UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

Instituto de Biociências
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ÁCIDOS GRAXOS DERIVADOS DE GELEIA REAL COMO FONTE DE INIBIDORES DE DESACETILASES DE HISTONAS HUMANAS: PERSPECTIVAS PARA A TERAPIA EPIGENÉTICA DO CÂNCER DE MAMA

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Dissertação apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, como parte dos pré-requisitos necessários para obtenção do título de Mestre junto ao Programa de Pós-Graduação em Ciências Biológicas (Genética).

BOTUCATU – SP
2021

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSEMEIRE APARECIDA VICENTE-CRB 8/5651

France, Fernanda Aparecida dos Santos. Ácidos graxos derivados de geleia real como fonte de inibidores de desacetilases de histonas humanas: perspectivas para a terapia epigenética do câncer de mama / Fernanda Aparecida dos Santos France. - Botucatu, 2021

Dissertação (mestrado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências de Botucatu
Orientador: Cláudia Aparecida Rainho
Capes: 20200005

1. Mamas - Câncer. 2. Epigenética. 3. Inibidores de histona desacetilases. 4. Geleia real.

Palavras-chave: 10-HDA; Câncer de mama triplo negativo; Geleia real; HDAC; Terapia epigenética.

Dedicatória

Dedico este trabalho aos meus pais: Francisco e Maria. Tenho orgulho de ser filha de vocês, sou grata pelos privilégios, pelo apoio, pelo amor e por sempre ajudarem na realização dos meus (e nossos) sonhos. Negra, da periferia, filha de técnica em enfermagem e pedreiro, sou a primeira da família a obter ensino superior completo e mestrado. E é graças a vocês.

Agradecimentos

Agradeço aos meus pais, pelo apoio incondicional. Amo vocês.

Agradeço à minha orientadora Profª. Drª. Cláudia Aparecida Rainho, pelo orientação, pelos ensinamentos, pelo apoio e por todas as oportunidades de crescimento e aprendizado. Agradeço pela confiança e pelo carinho.

Agradeço à nossa equipe do Laboratório de Epigenética: João, Diogo, Barbara, Naiade, Francieli e Marcos. Sempre aprendo muito com vocês.

Agradeço aos integrantes da banca do prévio Exame Geral de Qualificação pelas contribuições extremamente pertinentes, os professores Rafael Henrique Nóbrega (Departamento de Biologia Estrutural e Funcional, IBB-UNESP), Valéria Cristina Sandrim (Departamento de Biofísica e Farmacologia, IBB-UNESP e Roberto da Silva Gomes (Department of Pharmaceutical Sciences, North Dakota State University, NDSU-USA). Aos integrantes da banca de defesa: Profa. Dra. Adriana Camargo Ferrasi (Departamento de Clínica Médica, FMB-UNESP) e Prof. Dr. Lucas Tadeu Bidinotto (Faculdade de Ciências da Saúde de Barretos Dr. Paulo Prata – FACISB) por terem aceitado o convite para avaliar a versão da dissertação e comporem a banca.

Agradeço aos meus amigos, que estiveram sempre acreditando em mim. Vocês são pessoas incríveis, amo vocês.

Por fim, agradeço à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil - CAPES pela bolsa de mestrado concedida, processo nº 88882.432974/2019-01 e pelo auxílio para a capacitação no exterior com financiamento de bolsa de estudos, no âmbito do Programa Capes-PrInt, processo nº 88887.310463/2018-00, mobilidade nº 88887.570051/2020-00.

Epígrafe

“Life is not easy for any of us. But what of that? We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something and that this thing must be attained”.

– Marie Curie

“A vida não é fácil para qualquer um de nós. Mas e aí? Devemos perseverar e acima de tudo, confiar em nós mesmos. Devemos crer que temos talento para alguma coisa e buscar alcançá-la”.

Tradução livre e recorte de:
https://www.mariecurie.org.uk/who/our-history/marie-curie-the-scientist

Resumo

O câncer consiste em um conjunto de doenças heterogêneas, responsáveis for notória mortalidade e morbidade no mundo, sendo a segunda maior causa de morte antes dos 70 anos de idade no Brasil. Particularmente, o câncer de mama é o segundo tipo mais incidente e o mais prevalente entre as mulheres (excluindo-se o câncer de pele do tipo não melanoma). O câncer de mama é uma doença complexa que envolve diversas alterações genéticas e epigenéticas. A desregulação epigenética tem um papel essencial no desenvolvimento e progressão tumoral, bem como na aquisição de resistência à quimioterapia. A terapia epigenética é uma ferramenta promissora para o tratamento ou sensibilização de tumores aos protocolos clássicos, melhorando sua eficiência. Dentre as diferentes estratégias para a terapia epigenética, as desacetilases de histonas (HDACs) são os alvos mais estudados em ensaios clínicos, geralmente em combinação com radioterapia ou quimioterapia citotóxica/genotóxica, imunoterapia, hormonioterapia ou direcionadas a alvos específicos. Dados prévios do nosso grupo de pesquisa indicam o potencial de que o ácido 10-hidróxi-2-decenóico (10-HDA), derivado da geleia real, seja um possível inibidor de desacetilases de histonas (HDACi). Neste contexto, o presente estudo foi delineado com o objetivo de identificar novos HDACi dentre os ácidos graxos derivados da geleia realO estudo será apresentado em duas partes (capítulos). O primeiro capítulo apresenta as bases teóricas da terapia epigenética, seu potencial uso no câncer de mana e a evidências sobre o 10-HDA como um potencial HDACi. O segundo capítulo corresponde à versão premilinar do manuscrito contendo: a análise de expressão diferencial determinada por RNA-Seq dos genes codificadores de *HDAC*s recuperados do projeto *The Cancer Genome Atla*s (TCGA Research Network: https://www.cancer.gov/tcga); a descrição dos ácidos graxos derivados da geleia real quanto suas características estruturais, físico-químicas e principais componentes compartilhados entre as moléculas (chamados de *cores*); *in silico screening* com os ácidos graxos derivados da geleia real por meio do método *docking* molecular para determinar as possíveis interações com a proteína humana modelo HDAC2, comparada com o inibidor conhecido, ácido hidroxâmico suberoilanilida (SAHA). Globalmente, os resultados indicam que os ácidos graxos da derivados da geleia real podem ocupar o domínio catalítico da HDACs de modo semelhante ao inibidor conhecido (SAHA) e indicam as melhores moléculas candidatas para os estudos futuros baseados em ensaios bioquímicos de inibição de HDACs, bem como ensaios funcionais *in vitro* para investigar o potencial efeito de ácidos graxos na sensibilização de linhagens celulares resistentes à quimioterápicos comumente utilizados na prática oncológica.

Palavras-chave: 10-HDA; Câncer de mama triplo negativo; Geleia real; HDAC; Terapia epigenética.

Abstract

Cancer consists of a heterogenous group of diseases, responsible for notorious mortality and morbidity worldwide, considered the second main cause of death before 70 years old in Brazil. Particularly, breast cancer is one of the most incidents and more prevalent among women (excluding non-melanoma skin cancer). Breast cancer is a complex disease that involves several genetic and epigenetic alterations. The epigenetic dysregulation has an essential role in tumor development and progression, as well as chemotherapy resistance acquisition, therefore epigenetic therapy of cancer is promising tool for treatment or sensitization of tumors to the classical therapeutic protocols, increasing efficacy of response. In the different possible strategies for epigenetic therapy of cancer, the histone deacetylases (HDACs) are the most studied targets in clinical trials, usually combined with radiotherapy or cytotoxic/genotoxic chemotherapy, immunotherapy, hormonal therapy or targeted therapies. Previous data of our research group indicates the potential of the 10-hydroxy-2-decenoic acid (10-HDA), derived from royal jelly, as a possible new HDAC inhibitor (HDACi). This present work is composed of two parts (chapters). The first is an introduction to the theorical basis of epigenetic therapy of breast cancer and its possible use in breast cancer, the current evidence regarding 10-HDA as a potential HDACi. The second chapter is the preliminary version of the manuscript that is yet to be finished and submitted, with our preliminary data: differential expression analysis (RNAseq data) of HDAC coding genes in human cancers from *The Cancer Genome Atlas* (TCGA Research Network: https://www.cancer.gov/tcga); structural and physicochemical description of royal jelly derived fatty acids and their core structures; *in silico screening* with the fatty acids using the *molecular docking* method to determinate the possible interactions with human HDAC2 model, with a well-stablished inhibitor, suberoylanilide hydroxamic acid (SAHA). Overall, the results show that the royal jelly derived fatty acids might occupy the catalytic domain of HDACs, similarly to the known inhibitor (SAHA) and suggest candidate molecules for future assays based on biochemical inhibition of HDACs, as well as functional assay in vitro in order to investigate the potential effects of royal jelly derived fatty acids in sensitization of breast cancer cell lines resistant to chemotherapeutical agents routinely used in oncological practice.

Keywords: 10-HDA; triple negative breast câncer; royal jelly; HDAC; epigenetic therapy.

