqMan system (Applied Biosystems, Foster City, CA, USA). Each tube contained 2 µL of cDNA template, 5 µL of TaqMan 2X Master Mix (Applied Biosystems), 2.5 µL of water and 0.5 µL of 20× primers/probes (Assays-on-Demand gene expression products, Applied Biosystems). β-actin was used as a housekeeping gene. The reaction was performed using the following thermal cycling conditions: 94ºC for 10 min, followed by 40 cycles of 94ºC for 30 s and 60ºC for 1 min. Fluorescence data were collected during each annealing/extension step. The reactions were performed in triplicate at 21 hours after AITC treatment using the 7500 FAST Real-Time PCR System (Applied Biosystems) and SDS software, version 1.2.3 (Sequence Detection Systems 1.2.3, 7500 Real-Time PCR Systems, Applied Biosystems). The relative gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ method [30]. A gene interaction network was created using String 9.05 software (http://string-db.org/newstring_cgi/ show_input_page.pl) and MCL clustering algorithms.

3.5 STATISTICAL ANALYSES

Statistical analyses were performed using SigmaStat 3.5 and SAS software, version 9.2 (Statistical Analysis System, SAS Institute; Cary, NC, USA). Gene expression data, cell

viability and cell cycle kinetics were analyzed using a one-way ANOVA followed by Tukey's test. The results were considered statistically significant at $p < 0.05$.

4. RESULTS

4.1 CELL MORPHOLOGY

Microscopic evaluations indicated that the RT4 and T24 cells exhibited morphological alterations (elongated cells) following AITC treatment (Figures 1 and 2). Fewer scattered cells were observed in the AITC-treated cultures than in the controls.

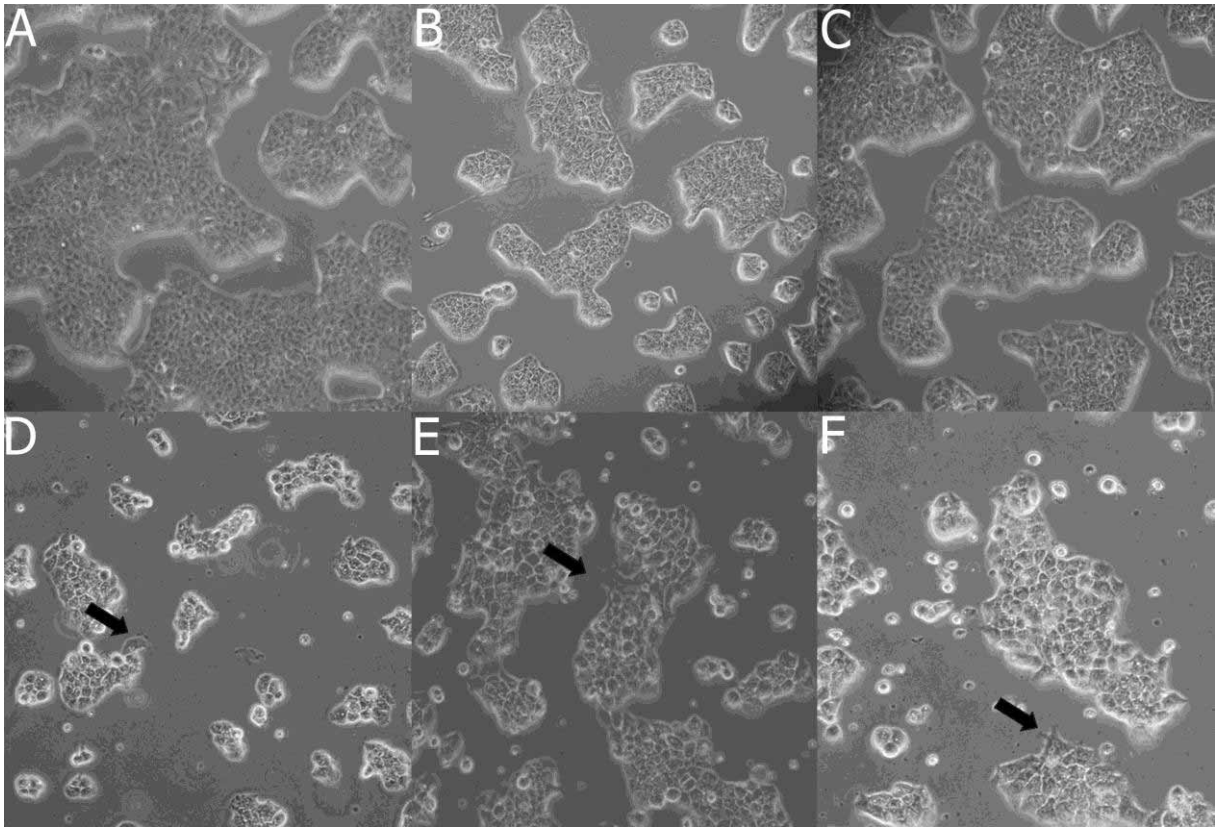

Figure 1: Photomicrograph at 1000× magnification of RT4 (wild type *TP53*) cells before and after treatment with allyl isothiocyanate (AITC). A - Negative control; B - 2% Tween-20 (AITC vehicle control); C, D, E and F - 0.005, 0.0625, 0.0725 and 0.0825 µM AITC, respectively. Arrows indicate morphological alterations after AITC treatment.