Abbreviations List

A	*Alanine*
Am	*Apis mellifera*
BIRC5	*Baculoviral IAP repeat containing 5*
C	*Cysteine*
CaM	*Calcium/calmodulin-dependent protein kinase*
CCND1	*Cyclin D1*
CCNB1	*Cyclin B1*
CCNE1	*Cyclin E1*
CK5	*Cytokeratin 5*
CSC	*Cancer Stem Cell*
D	*Aspartic Acid*
DFS	*Disease Free Survival*
DNA	*Deoxyribonucleic Acid*
DNMT	*DNA methyltransferase*
E	*Glutamic Acid*
EGCG	*(-)-Epigallocatechin gallate*
EGFR	*Epidermal growth factor receptor*
EMT	*Epithelial-mesenchymal transition*
ER	*Estrogen Receptor*
ERBB2	*Erb-b2 receptor tyrosine kinase 2*
ERBB3	*Erb-b2 receptor tyrosine kinase 3*
ESR1	*Estrogen receptor 1*
ESR2	*Estrogen receptor 2*
EZH2	*Enhancer of zeste 2 polycomb repressive complex 2 subunit*
F	*Phenylalanine*
FDA	*Food and Drug Administration (in United Stated of America)*
FOXM1	*Forkhead box M1*
G	*Glycine*
GAS2L2	*Growth arrest specific 2 like 2*
GATA3	*GATA binding protein 3*
GLOBOCAN	*Global Observatory of Cancer*
GRB7	*Growth factor receptor bound protein 7*
GTEX	*The Genotype-Tissue Expression*
H	*Histidine*

HAT	*Histone Acetylase*
HATi	*Histone acetyltrasferase inhibitors*
HDAC	*Histone Deacetylase*
HDI	*Human Development Index*
HDLP	*Histone deacetylase-like protein*
HER2	*Human Epidermal Growth Factor Receptor 2*
HRs	*Hormone Receptors*
Hs	*Homo sapiens*
HSP90	*Heat shock 90kDa proteins*
I	*Isoleucine*
IARC	*International Agency for Research on Cancer*
IDC	*Invasive Ductal Carcinoma*
ILC	*Invasive Lobular Carcinoma*
INCA	*Instituto Nacional de Câncer José Alencar Gomes da Silva*
INPP4B	*Inositol polyphosphate 4-phosphatase type II*
JAK	*Janus kinases*
JmjC	*Jumonji-C domain*
K	*Lysine*
L	*Leucine*
M	*Methionine*
MAPK	*Mitogen-activated protein kinases*
MDL	*Mixed Ductal-lobular Carcinoma*
MKI67	*Marker of proliferation Ki-67*
Mod	*Moderate*
mTOR	*Mechanistic target of rapamycin kinase*
MYBL2	*MYB proto-oncogene like 2*
N	*Asparagine*
NA	*Sodium*
NCBI	*National Center for Biotechnology Information*
NF-κB	*Nuclear factor kappa-light-chain-enhancer of activated B cells*
NOTCH3	*Notch receptor 3*
ORC6L	*Origin recognition complex subunit 6*
OS	*Overall Survival*
P	*Proline*
PAM50	*Prediction Analysis of Microarray with a 50-gene signature*
pCR	*Pathological complete response*

PDB	*Protein Data Bank*
PDBQT	*Protein Data Bank Partial Charge &Atom Type format*
PI3K	*Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha*
PIK3CA	*Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha*
PR	*Progesterone Receptor*
PRC2	*Polycomb Repressive Complex 2*
PTEN	*Phosphatase and Tensin Homolog*
PTM	*Post-translational modification*
Q	*Glutamine*
R	*Arginine*
RNA	*Ribonucleic Acid*
S	*Serine*
SAHA	*Suberoylanilide Hydroxamic Acid or Vorinostat*
STAT	*Sterol O-acyltransferase*
T	*Threonine*
TCGA	*The Cancer Genome Atlas*
TGF- β	*Transforming growth factor beta*
TNBC	*Triple Negative Breast Cancer*
TNPO1	*Transportin 1*
TOPO2	*DNA topoisomerase II alpha*
TP53	*Tumor protein p53*
TSA	*Trichostatin A*
TSG	*Tumor Supressor Gene*
UCSF	*University of California, San Francisco*
V	*Valine*
VEGF	*Vascular endothelial growth factor*
v-MYB	*Transforming protein Myb*
W	*Tryptophan*
WHO	*World Health Organization*
WNT	*Wingless-type MMTV integration site family*
XBP1	*X-box binding protein 1*
Y	*Tyrosine*
ZBG	*Zinc Binding Group*
Zn	*Zinc*

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Summary

Chapter I: an overview about cancer and the emerging role of epigenetic therapy in breast cancer treatment

1. Background

1.1. Epidemiology

According to the most recent data from the Global Observatory of Cancer (GLOBOCAN, International Agency for Research on Cancer, World Health Organization), 19.3 million new cancer cases and 9.9 million cancer deaths were estimated worldwide in 2020 (1). A few types present heterogeneous profile of geographic distribution, like thyroid and bladder cancers, while others exhibit a trend to aggregate in certain high-risk regions, such as malignant tumors of the mouth and oral cavity in South Asia and Kaposi sarcoma in East Africa (1,2). The global incidence of the ten most frequent cancers is available in **Figure 1**.

Cancer incidence and mortality are rapidly growing worldwide. The reasons are complex but reflect both aging and growth of the population, as well as changes in the prevalence and distribution of the main risk factors for cancer, several of which are associated with socioeconomic development (2–4). In terms of cancer incidence trends over time, analysis of cancer registry data draws attention to aspects of the global cancer transition: a tendency towards increases in breast, colorectal, and prostate cancers exist in the very high Human Development Index (HDI), high HDI, and medium HDI regions (5,6). This occurs due to the tendency of lifestyle, dietary, and environmental changes to shift the profiles of non-communicable diseases (6). Cancer transitions are also striking in emerging economies where cancer profiles are paralleled by increasing of the global disease frequencies. A recurring observation is the ongoing displacement of infection-related and poverty-related cancers by those cancers that are highly frequent in developed countries (*e.g.*, in Europe, North America, and high-income countries in Asia and Oceania). These cancers are ascribed to a so-called *westernization* of lifestyle, including urbanization-related habits such as sedentarism and inappropriate diet (7,8).

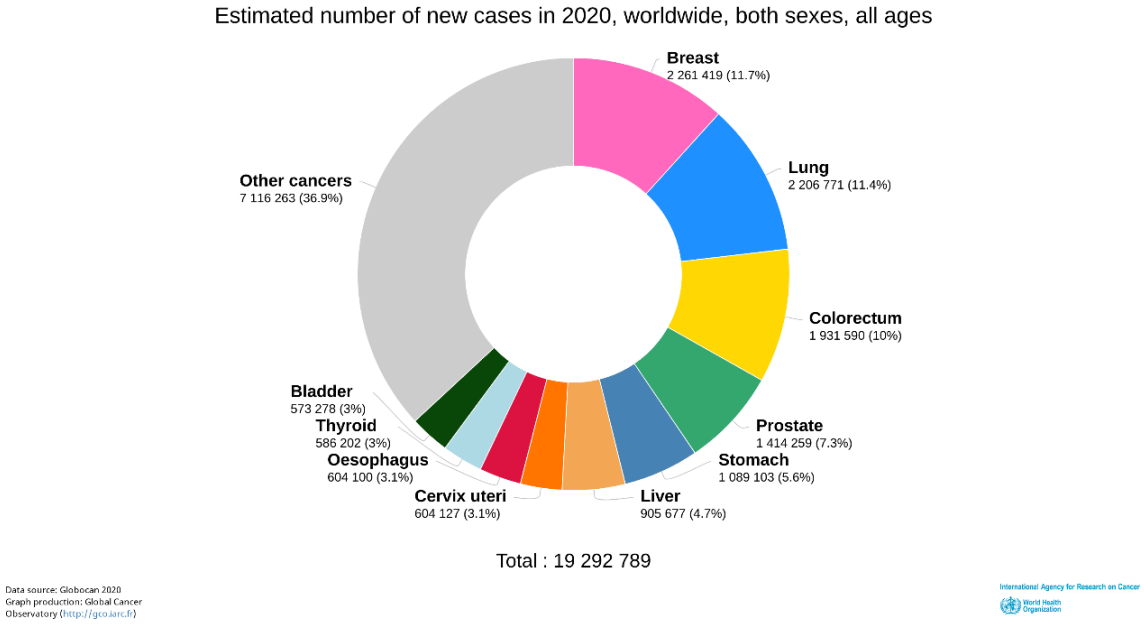

Figure 1. The estimated number of cancer new cases, all ages, both sexes, all cancers (non-melanoma skin cancer included in other cancers and rectal cancer included in colon – colorectum), in the world. Source: International Agency for Research on Cancer – World Health Organization, GLOBOCAN 2020 (available at https://gco.iarc.fr/>, last accessed on October 21st).

In Brazil, cancer is the second leading cause of death before 70 years (1). The Brazilian National Cancer Institute (INCA) estimates for each year of 2020, 2021, and 2022 that there will be 625 thousand new cancer cases (450 thousand, excluding non-melanoma skin cancer). The non-melanoma skin cancer will be the most incident (177 thousand), followed by breast and prostate cancers (66 thousand for each), colon and rectum (41 thousand), lung (30 thousand) and stomach (21 thousand). The Southeast region is going to have more than 60% of all new cases, followed by the Northeast (27.8%) and South (23.4%) regions. However, while in the South and Southeast regions the prostate and breast cancer, as well as lung and colon cancer are going to predominate, in the Midwest region is expected the increase of cervical and stomach cancers. Furthermore, in regions North and Northeast the cervical cancer and stomach cancers are going to have great impact, although the North region is the only one where breast cancer and cervical cancer have the same incidence.