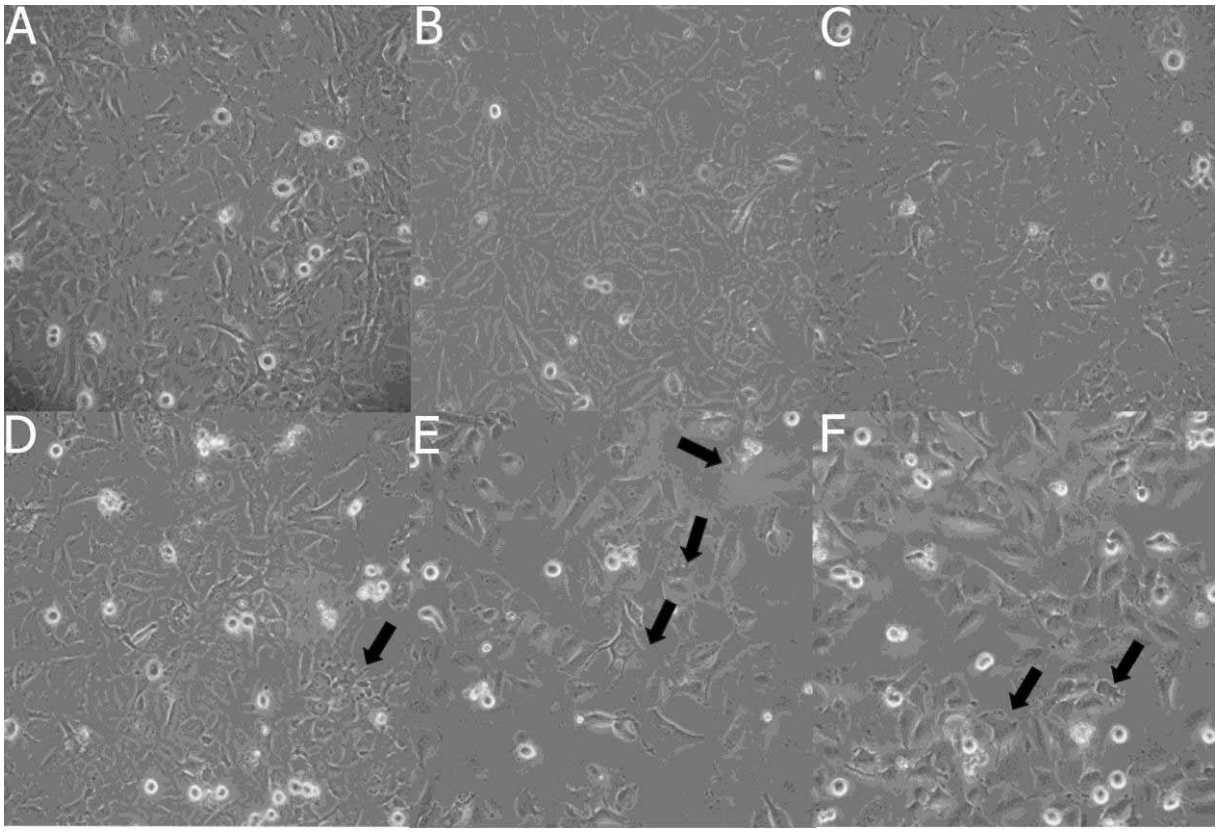

Figure 2: Photomicrograph at 1000× magnification of T24 (mutant *TP53*) cells before and after treatment with allyl isothiocyanate (AITC). A - Negative control; B - 2% Tween-20 (AITC vehicle control); C, D, E and F - 0.005, 0.0625, 0.0725 and 0.0825 µM AITC, respectively. Arrows indicate morphological alterations after AITC treatment.

4.2 CELL CYCLE KINETICS AND CELL VIABILITIES

In agreement with the microscopic findings, the cell numbers (RT4 and T24) of the AITC-treated cultures were generally lower than those of the controls (Figures 3 and 4). Inhibition of RT4 proliferation was observed at 45 and 69 hours following AITC treatment at concentrations of 0.0625, 0.0725, 0.0825 and 0.0925 µM. No changes in T24 cells were observed at 21 hours, but decreased cell numbers were detected at 45 (0.0625, 0.0725, 0.0825 and 0.0925 µM AITC) and 69 hours (0.005, 0.0625, 0.0725, 0.0825 and 0.0925 µM AITC). No differences in RT4 and T24 cell viability were observed at 21 hours following AITC treatment in the trypan blue assay (Table I).

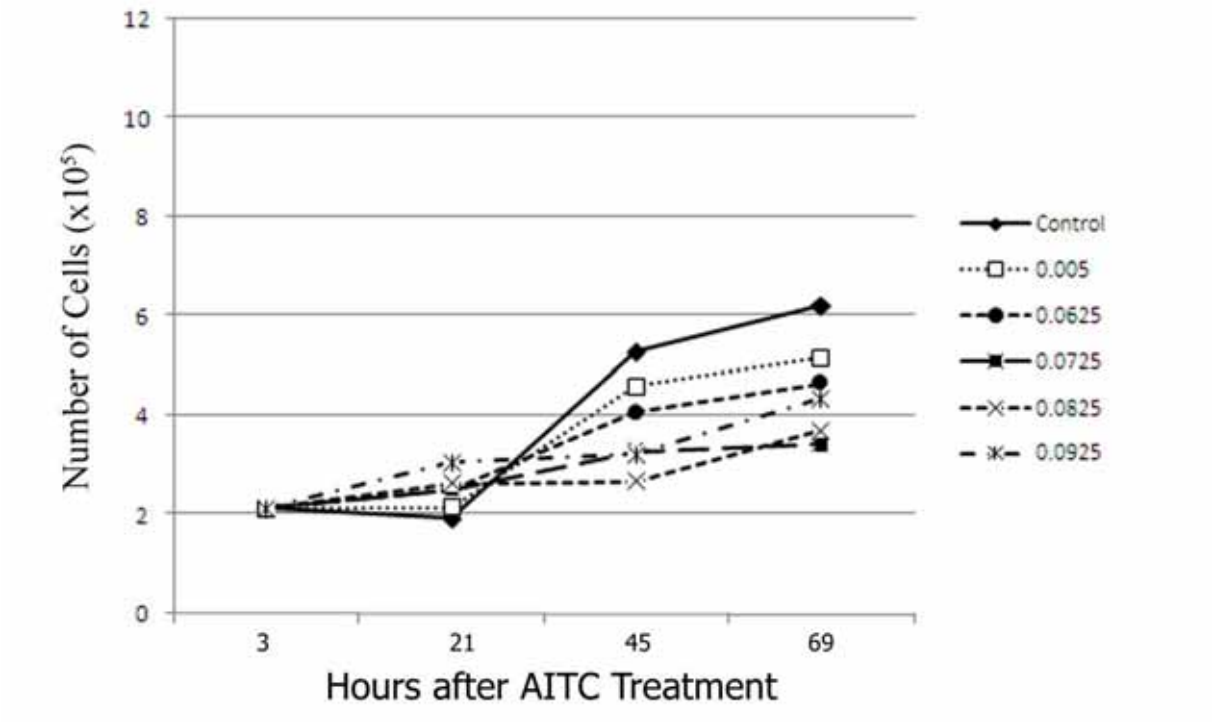

Figure 3: Cell cycle kinetics of RT4 cells after treatment with allyl isothiocyanate (AITC). Each point represents the mean value obtained from three independent experiments.