1.2. Breast cancer

Breast cancer is one of the most incident and prevalent cancer in the world, being the fifth in the overall cancer mortality ranking. Particularly, breast cancer represents 25.8% of cancer new cases diagnosed in women (excluding non-melanoma skin cancer) (1). According to the WHO, breast cancer is responsible for a deduction of 0.3 years of life expectancy in women compared to men (9). In Brazil, INCA estimatesa risk of 81,06 per 100 thousand inhabitants in the Southeast region (8). More than 95% of the diagnosed breast malignancies are adenocarcinomas, detected as carcinomas *in situ* or invasive carcinomas (8).

At molecular, histopathological, and clinical levels, breast cancer is a heterogeneous disease (**Figure 2**). Invasive ductal carcinoma (IDC) is the most common histological subtype followed by invasive lobular carcinoma (ILC) (10), being very different from each other in several histological and clinical features (10). However, the category of mixed ductal-lobular (MDL) is considered a single entity, not a collision tumor, because of its own phenotypic and genomic characteristics (11).The WHO includes 21 special subtypes with distinct morphologic characteristics, and some of them are rare and differ significantly concerning prognosis and response to adjuvant treatment (12). The histological grade of breast carcinoma is calculated based on a 3-tier score of intensity (varying from low to high) system. Histologically, it is observed the percentage of the tumor located in each part of the mammary gland – the acini or tubular structures, anisokaryosis, and proliferation rate (13,14).

Similarly to other epithelial tumors, breast cancer is evaluated using the TNM (tumor, node, metastasis) system (12). The TNM staging system is widely used for clinical stratification epithelial solid tumors. T stands for the primary tumor size, N for local lymph node infiltration, and M for distant metastasis (15). Histopathological features are relevant, referring to cellular subtyping and histological grade, indicating the differentiation status ranging from highly differentiated (G1) to poorly differentiated (G4) (16). Importantly, biomarkers are routinely accessed by immunohistochemistry, including the status of hormonal receptors of estrogen (ER) and progesterone (PR) as well the human epidermal growth factor receptor 2 (also named HER2 or ERBB2, erb-b2 receptor tyrosine kinase 2). These biomarkers are established prognostic and predictive factors and their expression in breast carcinomas are critical in guiding patient stratification and treatment (13). Genomic profiling of breast tumors has shown that they can be classified into five intrinsic molecular subtypes according to the PAM50 system

(a Prediction Analysis of Microarray based on 50-gene signature): Luminal A, Luminal B, Her2-enriched, Basal-like and Normal-like (17,18). Although there are other proposals for breast tumor classification based on new evidence (19,20), the PAM50 remains widely used classifier for prognosis and prediction of benefit from adjuvant tamoxifen (a selective estrogen receptor modulator). The PAM50 system is detailed in **Figure 2 and Table 1** (21).

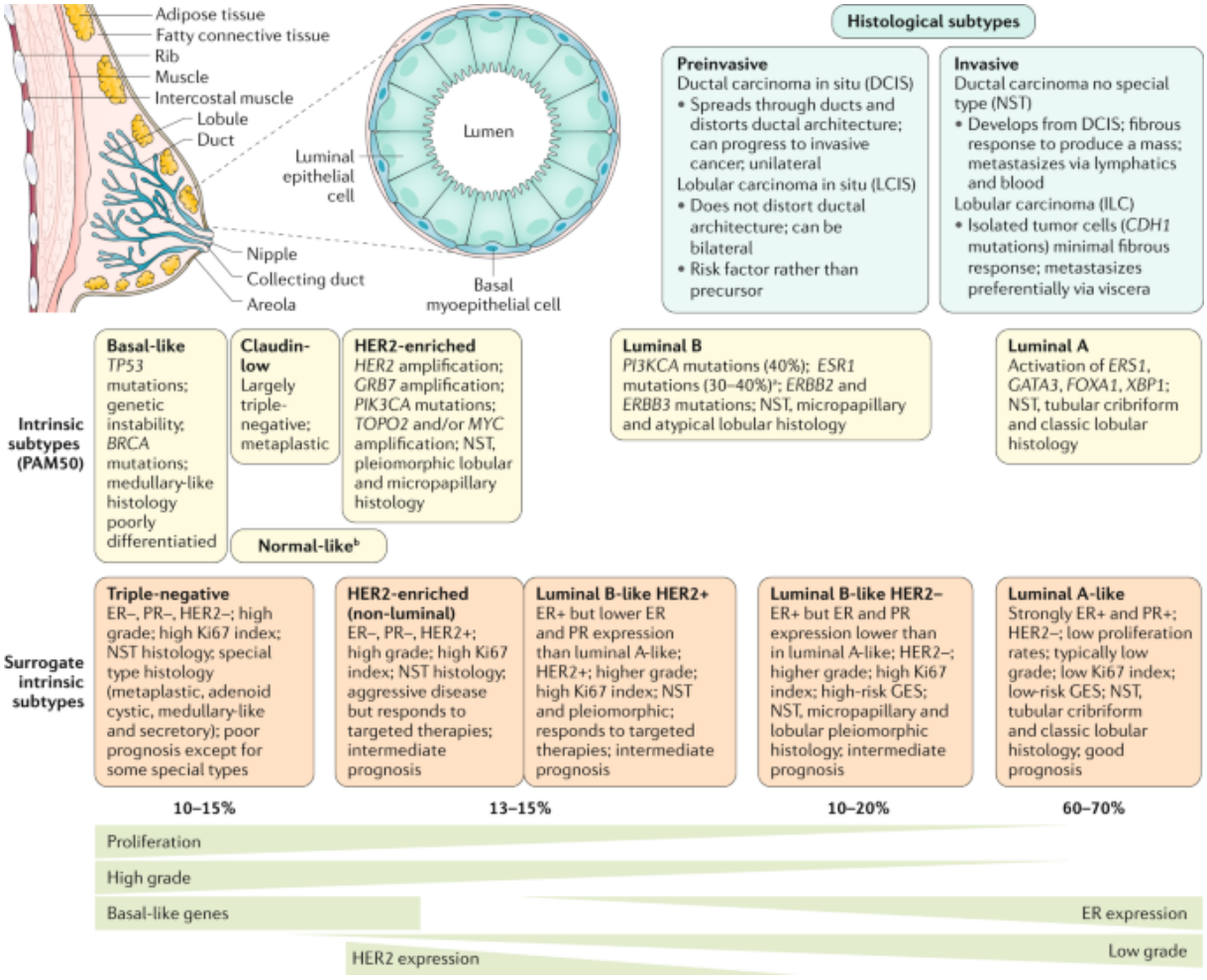

Figure 2. The histological and molecular characteristics of breast cancer. There are three main classification systems at morphological (histological subtypes) and molecular levels (intrinsic PAM50 signature and the surrogate intrinsic subtypes). Source: Harbeck *et al*., 2019 (22).

Table 1. Summary of molecular and pathological characteristics, prognosis, and gene-expression changes of breast cancer subtypes according to the 50-gene intrinsic subtype classifier (PAM50).

Subtype	Incidence (%)	Biomarker profile					Prognosis			Gene expression changes	Treatment modalities
		ER	PR	HER-2	Ki67	Other	OS (%)	5 Years DFS (%)	10 Years DFS (%)		
Luminal A	50-60	+	+	–	Low	Luminal epithelial cytokeratins 8 and 18 Low histological grade	89-95	79-85	70-78	Increased expression in genes associated with ER function: *FOXA1*, *PgR*, *BCL2*, *EsR1*, *LIV1*, *ZIP6*, *SLC39A*, *XBP1*, *GATA3*, *ERBB3/4*, and *TFF1*.	Hormonal therapies +/- chemotherapy
Luminal B	15-20	+	+	–	Mod	Luminal epithelial cytokeratins 8 and 18 High histological grade	71-85	60-75	50-60	Increased expression in genes associated with ER function: FOXA1, PgR, BCL2, EsR1, and GATA3 Increased expression of proliferative genes *CCNB1*, *CCND1*, *CCNE1*, *MYBL2*, *MKI67*, and *v-MYB*	Poorer outcomes from hormone therapy (Low levels of HRs); better pCR to neoadjucant chemotherapy
Her-2	15-20	–	–	+	High	Luminal cytokeratins	43-78	41-65	45-51	Amplification of *ERBB2* and GRB7 PI3K pathway activation (AKT, pS6, and p4EBP1) correlated with *INPP4B* and *PTEN* loss Increased expression of proliferative genes *BIRC5*, *CCNE1*, *CCND1*, *ORC6L*, *MYBL2* and *MKi67*	HER-2 targeted therapy and chemotherapy
Basal-like	15-20	–	–	–	High	Basal cytokeratins 5, 14 and 17 High EGFR	53-73	48-72	48-65	Increased expression of EGFR Dysregulation of MAPK/AKT/PI3K and Ras/Raf and JAK/STAT Increased expression of *FOXM1*, *cMYC*, *CCNE1*, *CCND1*, *CDC20*, *CDC6*, *BIRC5* and *ORC6L*	Chemotherapy
Normal-like	5-10	–	–	–	High	Negative for CK5 and EGFR	~93	79-87	~85	Loss of tight junction proteins*: claudin 3, 4, 7* *E-cadherin*	Chemotherapy

Mod = moderate; gene names shown in italic; data source Bernhardt *et al.*, 2016

In Brazil, approximately 40% of women with breast cancer are diagnosed at advanced stages (III and IV), which means a worse prognosis and lower survival rates (23). Consequently, this disease remains a challenge to be screened and properly treated, particularly in cases of aggressive phenotypes, such as triple-negative breast cancer (TNBC) and advanced, metastatic, and multidrug resistant tumors.