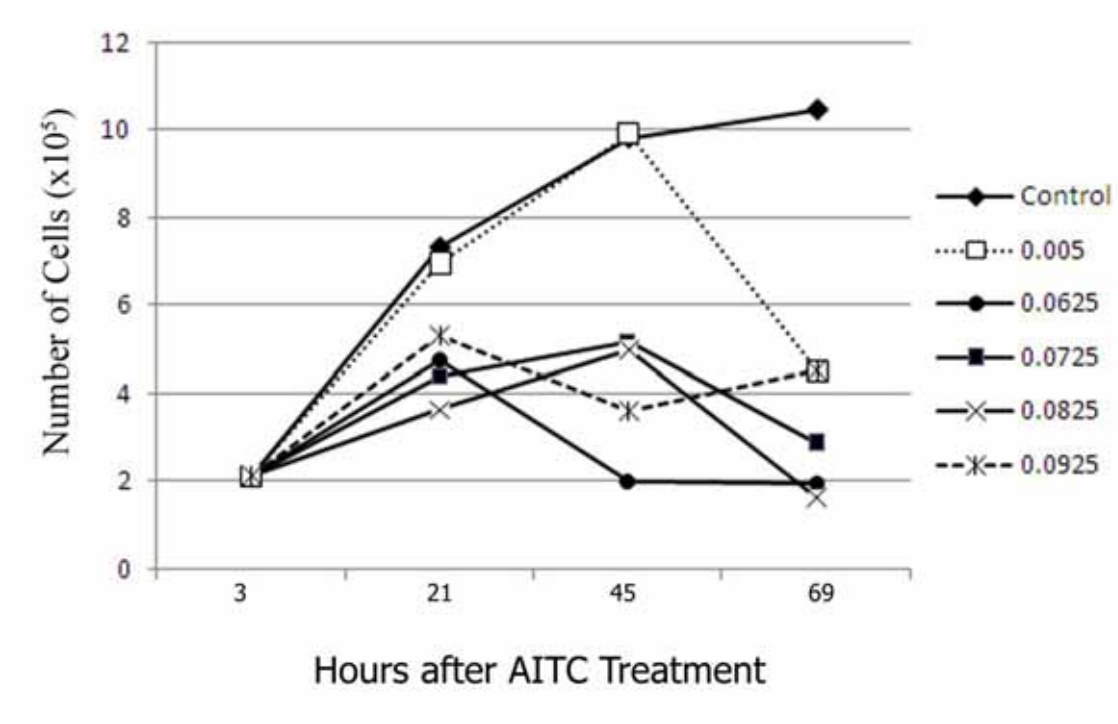

Figure 4: Cell cycle kinetics of T24 cells after treatment with allyl isothiocyanate (AITC). Each point represents the mean value obtained from three independent experiments.

Table I: Percentage of viable cells (RT4 and T24 cell lines) at 21 hours after treatment with allyl isothiocyanate (AITC)

Cell line	Negative control[1]	Control Tween[2]	AITC (μM)			
			0.005	0.0625	0.0725	0.0825
RT4	89.6 ± 5.8	88.4 ± 6.0	87.9 ± 20.2	87.8 ± 4.3	78.3 ± 2.2	77.7 ± 1.6
T24	75.2 ± 5.3	87.3 ± 12.2	89.8 ± 7.0	87.1 ± 3.4	90.4 ± 5.6	89.3 ± 9.0

[1]No treatment; [2]cells treated with 2% Tween-20 (vehicle control); data are presented as the median ± SD

4.3 ANLN, S100P, SMAD4, BCL2, BAX AND *CDK1* GENE EXPRESSION

ANLN, BCL2 and *BAX* were upregulated and *S100P* was downregulated in T24 cells after treatment with AITC at concentrations of 0.0625, 0.0725 and 0.0825 μM. By contrast, in RT4 cells, *BAX* (0.0625, 0.0725 and 0.0825 μM AITC) and *S100P* (0.0825 μM AITC) were upregulated, and *BCL2* was downregulated following AITC exposure (0.0625, 0.0725 and 0.0825 μM) (Figures 5 and 6). Figure 7 depicts the interaction network involving the investigated genes, which were clustered in the apoptosis pathway (*SMAD*, *BAX*, *BCL2* and *TP53*) and the cell cycle pathway (*CDK1* and *ANLN*).

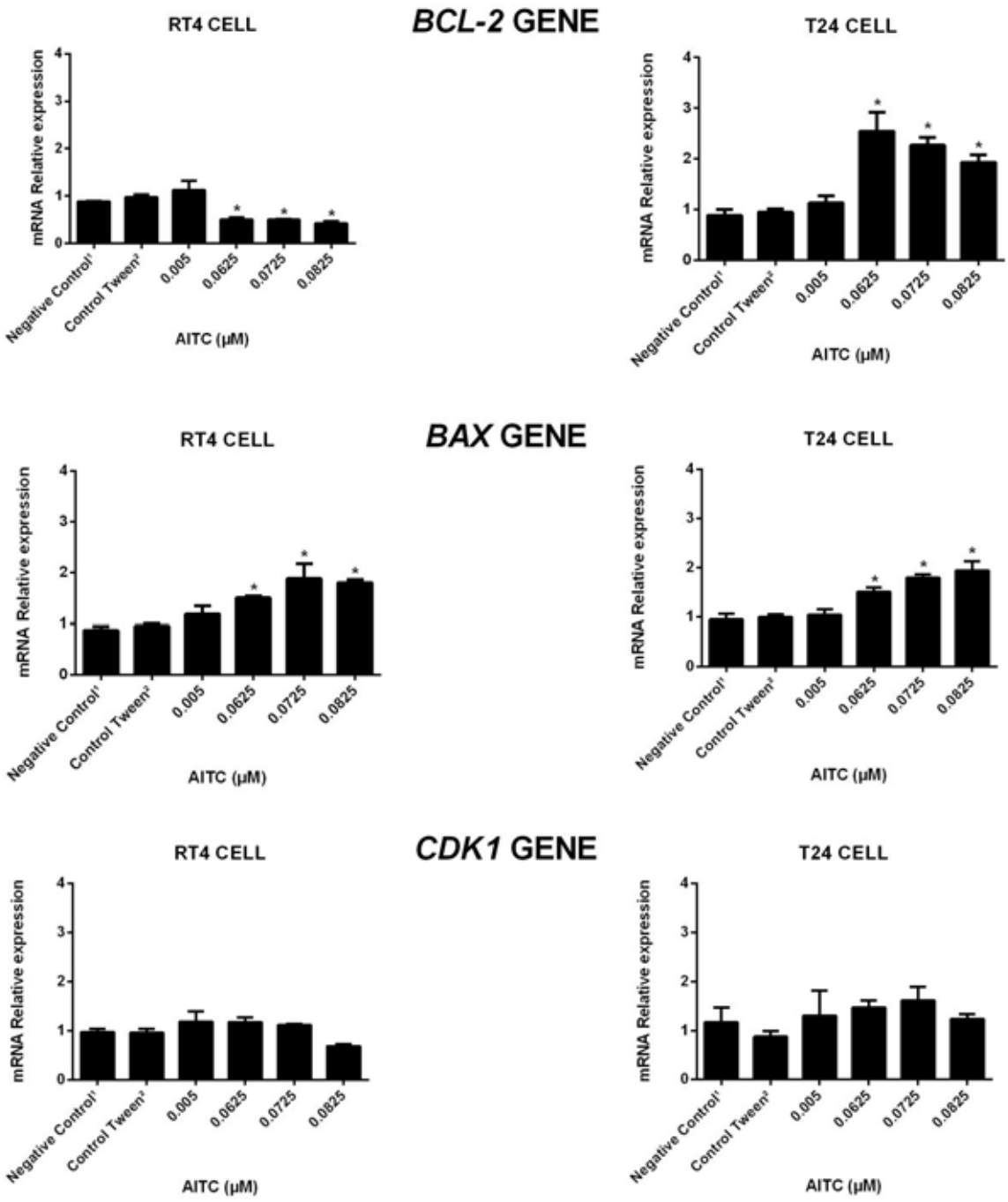

Figure 5: Relative mRNA levels of *BAX*, *BCL2* and *CDK1* in RT4 and T24 cells after treatment with allyl isothiocyanate (AITC). Negative control - no treatment; Control Tween - treated with 2% Tween-20 (AITC vehicle control); β-actin (endogenous RNA control). * $p < 0.05$ (compared to Tween control). Cell cultures were performed in triplicate.