1.3. Triple negative breast cancer

Triple-negative breast cancer (TNBC) accounts for about 10-15% of all breast cancers. This molecular subtype of the disease is characterized by the absence of expression of ER, PR or HER2 proteins and consists in a heterogeneous group regarding histological, genetic, prognostic and treatment response (13). There is significant overlap with the basal-like molecular subtype, however currently it is recognized that they are not a single entity (24). Because of their lack of response to endocrine and targeted therapy, surgery is the therapeutic modality frequently chosen associated with radiotherapy and/or chemotherapy (neoadjuvant and/or adjuvant). Interestingly, during early treatment, TNBCs are usually highly sensitive to chemotherapy (compared to other types of breast cancer), which is named "the TNBC paradox" (25), due its worse prognosis and a higher chance of recurrence. Approximately, 30-40% of the patients treated with standard chemotherapy regimen of anthracyclines and taxanes responds to treatment (26) (achieving the pathological complete response – pCR). According to medical guidelines in Brazil, the mentioned treatment options are recommended to intermediate to high-risk patients (27).

Most deaths of breast cancer patients occur because of the metastatic behavior of the disease and instead of due to the primary tumor growth (28). The development of metastasis in TNBC is a complex and poorly understood process, that includes multiple steps of acquired genetic, epigenetic and angiogenesis alterations, microenvironment interactions, basal lamina invasion, cell survival in circulation and distal sites colonization (28,29). Around 30-40% of patients with metastatic breast cancer who are treated with anthracyclines survive within 22 months; however, anthracyclines are associated with irreversible cardiotoxicity (28). Given the intratumoral (within a same tumor) and intertumoral (different tumors among different patients) heterogeneity, often there is a subset of TNBC cells that is sensitive to chemotherapy. On the other hand, many cells may convert to a resistant phenotype during the chemotherapy or present intrinsic resistance. Both mechanisms, *i.e*. intrinsic or acquired chemoresistance, are probably implicated in the treatment failure in human cancers (24). Further, intrinsic and/or acquired drug resistance accounts for 90% of treatment failures in patients with metastatic disease (30). The mechanisms that dictate the rise of resistance in cancer cells include alterations of drug influx and/or mainly efflux, such as ABC transporters (ATP-binding cassette). Alterations in key pathways of cell signaling such as Notch-1/3/4, Wnt/β-catenin, *NFKB1* (nuclear factor kappa B

subunit 1), JAK/STAT proteins, *PTEN*/*PI3K*/*AKT*/*MTOR* genes as well as alterations in cancer stem cells (CSC) with regard to TGF- β receptor regulation (24) are remarkably frequent. Recent work in the literature shows the role of differential expression of non-coding RNAs (31,32) and clonal interactions among different subsets of cancer cells (33) in emerging resistance during chemotherapy.

In addition, there is evidence implicating epigenetic mechanisms in chemotherapy resistance. Epigenetic mechanisms are involved in the regulation and structural adaptation of chromosomal regions so as to register, signal or perpetuate normal/altered activity states (34). The key epigenetic mechanisms involved in gene regulation expression are DNA methylation, post translational modifications (PTM) on histones, chromatin remodelers and long non-coding RNAs (35). Noteworthy, the PTM on histones occur by the covalent addition of chemical groups at the N-terminal tails that project from the nucleosome. They are regulated by the writers, readers, and erasers (respectively, the enzymes involved in catalytic reactions of addition, detection and removal of these chemical groups). These functional groups contribute to the fine tuning of gene expression and regulation of chromatin structure. Acetylation and methylation are the most well studied histone marks. An example of the invovlement of PTM in cancer resistance is the lysine 27 methylation of H3 histone (H3K27me). The histone-lysine N-methyltransferase (encoded by the gene *EZH2*, enhancer of zeste 2 polycomb repressive complex 2 subunit), the catalytic subunit of Polycomb Repressive Complex 2 (PRC2) is the enzyme responsible for writing this mark. Considered as a proto-oncogene, *EZH2* overexpression results in the transcriptional repression of target *loci*, increased cell proliferation, and also cooperates to cisplatin resistance development (36) in colorectal and ovarian cancer cell lines. When EZH2 protein inhibitor is associated with histone deacetylase inhibitor (HDACi) Panobinostat, there is an induction of apoptosis of breast cancer cell lines MDA-MB-231 and MDA-MB-436 (both derived from TNBC) (37). The histone demethylases of H3K4 from JmjC family, namely KDM5A, have been suggested to be involved in resistance (38), notwithstanding, KDM5B has been referred to as promoting cell proliferation in breast cancer (36,39). Additionally, cell lines resistant to chemotherapy were sensitized using Trichostatin A (TSA) (38). TSA is a well know inhibitor of histone deacetylase (HDACi), indicating the key role of epigenetic machinery enzymes in the fine tuning of gene expression related to resistance.

1.4. Epigenetic therapy: histone deacetylase inhibitors as promising modulators for chemosensitization

Unlike genetic changes, epigenetic alterations are potentially reversible, which gives the opportunity to develop therapeutic resources aiming the correction of aberrant epigenetic marks linked to cancer origin and progression (40). Epigenetic therapies consist of an encouraging alternative to refine the response to traditional cancer treatment. Additionally, they may act in synergy with several other strategies and also be used with the purpose of chemosensitization to acquired resistance to chemotherapy (40–42). The most studied small molecules are those which interfere with the expression or function of epigenetic enzymes (activators, inhibitors or regulators). Histone deacetylase and DNA methylation inhibitors (HDACi and DNMTi, respectively) are used to re-express epigenetic silenced genes (43,44). In cancer treatment, this strategy is frequently used to reduce tumor growth, due to its inhibition of DNA methylation on promoter regions and deacetylation of tumor suppressor genes (TSG), previously silenced during tumorigenesis (45). Epigenetic regulation influences all of the recognized hallmarks of cancer (**Figure 3**) (46–49), and altogether with remarkable trials' results encouraged the US Food and Drug Administration (FDA) to approve (to date) seven epigenetic drugs for cancer treatment: 5-Azacytidine and Decitabine (DNMTi); Vorinostat, Romidepsin, Belinostat, Panobinostat (HDACi) (50) and Tazemetostat, an EZH2 inhibitor recently approved (51,52).

The histone deacetylases remove the acetyl group from the N-terminus of lysine in histones (preferentially H3 and H4) , increasing the positive charges of these residues of histones, strengthening their interaction with DNA, thus preventing the transcription machinery from accessing a *locus*-specific region of the genome (42,53). HDACs also target non-histone substrates, such as the proteins p53, STAT3, X-ray repair cross-complementing protein 6, Nuclear factor NF-kappa-B p105 subunit, HSP90, and α-tubulin (54). The acetylation/deacetylation of proteins can increase or reduce their affinity with DNA, their transcriptional activation, their stability, and influence protein-protein interactions (55,56).

HDACs are part of a family of proteins with conserved domains in various species. The known sequences and structures organization for the mammalian HDAC proteins are commonly categorized into classes based on sequence similarity to yeast proteins Hda1 and Rpd3 from *Saccharomyces cerevisiae* and their catalytic mechanism, based on *Aquifex aeolicus* (bacteria) models. Particularly in humans, classical HDACs are grouped into 3 classes: HDAC1-3 and HDAC8 are part of class I; HDAC4, HDAC5, HDAC7, and HDAC9 are class IIa enzymes;

HDAC6 and HDAC10 are grouped into class IIb, and HDAC11 is the sole member of class IV (**Figure 4** presents a schematic diagram of the HDAC proteins). The class III enzymes are grouped into another subfamily, the sirtuins (SIRT1-7), due to a distinct catalytic mechanism and domain organization (53,57).

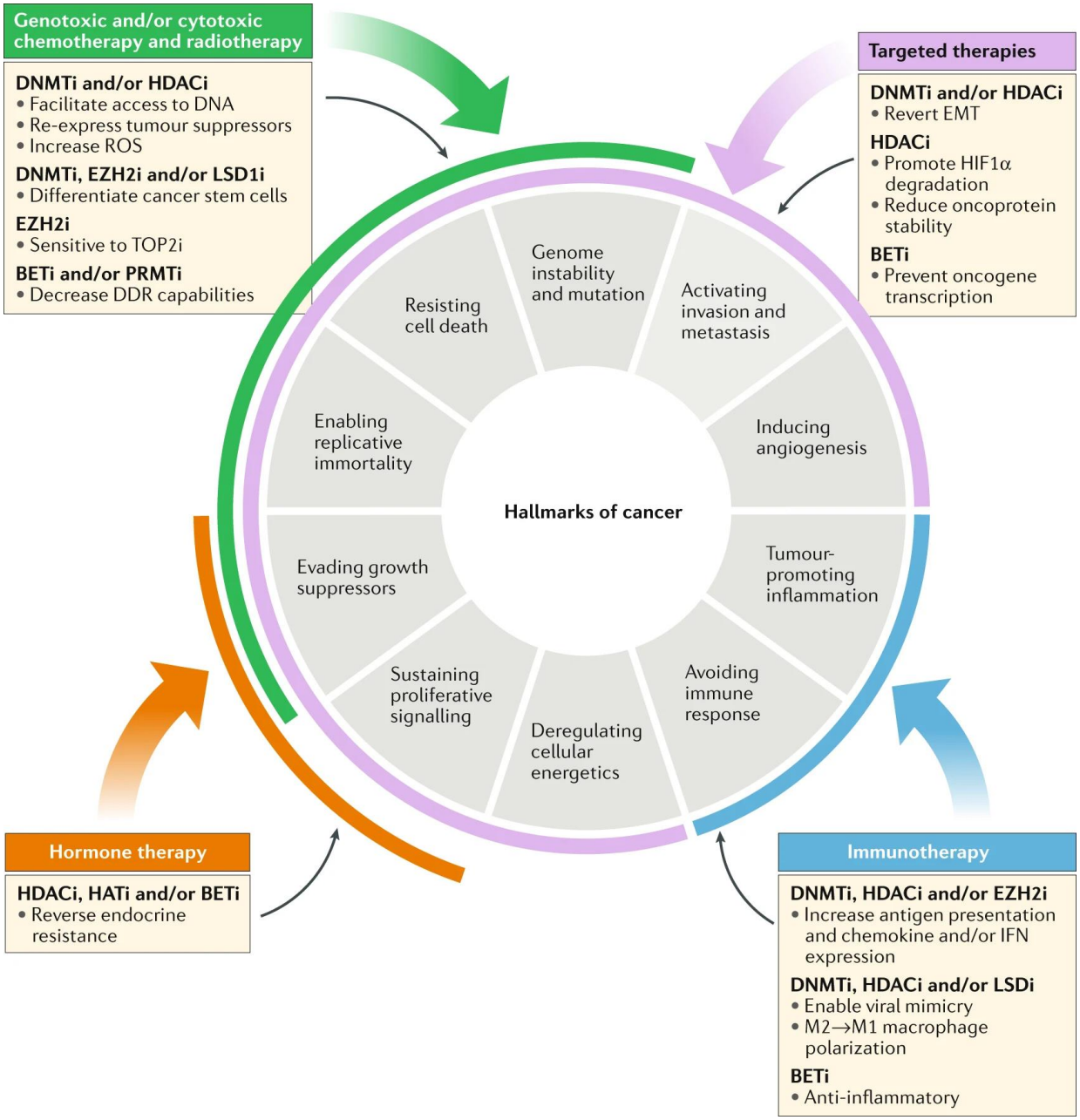

Figure 3. Histone Deacetylase Inhibitors (HDACi) are studied in combination with other therapeutic strategies to counteract all hallmarks of cancer. Source: Morel *et al*., 2019 (46).

Regarding the protein domain organization, mammalian HDACs share some similarities with each other (58). All family members are characterized by the presence of an acetyl-binding catalytic domain *Hist_deacetyl* (PF00850) (59), and its sequence and position are conserved within each class. The catalytic domain of the class I and IV enzymes corresponds to a great percentage of the total protein sequence, although enzymes of class IIa have their active site located at the C-terminal region (58). Class IIa HDACs also may show an additional zinc binding

motif conserved (its putative roles are believed to be to regulate catalytic activity and/or the specificity of the active site) (60). The N-terminal region of class IIa members contains several serine (S) residues, targets of CaM kinases and are involved in the process of its exportation to the cytoplasm (58,61). Class IIb enzymes, *i.e.*, HDAC6 and HDAC10 have two catalytic domains, but the second domain of this last one is inactive (62,63).

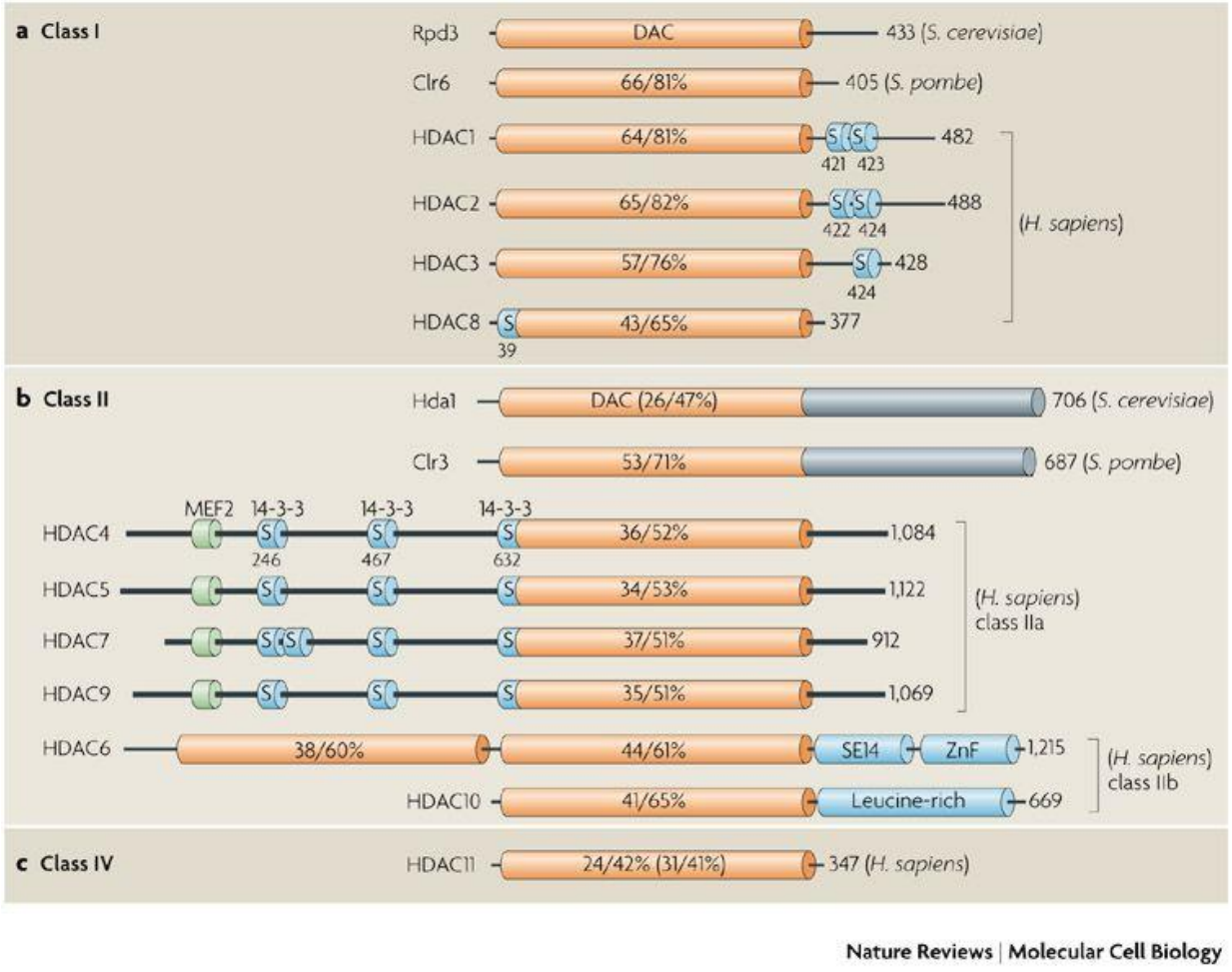

Figure 4. Diagram representing the conserved domains of class I, II, and IV HDACs. The histone deacetylase family is grouped into different classes according to sequence similarity to yeast prototypes. Source: Yang and Seto, 2008 (61).

The firstly elucidaded structure of the HDAC catalytic domain was built based on the bacterial *Aquifex aeolicus* HDAC ortholog, known as histone deacetylase-like protein (HDLP). This protein enabled comparisons with HDAC8 and was used as reference to describe the catalytic mechanism of this enzyme (53,58), as illustrated in **Figure 5A**. A zinc ion (Zn^{2+}) is essential as a cofactor for the catalytic reaction: it tetrahedrally coordinates to the side chains of amino acids residues of two aspartate and one histidine. It then stabilizes the binding of the substrate using a water molecule. The Zn^{2+} cation coordinates the polarization of the carbon-oxygen bound of the acetyl unit, turning the carbon more susceptible to the nucleophilic attack by the water and the histidine. The catalysis proceeds with the actions of a tyrosine and another histidine residue towards the substrate to displace the acetyl group (this tyrosine residue is

replaced by histidine in vertebrates class IIa) (53,64). The amino acids that participate the catalytic process are highly conserved considering the structures of all classic HDACs among species. The conservation of the main HDAC catalytic amino acids is illustrated in **Figure 5B**.