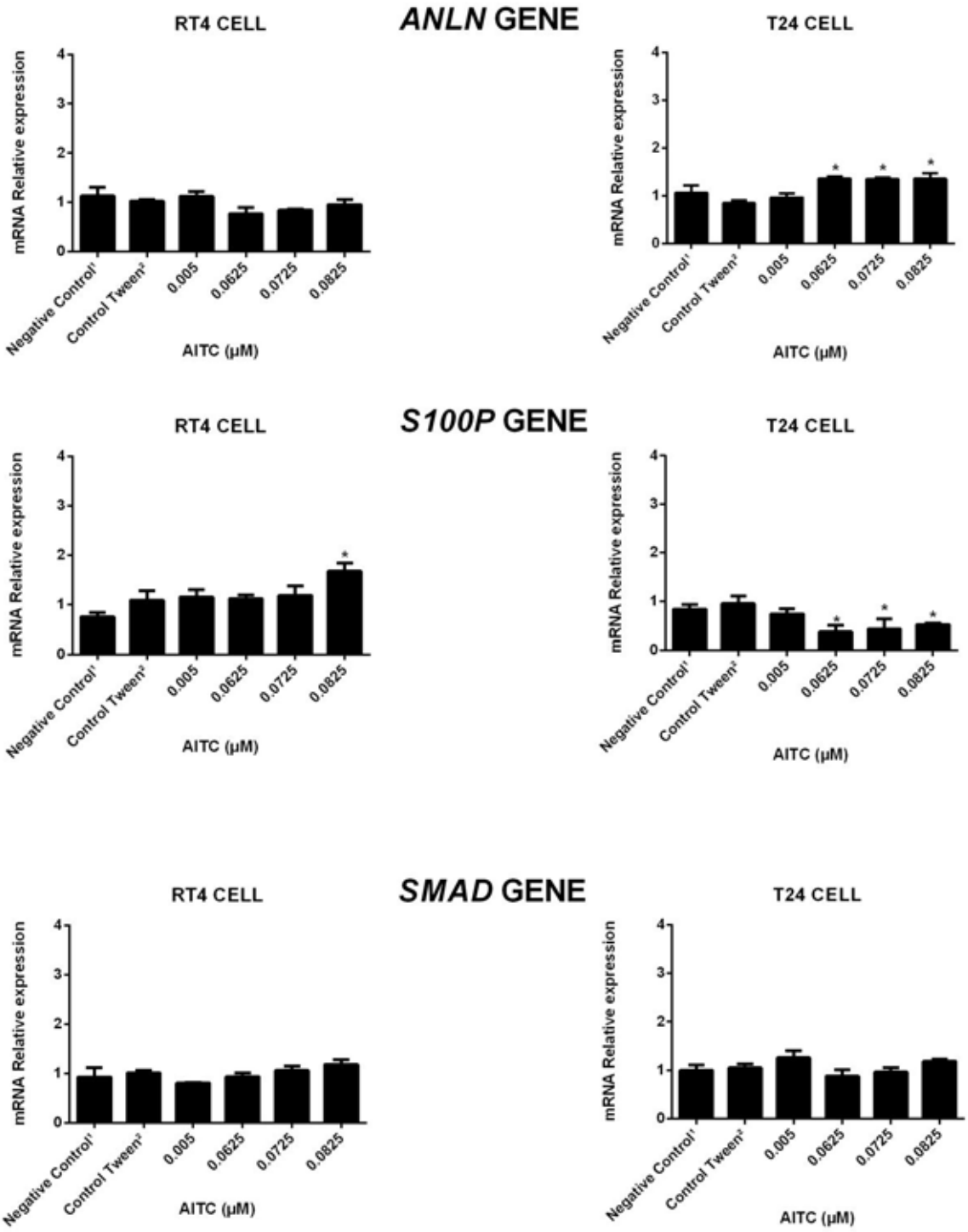

Figure 6: Relative mRNA levels of *ANLN, S100P,* and *SMAD4* in RT4 and T24 cells after treatment with allyl isothiocyanate (AITC). Negative control - no treatment; Control Tween - treated with 2% Tween-20 (AITC vehicle control); β-actin (endogenous RNA control). * $p < 0.05$ (compared to respective Tween controls). Cell cultures were performed in triplicate.

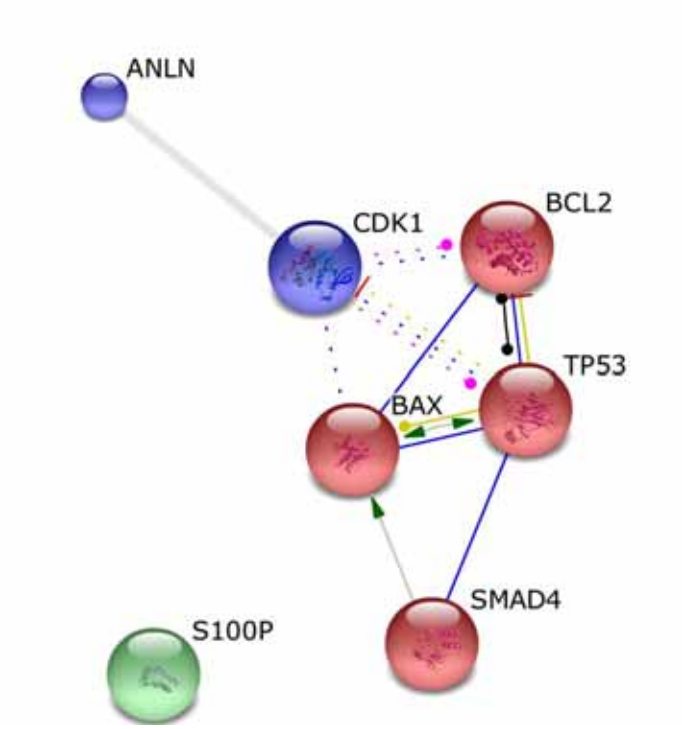

Figure 7: Gene interaction network created using String 9.05 software and the MCL clustering algorithm. Red balls indicate genes clustering exclusively with apoptotic processes; blue balls depict genes clustering with cell cycle processes. The green ball indicates genes related to a variety of cell processes. Blue lines represent interaction, yellow lines indicate gene inhibition, and dark green arrows depict gene activation.

5. DISCUSSION

Despite improvements in chemotherapeutic protocols, patient outcomes for bladder cancer have improved little in recent decades, resulting in the increased interest in natural medicines to improve the quality of life in afflicted individuals [9, 10]. Medicinal plants may be more compatible with the human body and have lower rates of side effects than typical chemotherapeutic drugs [31]. Furthermore, some plant-derived compounds not only possess antiproliferative activities but also might preferentially destroy malignant cells with low levels of toxicity to non-neoplastic cells. For example, Bhattacharya et al. [32] demonstrated that the delivery of AITC through urinary excretion to bladder tissues in a rat orthotopic model

inhibited cancer development and muscle invasion. Other *in vitro* and *in vivo* studies have also demonstrated the anticarcinogenic potential of some isothiocyanates (ITC) [17, 33, 34].