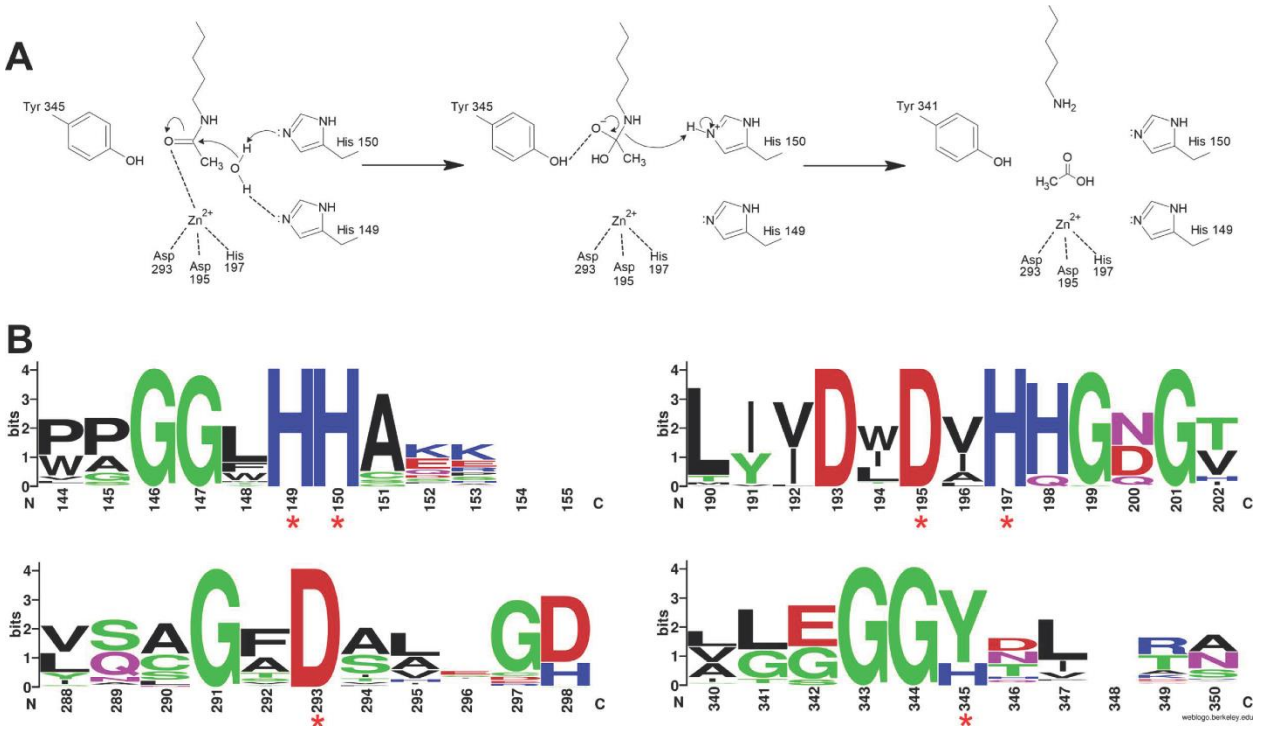

Figure 6. Catalytic domain of HDACs. A) Catalytic reaction of lysine deacetylation of HDACs. **B)** Sequence conservation in four different sites of the active domain, the most important amino acids are highlighted with red asterisks. Calculation of conservation between 25 species analyzed by the authors of the original article. Source: Milazzo *et al*., 2020 (58).

The HDACi may selectively inhibit one specific class of HDACs (HDAC isoform-specific inhibitors) or all types of HDACs (pan HDACi). The current HDACi can be classified within four classes of chemical substances: hydroxamic acids; short chain fatty acids; benzamides, and cyclic tetrapeptides (65). The indispensable pharmacophoric characteristics for HDACi are three chemical groups. First, a hydrophobic cap group that interacts with the external part of the active tunnel. Second, a Zn-binding group (ZBG) that chelates the metal. Third, a linker group that occupies the tunnel connecting the ZBG and the capping unit (66–68).

The wide range of effects and cellular processes that can be modulated by HDACis is reflected by the current clinical trial protocols and different combination strategies with immunotherapy, hormone therapy, radiotherapy, chemotherapy, as well as targeted therapy (46). Key processes of cancer cells can be modulated by HDACi: apoptosis, DNA damage repair, cell cycle, autophagy, angiogenesis, and metastasis, besides non-coding RNA expression and immune response (65,69). Recent evidence supports that immune response can be explored to TNBC treatment due to the modulation of PD-L1 expression (70), similarly to what has been

reported for ovarian, colorectal, and lung cancer *in vitro* models (71–73). There are studies that also suggest that dual HDACi and DNMTi could resensitize thyroid cancer cells to chemotherapy (74), the combination of different HDACi and DNMTi has been used in ovarian (75) and osteosarcoma (76) cell lines, and combination of epi-drugs with chemotherapy to overcome resistance in breast cancer, lung and ovarian cell lines (77). However, the previous reports of the association of HDAC inhibition with chemotherapy sensitivity in breast cancer are contradictory. *In vitro* experiments have shown that different classes of HDACi might induce cell death, and simultaneously, promote cell migration in a variety of cell lines (liver, lung, and gastric cancers), including MCF-7 cells of luminal subtype (78). Contrarily, the same research group also described that the treatment with TSA promoted cell migration and metastasis in liver cancer xenografts (78). Nonetheless, HDACi utilized as adjuvant chemotherapy prevented local recurrence and metastasis in xenograft model of breast cancer induced by MDA-MB-231 cells (derived from human TNBC as well as in 4T1 cell line from mouse breast cancer) (79). The combined treatment with anthracyclines and HDACi can induce synergic effects *in vitro* (80,81). Also, the combined treatment of HDACis with epirubicin (an anthracycline topoisomerase II inhibitor) intensified DNA damage (double strand breaks) and increased cell death (57), including chromatin decondensation in breast cancer cell lines (82). High-throughput chemical library screen identified the combination of HDACi with doxorubicin as effective to synergistically target both CSC and other cancer cells in 3D culture models (83). *In vitro* culture of cells derived from of renal, lung, and breast cancer treated with HDACi alone and in combination with other drugs suppressed the epithelial-mesenchymal transition (EMT) and compromised the DNA integrity of cancer cells (84).

The first phase I clinical trial designed to dosage choice of the combination of valproic acid (a well-known HDACi) with epirubicin in solid tumors resulted in a good tolerance and a response rate of 22-39% leading to stabilized disease in oncological patients. Accordingly, patients with anthracycline-resistant tumors (previously treated) demonstrated a notable degree of antitumor activity (ClinicalTrial.gov: NCT00246103) (57,85). In patients bearing metastatic melanoma who undergone progression after chemotherapy treatment, 18% presented partial responses, and 18% did not had advanced disease. Further, patients carrying anthracycline-resistant breast cancer, cervical cancer, non–small-cell lung cancer, and small-cell lung cancer had partial response (85). In an dose expansion of cohort, 64% of breast cancer patients responded (57,81). Notwithstanding, advanced or metastatic tumors presented a modest effect to

Vorinostat treatment (suberoylanilide hydroxamic acid or SAHA), a pan-HDACi used in association with doxorubicin.

The role of HDAC2 as a biomarker and promising specific target has been suggested (57,86). The potential of HDAC to reverse endocrine resistance in luminal tumors has been highlighted by clinical trials (87–90). The main clinical trials involving targeted therapy with epi-drugs were recently reviewed elsewhere (46,58,68,91).

The activity of established and investigational epigenetic therapies in well-defined clinical contexts has provided evidence that this strategy can be effective. Given the vast number of potential targets, a systematic approach that identifies and validates potential drug targets is needed to focus on drug development and achieve the promise of this strategy (92).

1.5. *In silico* approaches for identification of small molecule for epigenetic therapy (epi-informatics)

Drug research and development (R&D) are nowadays mainly based on the discovery of low molecular weight compounds that could interact with macromolecules related to diseases (referred as receptors or targets). Although the number of estimated targets pharmacologically modulable is estimated in 10,000 gene products, about only 4% of them are being investigated in drug discovery programs. Given the unexplored potential of this field, it is crucial to understand the fundamentals and interactions between possible ligands and receptors (93,94). In this context, virtual screening (VS) is a rational and straightforward method and costs less than high-throughput screening (HTS) experiments with highly information capability (95). The VS can be classified into two strategies: ligand based, such as pharmacophore modeling and quantitative structure activity relationship (QSAR) methods, and structure based (such as molecular dynamics and molecular docking) (95,96).

The molecular docking approach can be used to test the putative interaction between a compound and a protein. It allows the modeling of the possible binding modes, revealing the nature of the biochemical interaction. Two basic steps are involved: prediction of the ligand conformation in the domain of the target protein, and the calculation of the binding affinity. These fundaments are related to the scoring scheme utilized by the docking algorithm (95,96).

Currently, few studies focused on the identification and characterization of new HDACi derived from natural products, reveling a gap to be explored. Indeed, the use of bioinformatics tools for the discovery of epigenetic drugs is continuously being expanded and improved (50), as well as the chemical description of natural products, given their complexity related to biological resources, variable composition, and/or complex reaction products, that may include partially unknown constituents (97). Our research group has been designing an *in silico* screening pipeline in recent years (98–100). We utilize literature evidence of the biological effects of bee products in order to theorize if the epigenetic reprogramming is the factor that underlies the phenotypic plasticity observed during bee development and could serve as a source of new epigenetic drugs discovery for cancer treatment (101–103). For instance, naturally derived polyphenols such as EGCG, flavonoids (such as quercetin), quinones (such as laccaic acid), lycopene, and boswellic acid have been reported to function as DNMTi (104) and Trichostatin A and Romidepsin are microbial-derived HDACi (105).

As reviewed elsewhere (106–114), phytochemicals and dietary compounds have been widely studied to investigate their possible modulation of the epigenetic enzymes involved in

cancer progression. In this context, many biological properties have been detected in honeybee products, as nutraceuticals, pharmaceuticals, and cosmetic ingredients (114). Honeybees are social hymenopteran insects, characterized by the production and storage of honey, royal jelly, propolis, bee venom, bee pollen, and beeswax. These products may benefit humans with different promising uses, and some of the biological properties of those products have been described in the literature (115). In this context, royal jelly offers opportunities for possible molecules with biomedical interest.

Royal jelly is a complex product containing water, sugar, protein and amino acids, lipids, and at minor concentrations, it includes enzymes, vitamins, phenolics, and minerals (114). It is secreted by hypopharynx and mandibular salivary glands of worker bees, which are responsible for the nursing of *Apis mellifera* larvae that might develop into future queens or workers (116). The nutrition of larvae controls the caste switching: although some female larvae share the genome, the amount and duration of royal jelly that is fed to them determines the development fate. Larvae that will become a queen receives a diet rich in royal jelly for longer periods of days, while the ones to become workers receive royal jelly only at initial days, then their diet is changed to honey and other compounds (101,117). Spannhof and colleagues suggested that the epigenetic regulation that controls the phenotypic plasticity for queens is in part mediated by the HDACi activity of the (E)-10-hydroxy-2-decenoic acid, a fatty acid exclusively found in royal jelly (detected as a concentration of up to 5%) (102). Sequencing of the honeybee genome has shown conservation of histone sequences and chromatin modifying machinery with the human ones. For example, PTM on histone tails interfere the PTMs of histones H3.1, H3.3 and H4 in *Apis mellifera* have been characterized (118), and the histone methyltransferases and demethylases have been described (119). In addition to the modulation of caste switching (120), involvement of histone acetylation in aversive memory (121) and behavior have been described in honeybees (122). A more recently published article indicates that 10-HDA, zinc and potassium concentrations have a strong correlation with caste differentiation among other control mechanisms (103), such as microRNAs (123–125) and methionine concentration (126).

Royal jelly has been used since ancient times and is currently used in pharmaceutical and cosmetic fields (114). Although there are no crystal structures of the *Apis mellifera* HDACs on public databases, there are histone deacetylases encoded in the honeybee genome, similar to the human class I and class II enzymes (102). If 10-HDA inhibits class I and class II HDACs on honeybees, and the recombinant HDACs tested by Spannhof *et al.*, it is reasonable to

hypothesize that 10-HDA could inhibit human HDACs similarly. Carboxylic acids are a subset of HDACi, and even though it has a larger chain, the 10-HDA can be classified within this group, which includes valproic acid and butyric acid (102,127,128).

Interestingly, Royal Jelly has shown bioactive effects on *in vitro* models using human breast carcinoma cells (114,129–134) and in a recently published clinical trial of renal cell carcinoma (132). Also, perspectives for epigenetic therapy of cancer have emerged from evidences of its role in the regulation of histone deacetylases (102,103,135). Given the key role of HDACs in breast cancer, we hypothesize that 10-HDA interact with human HDACs and inhibits its activity, being a new HDACi candidate to epigenetic therapy.

2.1. General Objetive:

- This study was delineated with the objective of identify new histone deacetylase inhibitors among the fatty acids derived from royal jelly.

2.2. Specific Objectives:

- Identify the differentially expressed histone deacetylase genes in human cancer, focused on the overexpressed HDACs in breast cancer

- Create a dataset of royal jelly derived fatty acids with known structure, chemical properties and to understand their similarity with 10-HDA;

- Perform molecular docking to analyze the possible conformations, free energy values and interactions of the fatty acids used as ligands to dock into the HDACs active site;

- Use refinement tools to predict the best HDACi candidates among the royal jelly derived fatty acids, to future experimental validation investigation.

3. Conclusions

By using *in silico* approaches, the present study contributed to the scientific literature with new insights in drug development and discovery from products of natural origin. Overall, the findings suggest that fatty acids derived from royal jelly can inhibit human HDACs.

Among the differentially expressed genes encoding HDACs, there is a trend of the ubiquitous HDACs (class I) to be up-regulated in most cancers. *HDAC2* gene is one of most frequently overexpressed in human cancer, including breast cancer.

Fatty acids derived from royal jelly are very similar to the 10-HDA, its major and unique fatty acid, which suggests that they may have similar biological functions in royal jelly, notably considering the analysis of core structures that indicated three key clusters: 3-hydroxydecanoic acid and Methyl 3-hydroxydecanoate in the first; Trans-10-acetoxydec-2-enoic acid and 10-hydroxy-2-decenoic acid in the second; and Octanoic acid and 2-decene-1,10-dioic acid in the third cluster. .

Using this information, it was demonstrated that these fatty acids might interact with the catalytic domain of human HDAC2, similar to suberoylanilide hydroxamic acid (SAHA), a well-known HDACi. The aforementioned data strengths the hypothesis of an additive effect of these fatty acids in royal jelly.

These findings support the design of future experimental approaches devoted to validating the inhibitory activity of fatty acids on HDACs and the potential use of this chemical compounds in pre-clinical studies for the identification of new drugs for epigenetic therapy.

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Chapter II: Research article (in preparation)

Background: This chapter consists of the primary version of the manuscript derived from the present master's degree study. The journal for submission is Chemical Biology & Dug Design (Oxford: Wiley-Blackwell, Online ISSN:1747-0285). Given that the text is not finished, the co-authors list will be updated by the time for submission. We declare no conflicts of interest.

Provisory title:

Royal jelly as source of new histone deacetylase inhibitors for epigenetic therapy of cancer

Abstract

Cancer is a collection of heterogenous diseases responsible for great mortality and morbidity worldwide, remarkably characterized by complex genetic and epigenetic alterations. Since epigenetic dysregulation has major roles in tumor development, progression and acquisition of therapeutic resistance, epigenetic therapy is a promising tool to treat or sensitize tumors to classic therapeutic protocols, improving efficiency. Among epigenetic therapy strategies, the histone deacetylases (HDACs) are the most studied targets in clinical trials, usually tested in combination with cytotoxic and/or genotoxic chemotherapy and radiotherapy, hormone therapy, immunotherapy, or targeted therapies. Previous data of our research group indicate the potential of the 10-hydroxy-2-decenoic acid (10-HDA), derived from royal jelly, as a possible inhibitor of HDACs (HDACi). Herein, we demonstrate de differentially expressed HDAC coding genes across human cancers (TCGA-PANCAN), to identify possible targets for HDACi. Subsequently, we described royal jelly derived fatty acids as possible new naturally derived HDACi. To predict their interactions with human HDACs we performed an *in silico* screening using molecular docking to predict binding affinity and positioning. We are going to perform in vitro study of HDAC inhibition by 10-HDA using breast cancer derived cell lines. The present work demonstrates the structural and physicochemical diversity of royal jelly derived fatty acids and indicates that these compounds can be used for drug design of HDACi for further biochemical validation.

Keywords: epigenetic therapy, HDAC, royal jelly, 10-HDA, chemical space.

1. Introduction

The key epigenetic mechanisms involved in gene expression regulation are chromatin remodelers, DNA methylation, long non-coding RNAs and post translational modifications (PTM) on histones [1]. Noteworthy, the PTM on histones have been indicated to be cancer epigenetic biomarkers for use in the development and evaluation of epigenetic drugs for cancer therapy [2]. Particularly histone acetylation/deacetylation, represent a major epigenetic regulatory process and play an essential role in human cancers. Histone deacetylation is a critical regulator of gene transcription involving the removal of acetyl groups and is regulated by a group of enzymes termed histone deacetylases (HDACs), often associated with gene silencing complexes [3]. The HDACs can be classified into four classes: the classes I, II and IV compose the classical histone deacetylases whereas the class III consists of sirtuins, which differ from the first in domain structure and enzymatic reaction. HDACs 1, 2, 3 and 8 belong to class I, HDACs 4, 5, 7 and 9 belong to subclass IIa, HDACs 6 and 10 belong to subclass IIb, and HDAC11, class IV [4]. The human enzymes are well characterized and have known sequences and structure organization, particularly for the interest to develop HDAC inhibitors (HDACi) for epigenetic therapy [5]. To date, there are four HDACi approved for cancer treatment by the US Food and Drug Administration (FDA): Vorinostat – also known as suberoylanilide hydroxamic acid or SAHA, Romidepsin, Belinostat, and Panobinostat. There are several clinical trials involving targeted therapy with epi-drugs, as reviewed elsewhere [6–9]. The effect of HDACi in solid tumors alone is usually limited, which leaves a lack in the development of new HDACi and new strategies for combined treatments and sensitization to tumor resistance. Currently, few studies focused on the identification and characterization of new HDACi derived from natural products, reveling a gap to be explored. Indeed, the use of bioinformatics tools for the discovery of epigenetic drugs is continuously being expanded and improved [10], as well as the chemical description of natural products, given their complexity related to biological resources, variable composition, and/or complex reaction products, that may include partially unknown constituents [11]. There is evidence that royal jelly contains a unique fatty acid, the (E)-10-Hydroxy-2-decenoic acid (10-HDA), a possible HDACi that might contribute to the caste switching in honeybee (*Apis mellifera*). although some female larvae share the same genome, the amount and duration of royal jelly that is fed to them that determines the development fate [12]. Larvae that will become queen receives a diet rich in royal jelly for longer periods of days, while the ones to become workers receive royal jelly only at initial days, then their diet is changed to honey and other compounds [13,14]. This evidence was recently

restated by the discovery that honeybee histone proteins are extensively post-translationally modified and show caste-specific signatures [15], reinforcing the hypothesis that chromatin modifications play a key role in the establishment and maintenance of caste-specific transcriptional programs in the honey bee [16]. Additionally, two independent research groups suggested similar effects of 10-HDA in recombinant human HDACs [17,18].

Here, we will compare the known genes and protein structures of the *Apis mellifera* histone deacetylases (Hdacs), describing the similarities with the human orthologs. Given that fatty acids such as phenyl butyrate [19] and valproic acid [20] are known HDACi, and that 10-HDA has experimental evidence as a promising mid-chain fatty acid to be an HDACi implicated in the caste switching in bees [17,18], we hypothesize that other fatty acids in royal jelly could be similar to 10-HDA in structure and/or HDACi activity. We aim to perform an *in silico screening* of the royal jelly derived fatty acids to compare their docking solutions with the binding mode of the widely studied HDACi, SAHA , based on previous protocols from our research group [21–23]. We hypothesize that human HDACs may interact with the 10-HDA, thus supporting future investigation about their putative interaction. Further, since HDAC family members are differentially expressed in human cancer, we also investigate the expression profile of HDAC genes of human primary tumor samples (33 different tumor types, a pan-cancer analysis (PANCAN) from TCGA database (The Cancer Genome Atlas) available at portal.gdc.cancer.gov [24,25]. Thus, we aim to better evaluate the putative targets for inhibition, under a perspective of the epigenetic therapy.