In this study, we investigated the activity of AITC in urothelial carcinoma cell lines carrying a wild type (RT4) or mutated (T24) *TP53* gene. These cell lines were chosen because *TP53* is the most frequently mutated gene in invasive bladder tumors [21] and because the relationship between the response to anticancer drugs and *TP53* status has not yet been fully elucidated. Initially, we detected morphological alterations with no changes in cell viability in both RT4 and T24 cells at 24 hours after AITC treatment. The induction of morphological alterations by AITC has also been observed in glioma cells (GBM-8401) [14]. Gonçalves et al. [35] also reported morphological changes in Vero cells following treatment with cisplatin and suggested that irregular cell morphologies might indicate compromised polymerization of the actin cytoskeleton and/or activities of actin-binding proteins. However, Janson et al. [36] suggested that early-phase morphological changes were not representative of the cytotoxic and apoptosis-inducing effects of a compound. Nevertheless, in addition to morphological alterations, our data indicated scattered and decreased numbers of RT4 and T24 cells following AITC treatment, suggesting that AITC possesses antiproliferative effects that are independent of *TP53* status. These findings are in agreement with our previous observation of increased apoptosis rates and G2 phase cell cycle arrest in AITC-treated RT4 and T24 cells, respectively [37].

To better understand the mechanisms of action of AITC, we also evaluated its effects on the gene expression profile. Overexpression of *BAX* (a proapoptotic gene) and decreased expression of *BCL2* (an anti-apoptotic gene) were observed in RT4 cells. Similarly, Kumar et al. [17] reported growth inhibition via proapoptotic mechanisms in EAT cell cultures following treatment with AITC. The balance between *BCL-2* and *BAX* expression is typically regulated

by the *TP53* tumor suppressor gene [38, 39]. Our results for RT4 (wt *TP53*) cells support these findings. However, we detected not only *BAX* but also *BCL2* overexpression in T24 cells, although inhibited cell proliferation was also observed. Teijido et al. [40] reported that upregulation of Bcl2 stabilizes the loose binding of Bax to mitochondrial membranes, inhibiting cytochrome c release and, consequently, inhibiting apoptosis. Thus, we suggest that early apoptosis is the main mechanism underlying the inhibition of cell proliferation in T24 cells. These findings corroborate with and expand upon our previous observation of increased apoptosis in RT4 cells and G2/M cycle arrest in T24 cells [37].

We observed the upregulation of *S100P* in RT4 cells and downregulation in T24 cells following AITC treatment (at the highest concentration of 0.0825 μM). *S100P*, a signal-dependent Ca^{2+} mediator gene, plays important roles in several intra- and extra-cellular processes, including the regulation of protein phosphorylation, enzyme activity, apoptosis, gene transcription and cell proliferation and differentiation [41, 42]. Because the decreased numbers of mutated *TP53* cells that were observed by microscopy following AITC treatment cannot be explained by the classical *BAX/BCL-2* mechanism, we suggest that the downregulation of *S100P* may play a role. Notably, significant inhibition of cell growth has also been detected in *S100P*-knockdown colon cancer cells [43]. According to Darkee et al. [44], the upregulation of *S100P* is likely associated with drug resistance in cell lines. Further, Arumugam et al. [45] reported that *S100P* modulation may be related not only to drug resistance but also to metastasis and poor clinical outcome.

We chose to study *SMAD4* expression because this gene is also related to cell proliferation but acts via different mechanisms than the genes described above. *SMAD4* is a tumor suppressor gene that encodes a protein that plays important roles in the transduction of signals from transforming growth factor-β (TGF-β). TGF-β signaling pathways are involved

in the regulation of numerous cellular processes, including growth, development, division, death, oncogenesis and tumor progression [46]. Therefore, the antitumor effects of *SMAD4* depend on its potential to mediate the growth inhibition induced by TGF-β. Reports in the literature have indicated that *SMAD4* modulation is associated with tumor cell proliferation and contributes to invasiveness and metastatic phenotypes in a rat prostate cancer model [47, 48]. However, we did not detect any changes in *SMAD4* expression in either cell line following treatment with AITC, suggesting that this gene is not directly related to the main mechanisms by which AITC inhibits cell proliferation.

Because we previously observed G2/M cell cycle arrest in T24 cells after AITC treatment [37], we also chose to investigate the expression of *ANLN*. The main function of this gene is to organize the cytokinetic machinery, and it is essential for the structural integrity of the cleavage furrow and completion of cleavage furrow ingression [49]. Therefore, the actin-binding protein anillin, which is encoded by the *ANLN* gene, plays a special role in cell division [50]. We observed upregulation of *ANLN* in T24 cells (but not in wt RT4 cells), suggesting AITC might indirectly affect cell division. Ronkainen et al. [51] recently reported that cytoplasmic anillin expression might be a marker of favorable prognosis in renal cell carcinoma patients. The disruption of mitotic microtubules in BALB cells following treatment with sulforanes, another type of isothiocyanate compound, has also been described [52], indicating an additional manner in which this class of compounds may affect the cell cycle (via the blocking of cell division prior to the initiation of metaphase).

CDK1, the other cell cycle gene that was investigated, encodes the catalytic subunit of the highly conserved protein kinase complex known as M-phase promoting factor (MPF). This protein is essential for the G1/S and G2/M phase transitions of the eukaryotic cell cycle. Chen et al. [14] described an association between decreased CDK-1 protein levels and G2/M

phase arrest in GBM-8401 cells (human brain malignant glioma cells) after treatment with AITC. In contrast, we did not detect any changes in *CDK1* expression in either RT4 or T24 cells, suggesting that MPF is not involved in the inhibition of cell proliferation by AITC in bladder cancer cells.

In conclusion, the inhibition of cell proliferation by AITC is independent of *TP53* status. However, the molecular targets of AITC appear to differ. For example, while AITC acts via *BAX/BCL2* in RT4 cells, suggesting cell death by apoptosis, altered expression of *ANLN* and *S100P* were detected in T24 cells, suggesting the inhibition of proliferation due to cell cycle arrest.

6. AUTHOR CONTRIBUTIONS

All authors reviewed the manuscript. ALVS performed the experiments, interpreted the data and wrote the preliminary version of the manuscript. GNS contributed to the experimental design, data interpretation and the writing of the manuscript. DMFS contributed to the experimental design, data interpretation and the writing of the manuscript.

7. ACKNOWLEDGMENTS

The authors are grateful to Dr. José Eduardo Corrente for the statistical analyses.

8. FUNDING

This study was supported by FAPESP- Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil; CNPq - Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil; and CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil.

8. COMPETING INTERESTS

The authors declare that they have no competing interests.

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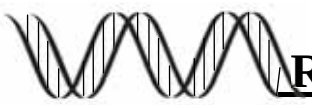
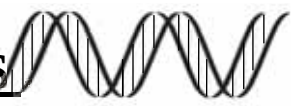


RESUMO DOS RESULTADOS



7. RESUMO DOS RESULTADOS

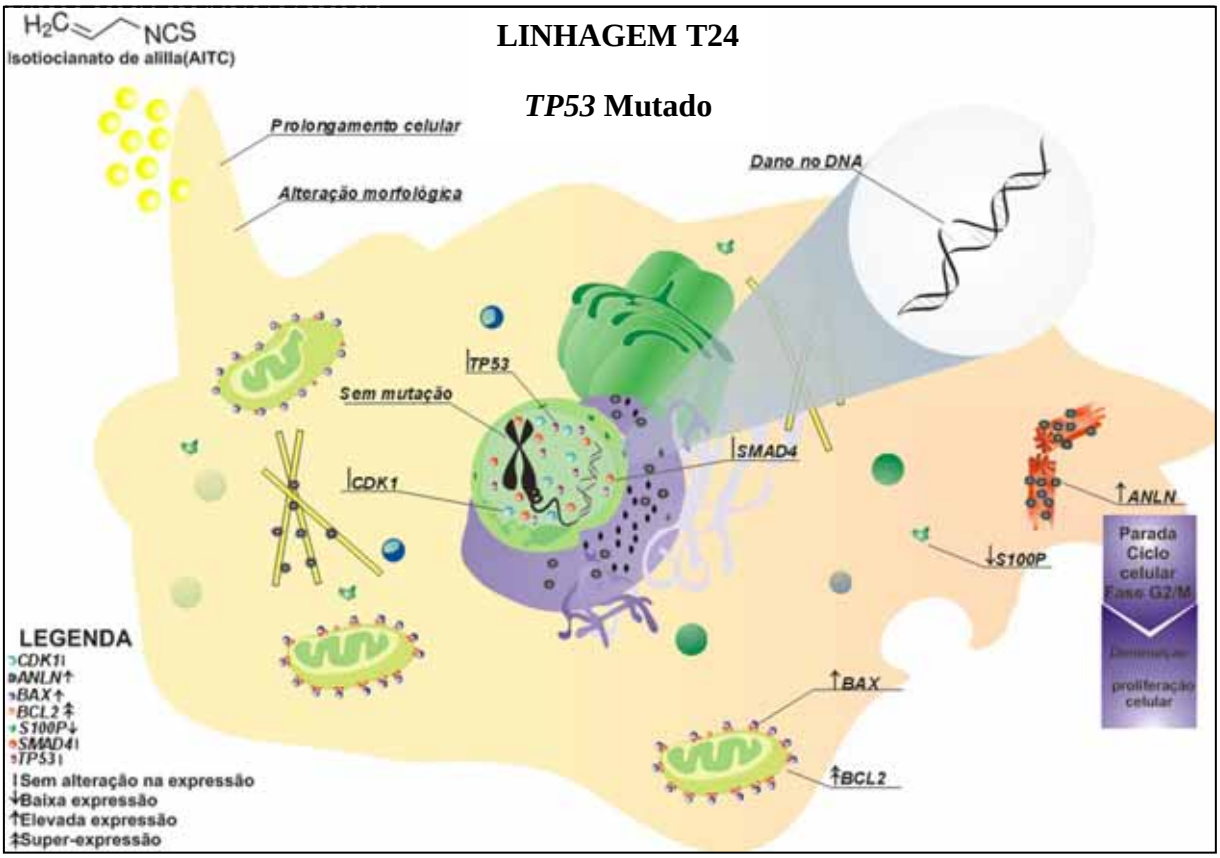


Figura 3: Desenho esquemático demonstrando as alterações observadas em células da linhagem T24 de carcinoma urotelial após tratamento com o isotiocianato de alila (AITC): danos primários no DNA, alteração na expressão dos genes *ANLN*, *BAX*, *BCL2* e *S100P* e os respectivos locais de ação das proteínas por eles codificadas. Foi detectada diminuição da proliferação celular com parada do ciclo na fase G2/M.

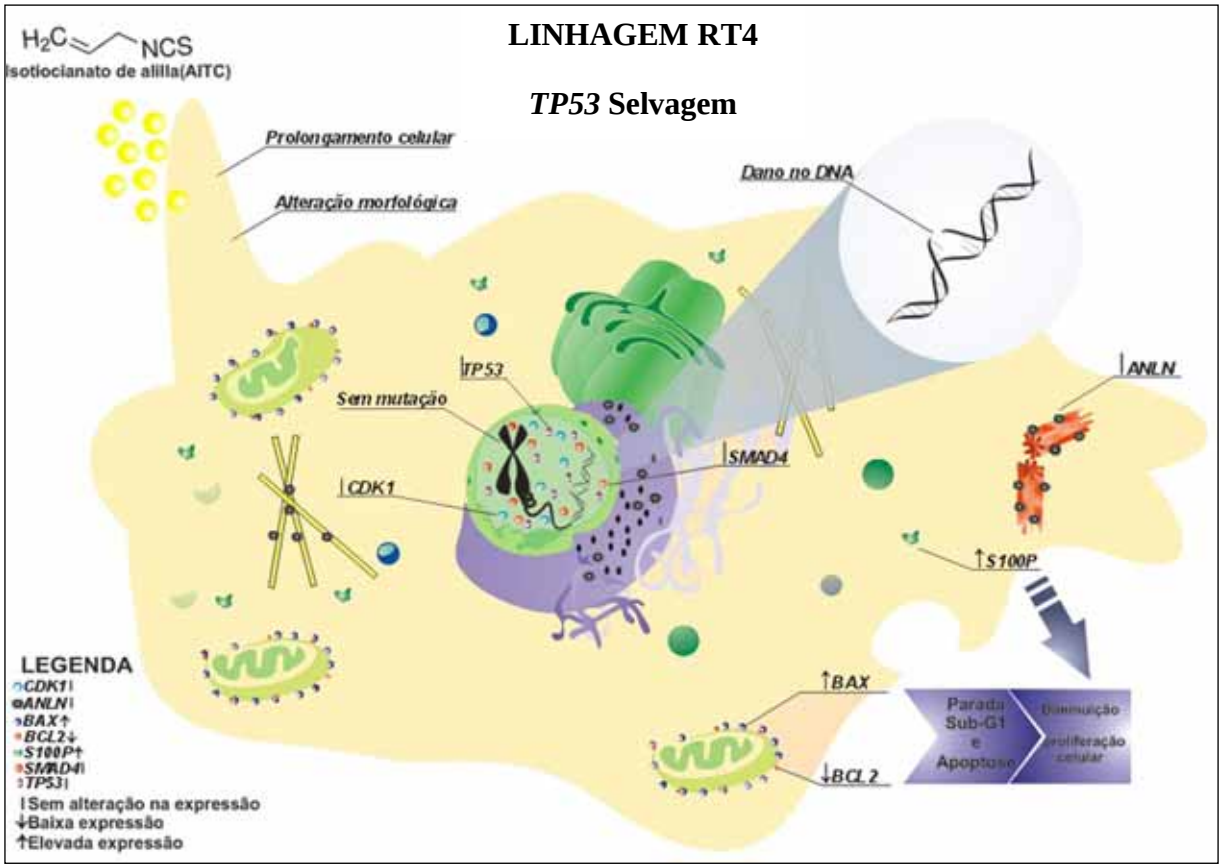


Figura 4: Desenho esquemático demonstrando as alterações observadas em células da linhagem RT4 de carcinoma urotelial após tratamento com o isotiocianato de alila (AITC): danos primários no DNA, alteração na expressão dos genes *BAX*, *S100P* e os respectivos locais de ação das proteínas por eles codificadas. Foi detectada parada do ciclo na fase G2/M com diminuição da proliferação celular e apoptose.

CONSIDERAÇÕES FINAIS

8. CONSIDERAÇÕES FINAIS

O câncer de bexiga caracteriza-se por diversas alterações genéticas: os tumores papilares de baixo grau estão especialmente associados a alterações nos genes *FGFR3* e *RHAS* (Jebar et al., 2005; Wu, 2005; Cheng et al., 2011); o carcinoma *in situ,* a mutações nos genes *PTEN e RB1* (Bakkar et al., 2003); e os tumores avançados a mutações no gene *TP53* (Nishiyama et al., 2008). Particularmente em relação ao gene *TP53,* este está envolvido no controle do ciclo celular na fase G_1/S (*checkpoint* celular) e tem papel fundamental na ativação das vias apoptóticas (Wiman, 2007; Sengupta, 2005). Por outro lado, estudos epidemiológicos têm demonstrado que vegetais da família dos crucíferos apresentam potencial antineoplásico para diversos tipos de câncer, como os de pulmão, mama, pâncreas e bexiga, muito embora seus mecanismos de ação não sejam completamente conhecidos. Portanto, este estudo objetivou avaliar a ação do isotiocianato de alila (AITC) em duas linhagens de células de carcinoma de bexiga, com diferentes *status* do gene *TP53:* a linhagem T24, com *TP53* mutado, e a linhagem RT4, com *TP53* selvagem.

Inicialmente, nossos resultados mostraram que, nas maiores concentrações (0,0625, 0,0725, 0,0825, 0,0925 µM) o AITC reduziu a taxa de proliferação celular 72 horas após o tratamento. Entretanto, o ensaio de sobrevivência clonogênica mostrou que o composto possui, também, a capacidade de inibir a formação de clones celulares até 15 dias após o tratamento, independente do *status* do gene *TP53*. Anteriormente, dados da literatura mostraram o efeito citotóxico do AITC em linhagens celulares de carcinoma de bexiga (UM-UC-3 e UM-UC-6; Geng et al., 2011), de glioma humano (GB 8401; Chen et al., 2010) e de câncer de pulmão (H596; Wang et al., 2011). No entanto, nenhum desses estudos avaliou a relação entre mutações no gene *TP53* e a maior ou menor capacidade de resposta ao composto.

Na tentativa de esclarecer os mecanismos de "bloqueio" da proliferação celular pelo AITC, realizamos a análise de morte celular por citometria de fluxo. Os resultados mostraram o aumento de apoptose precoce e necrose apenas na linhagem *TP53* selvagem. Com base nessa informação, avaliamos a expressão de outros genes também relacionados ao mecanismo de apoptose. Na linhagem *TP53* selvagem, o AITC induziu aumento da expressão do gene pró-apoptótico *BAX* e diminuição do anti-apoptótico *BCL-2*. Em conjunto, esses dados indicam que a linhagem *TP53* selvagem, tratada com o AITC, apresenta bloqueio da proliferação celular mediado pelo mecanismo clássico *BAX/BCL-2*. Portanto, nossos resultados corroboram dados da literatura que demonstraram que o AITC induz a apoptose de células de tumor ascítico de Ehrlich (EAT) por meio da modulação da expressão dos genes *BAX* e *BCL2* (Kumar et al, 2009). No caso da linhagem *TP53* mutada, embora tivesse ocorrido aumento da expressão do gene *BAX*, observamos a hiper-expressão de *BCL-2*, sugerindo um desequilíbrio na relação entre *BAX/BLC-2*, o qual teria reduzido a ocorrência de apoptose nessas células. Recentemente, Teijido et al. (2010) relataram que o aumento da expressão de *BCL-2* está associado ao bloqueio da ligação de *BAX* na membrana mitocondrial, evitando a liberação do citocromo C e, consequentemente, inibindo a apoptose.

Outros dois genes relacionados a apoptose foram também avaliados no presente estudo: o *S100P* e o *SMAD4*. Para o *S100P*, observamos aumento de expressão em células *TP53* selvagem e diminuição nas *TP53* mutadas. Novamente, nossos confirmam a maior taxa de apoptose detectada na linhagem *TP53* selvagem. De fato, o aumento da expressão do gene *S100P* tem sido associado à diminuição da taxa de sobrevivência celular (Zhao et al., 2013). Com relação ao *SMAD4*, gene supressor tumoral com papel importante na transdução de sinais a partir do fator de crescimento transformador-β (TGF - β), não observamos alterações na expressão após a exposição de ambas as linhagens celulares ao AITC.

Quanto aos efeitos do AITC sobre o ciclo celular, nossos dados mostraram que após o tratamento houve aumento de células na fase sub-G1 e na fase G2/M, respectivamente, para as linhagens *TP53* selvagem e *TP53*-mutada. Com o objetivo de entender o(s) mecanismo(s) relacionado(s) a estes achados, avaliamos a ação do AITC sobre a expressão dos genes *ANLN* e *CDK1*. O aumento da expressão de *ANLN* foi observado apenas nas células *TP53* mutadas, enquanto a expressão de *CDK1* permaneceu inalterada em ambas as linhagens. Estes resultados sugerem que o gene *ANLN*, responsável pela organização da maquinaria citocinética, pode estar envolvido na parada G2/M observada nas células *TP53* mutadas. Por outro lado, o gene *CDK1* não foi modulado pelo AITC, não sendo, portanto, o alvo de ação desse composto, independente do status de *TP53*.

Outros resultados importantes deste estudo foram aqueles relacionados ao potencial genotóxico e mutagênico do AITC. As células com o gene *TP53* mutado foram mais sensíveis aos tratamentos, uma vez que concentrações menores do composto foram capazes de induzir maior quantidade de lesões primárias no DNA. A menor sensibilidade das células *TP53* selvagem poderia ser explicada pela interação entre a proteína p53 e mecanismos de reparo do DNA, levando a maior sobrevivência celular. Cabe destacar que, embora o AITC tivesse sido capaz de induzir lesões moleculares em ambas as linhagens, essas não se refletiram em alterações cromossômicas como as detectadas pelo teste do micronúcleo, ou seja, eventos clastogênicos ou aneugênicos. No entanto, não se pode descartar a possibilidade da existência de mutações gênicas que não detectadas pelo teste do micronúcleo. No final da última década, Lamy et al (2009), de forma similar, observaram que os compostos 3–metiltiopropilo isotiocianato (MTPITC), 5– metiltiobutilo isotiocianato (MTBITC) e 4 – metiltiopentilo isotiocianato (MTPeITC) não apresentaram efeito mutagênico em células de hepatoma humano (HepG2).

CONCLUSÕES

9. CONCLUSÕES

Os resultados do presente estudo, sobre os efeitos toxicogenéticos e toxicogenômicos do isotiocianato de alila (AITC) em duas linhagens de carcinoma de células uroteliais (RT4, de grau 1 e com o gene *TP53* selvagem e T24, de grau 3, e com o gene *TP53* mutado), permitiram as seguintes conclusões:

1- o tratamento com AITC induz baixas taxas de sobrevivência celular, independente do *status* do gene *TP53*;

2- o AITC induz alteração no ciclo celular de forma dependente do *status* do gene *TP53*. Para a linhagem *TP53* selvagem (RT4) ocorre parada do ciclo celular na fase sub-G1 e apoptose, enquanto na linhagem *TP53* mutado (T24) ocorre aumento da parada na fase G2/M;

3- o AITC induz danos primários no DNA de ambas as linhagens, sendo mais sensíveis aquelas com mutação no gene *TP53*, porém tais lesões não se refletiram em aumento da frequência de micronúcleos;

4- o AITC induz alterações na expressão de genes relacionados a apoptose e a proliferação celular na linhagem *TP53* selvagem, enquanto na linhagem *TP53* mutado, induz aumento na expressão do gene *ANLN* e diminuição na expressão do *S100P*, genes relacionados a proliferação celular.

Finalizando, os resultados fornecem informações que poderão contribuir para a elucidação do mecanismo de ação do AITC como composto promissor para o tratamento do câncer de bexiga.

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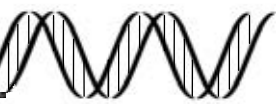
LISTA DE ABREVIAÇÕES



SIGLA	SIGNIFICADO
6-MTITC	6-(METHYLTHIO)HEXYL ISOTHIOCYANATE (6-METILTIOHEXIL ISOTIOCINATO)
A549	LUNG CANCER CELL (CÉLULAS DE CANCER DE PULMÃO
AITC	ALLYL ISOTHIOCYANATE (ISOTIOCIANATO DE ALILA)
AY-27	MOUSE BLADDER CELL CARCINOMA (CELULAS DE CARCINOMAS DE BEXIGA DE RATO)
BC	*BLADDER CANCER (CÂNCER DE BEXIGA)*
BCG	*BACILLUS CALMETTE GUÉRIN* (BACILO DE CALMETTE-GUÉRIN)
BEITC	BENZYL ISOTHIOCYANATE (BENZIL ISOTIOCIANATO)
CDK1	CYCLIN-DEPENDENT KINASE 1 (QUINASE DEPENDENDE DE CICLINA)
$_{C}$DNA	COMPLEMENTARY DNA (DNA COMPLEMENTAR)
CIS	*IN SITU* CARCINOMAS (CARCINOMA *IN SITU*)
COX-2	CYCLOOXYGENASE (CICLO-OXIGENASE-2)
DMSO	*DIMETHYLSULFOXID (*DIMETILSUFÓXIDO)
DNA	ACIDO DESOXIRRIBONUCLÉICO
EAT	*EHRLICH ASCITES TUMOR (TUMOR ASCÍTICO DE EHRLICH)*
FGFR3	*FIBROBLAST GROWTH FACTOR RECEPTOR 3 (RECEPTOR DO FATOR DE CRESCIMENTO DE FIBROBLASTOS 3)*
GBM 8401	*HUMAN BRAIN MALIGNANT GLIOMA CELLS (CÉLULAS MALÍGNAS DE GLIOMA HUMANO)*
HRAS	*HARVEY RAT SARCOMA VIRAL ONCOGENE HOMOLOG(HARVEY SARCOMA RATO VIRAL HOMÓLOGO ONCOGENE)*
H596	*CELL LUNG CARCINOMA (CÉLULAS DE CARCINOMA PULMONAR)*
HDAC	*HISTONE DEACETYLASES (HISTONA DEACETILASES)*
HELA	*CERVICAL CANCER (LINHAGEM DE CÂNCER DO COLO UTERINO)*
HEPG2	*HUMAN LIVER HEPATOCELLULAR CARCINOMA CELL LINE (CÉLULA DE CARCINOMA HEPATOCELULAR DE FÍGADO HUMANO)*
HL-60	HUMAN PROMYELOCYTIC LEUKEMIA CELLS (CÉLULAS DE LEUCEMIA PROMIELOCÍTICA HUMANA)
HT29	CELL COLON CARCINOMA (CÉLULAS DE CARCINOMA DE COLON)
ITC	ISOTHIOCYANATE(ISOTIOCIANATOS)
MCF-7	HUMAN BREAST ADENOCARCINOMA CELL LINE (CÉLULAS HUMANAS DE ADENOCARCINOMA DE MAMA)
MDA-MB-231	HUMAN MAMMARY GLAND ADENOCARCINOMA CELL LINE (CÉLULAS HUMANDAS DE DE ADENOCARCINOMA DA GLANDULA MAMARIA)

MRNA	MESSENGER RNA (RNA MENSAGEIRO)
MTBITC	4-METHYLTHIOBUTYL ISOTHIOCYANATE (4-METILTIOBUTILO ISOTIOCIANATO)
MTPEITC	5 METHYLTHIOPENTYL ISOTHIOCYANATE (5 – METILTIOPENTILO ISOTIOCIANATO)
MTPITC	3-METHYLTHIOPROPYL ISOTHIOCYANATE (3 – METILTIOPROPILO ISOTIOCIANATO)
MVAC	METHOTREXATE, DOXORUBICIN, VINBLASTINE AND CISPLATIN (METROTREXATO, VINBLASTINA, DOXORRUBICINA E CISPLATINA)
NQO1	QUINONE OXIDOREDUCTASE (QUINONA OXIDOREDUTASE 1)
NRF2	NUCLEAR FACTOR ERYTHROID -RELATED FACTOR 2
PBS	PHOSPHATE BUFFERED – SALINE (TAMPÃO FOSFATO SALINO)
PTEN	PHOSPHATASE AND TENSIN HOMOLOG (FOSFATASE E HOMÓLOGO ANGIOTENSINA)
PCR	POLYMERASE CHAIN REACTION (REAÇÃO EM CADEIA DA POLIMERASE)
PEITC	PHENETHYL ISOTHIOCYANATE (FENIL ISOTIOCIANATO)
PHITC	PHENYLHEXYL ISOTHIOCYANATE FENILHEXIL ISOTIOCIANATO
PIAS	PROTEIN INHIBITOR OF ACTIVATED STAT PROTEIN
RT4	BLADDER TRANSITIONAL CARCINOMA CELL “TP53- WILD-TYPE”/ CARCINOMA DE CÉLULAS DE BEXIGA “TP53-SELVAGEM”
RB1	RETINOBLASTOMA 1 (RETINOBLASTOMA 1)
SFN	SULPHORANE (SULFORANO)
T24	BLADDER TRANSITIONAL CARCINOMA CELL “TP53- MUTATED”/ CARCINOMA DE CÉLULAS DE BEXIGA “TP53-MUTADO”
TP53	TUMOR PROTEIN P53 (PROTEÍNA P53 TUMOR)
TGF-B	FACTOR TRANSFORMING GROWTH-B
UCC	UROTHELIAL CELL CARCINOMA (CARCINOMA DE CÉLULAS UROTELIAIS)
UM-UC-3 **UM-UC-6**	CELL BLADDER CARCINOMA (CÉLULAS DE CARCINOMA DE BEXIGA)
XTT	SÓDIO 3’-1- FENILAMINOCARBONIL- 3,4-TETRAZOLIUM-BIS 4-METOXI-6-NITRO (ÁCIDO BENZENO SULFÔNICO HIDRATADO)