2. Materials and Methods

2.1. Expression profile of classical histone deacetylases in human cancer

The coding genes for the eleven classical HDACs (zinc dependent) identification was retrieved as their standard names in <https://www.genenames.org/>. RNA sequencing data from the TCGA [25] were retrieved from 33 tumor types* (ACC, BLCA, BRCA, CESC, CHOL, COAD, DLBC, ESCA, GBM, HNSC, KICH, KIRC, KIRP, LAML, LGG, LIHC, LUAD, LUSC, MESO, OV, PAAD, PCPG, PRAD, READ, SARC, SKCM, STAD, TGCT, THCA, THYM, UCEC, UCS and UVM) and also from normal tissues data available at The Genotype-Tissue Expression (GTEx) [26,27] using the UCSC Xena Explorer (xena.ucsc.edu/) [28]. Since our focus is on carcinomas, the sarcomas, leukaemias, lymphomas, as well as the TCGA datasets PCPG, HNSC, and MESO were excluded. We only included in our study the TCGA datasets of tissues that could be compared with normal tissues from GTEX, considering that they should have at least 10% number of normal samples in comparison with the tumor samples for a valid comparison. The differentially expressed genes were defined with log2FoldChange > +1 or < – 1 and adjusted p-value (student t test) of <0.01 according to Binomial Negative Distribution (Pascal). All statistical analysis and data normalization were performed in accordance with manual of the DESeq2 package for R language [29], available on Bioconductor (v 3.8) (bioconductor.org/packages/release/bioc/html/DESeq2.html). We filtered primary tumor samples RNAm data and normalized HTSeq counts and RSEM expected counts by UCSC Xena Explorer (docs.gdc.cancer.gov/Data/Bioinformatics_Pipelines/Expression_mRNA_Pipeline). The volcano plot was built on GraphPad Prism 7 (San Diego, CA) software, and the alluvial diagrams on RawGraphs (app.rawgraph). The full report with log2FoldChange values for each gene is available at Supplementary File 1 (S1).

*Pan-Cancer analysis (PANCAN) was performed in the TCGA projects as listed by their official abbreviations: adrenocortical carcinoma (ACC), bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), endocervical adenocarcinoma (CESC), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), lymphoid neoplasm diffuse large B cell lymphoma (DLBC), esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), acute myeloid leukemia, lower-grade glioma (LGG), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), mesothelioma (MESO), ovarian (OV), pancreatic adenocarcinoma (PAAD), pheochromocytoma and paraganglioma (PCPG), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ), sarcoma (SARC), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), testicular germ cell tumors (TGCT), thyroid carcinoma (THCA), thymoma (THYM), uterine corpus endometrial carcinoma (UCEC), uterine carcinosarcoma (UCS), and uveal melanoma (UVM).

2.2. The histone deacetylase family in *Apis mellifera* and *Homo sapiens* genomes

We searched for genomic data for *Apis Mellifera* available at public databases with different evidence levels. At the Ensembl website (*metazoa.ensembl.org/Apis_mellifera*) there is information from BeeBase [30], where each consortium provides genomic data [31–33], including their respective records for UniProt [34] and ProtoBee [35], considering Amel_4.5 genome assembly. We also compared it to the most recent assembly, the Amel_HAv3.1 [31] to verify if the Ensembl, HymenopteraMine v1.4 and NCBI [36] provide identical records for the histone deacetylases. The representation of human *(Homo sapiens*, Hs) and Honeybee (*Apis mellifera*, Am) genomes are given in a proportion of 1:9 using hg38 assembly data retrieved by the circus repository with genomic data [37,38] and the coordinates for Amel_4.5 assembly [32] were manually entered. The amino acid sequences were retrieved from UniProt [34] and are available at Supplementary File 2 (S2). Phylogenetic Tree built with classic human HDACs and the honeybee orthologs on Clustal Omega [39] and is available in Supplementary File 3 (S3). Branch lengths, connections, nodes and labels are given as result of multiple protein sequence alignment that was also performed on Clustal Omega [39] and visualized with iTOL [40]. The same input file (S1) was used for multiple protein sequence alignment performed on Clustal Omega [39] and visualized with Jalview 2.11.1.3 [41] including the 11 human HDACs and the 4 honeybee orthologs. The pairwise comparison between Hs_HDAC1 and Am_Hdac1, Hs_HDAC2 and Am_Hdac1, Hs_HDAC3 and Am_Hdac3, and Hs_HDAC6 and Am_Hdac6 was calculated by Jalview 2.11.1.3 [41] software is available at Supplementary File S4. The curation of the deacetylase domain was performed using PFAM [42], InterProScan [43], and MOTIF <https://www.genome.jp/tools/motif/>.

2.3. Royal jelly derived fatty acids: characterization of physicochemical and structural properties

We performed a non-systematic review on the PubMed database (pubmed.ncbi.nlm.nih.gov) to list the fatty acids found in royal jelly (specifically those named by the authors as a unique entity). The given names were used for structure search on PubChem [44], then their compound identification number (CID) was manually curated to guarantee their identities – Supplementary File 4 (S4). The fatty acids list (**Table 2**) was uploaded on ChemMine Tools Web Server [45] to estimate the chemical diversity of the dataset. Subsequently, we used ChemMine Tools, an online service for small molecule data analysis. It provides a web interface to a set of cheminformatics and data mining tools that are useful for various analysis routines performed in chemical genomics and drug discovery [45] and runs using R packages [46] and C++ language [47]. The binning clustering tool was used with the 0.5 cutoff, with *cmp.cluster* software [45]. Hierarchical clustering was performed using *hclust, it* organizes compounds by similarity in a tree with branch lengths proportional to the item-to-item (compound-to-compound) similarities. The configurations chosen to generate the distance matrix heatmap were single linkage method, physicochemical properties from OpenBabel Descriptors [48], and Z-scores displayed (empty data removed). The chemical space was computed using the script from the literature [49], in order to determinate the cores of the fatty acids and further compare their docking interactions.

2.4. Molecular docking

Based on the aforementioned data, we chose the human HDAC model that was listed as a suitable template for the Am_Hdac1 and Am_Hdac3 by SWISS-MODEL – detailed description about the server and full reports available at Supplementary File 5 (S5) [50]. Therefore, we used the only model that accomplished our criteria: the PDB ID - 4LXZ, a high resolution x-ray crystallography of HDAC2 bound to SAHA [51], using the active domain (most conserved and the representative of *Apis mellifera*) as the area of interest for our *in silico* analysis of interactions with RJ derived fatty acids. Supplementary File S5 details the process of *in silico* screening. At PDB database (rcsb.org) [52] the chosen structure of HDAC2 is available under the accession code 4LXZ and its resolution is 1.85Å [53]. The model represents 3 chains of HDAC2 enzyme in complex with suberoylanilide hydroxamic acid (SAHA/Vorinostat/Zolinza®) and tetraethylene glycol. Chains B and C were removed, and all coordinates and models used in this work are relative to chain A. We also downloaded the 3D structure of SAHA available on PubChem [44] (PubChem CID: 5311). We proceeded with the default UCSF Chimera 1.14 software [54] settings, with adjustments recommended by the literature [55] attributing charges and keeping water molecules. The file was saved as MOL2 and converted to PDB and then to PDBQT using OpenBabel 2.3.2 software [48,56]. In order to evaluate the most similar pose to the crystallographic model, we calculated the RMSD using LigRMSD web server [57]. The compounds identification (CID) list (Supplementary File S4) was used as input on PubChem Download Service (https://pubchem.ncbi.nlm.nih.gov/pc_fetch/pc_fetch.cgi) [36,58,59]. A single SDF file containing all molecules was generated and split using OpenBabel 2.3.2 [48,56] and then each molecule was converted into PDBQT format. The *in silico* screening strategy was performed using the software's recommended script (http://vina.scripps.edu/manual.html#screening) and established as described in Supplementary File S6). To understand the impact of ligand ionization state on self-docking, the PubChem 3D file in SDF format was converted to SMILES using OpenBabel. We used the Dimorphite-DL 1.2.4 software [60] to predict the ionization state of SAHA in the pH range of 6.4 to 8.4. We used the UCSF Chimera 1.14 software [54] to visualize the poses and energy values. The putative interactions with the residues were visualized on BIOVIA Discovery Studio 2020 (Dassault Systèmes, San Diego) and the comparison between the main HDACi candidate, the 10-HDA and the SAHA crystallographic model is available at Supplementary File 7 (S7).

3. Results

3.1. Histone deacetylases are differentially expressed among human cancers

We performed a pan-cancer (PANCAN) analysis aimed to investigate the differential expression of HDAC genes across diverse types of human cancers, comparing them with normal tissues from GTEX. Our findings indicates that among the cancer types analyzed, at least one HDAC genes was up or down regulated. The *HDAC10* was down regulated in all projects, except for TGCT. *HDAC7* was down regulated in BRCA, COAD, KICH, LUAD, LUSC, PRAD, SKCM, and UCS. On the other hand, *HDAC7* is up regulated in TGCT. Interestingly, the class I HDACs are the most frequently overexpressed across several cancer types than class II enzymes. *HDAC2* is up regulated in BRCA, COAD, ESCA, GBM, LUSC, STAD, TGCT, UCEC, and UCS. The other class I genes (*HDAC1, 3* and *8*) are also up regulated in some datasets, although the proportion is lower than the overexpression of *HDAC9*. This latter one draws attention to the ~2.911 log2FoldChange in OV, the highest value among our data. COAD and GBM are particularly characterized by an increase in class I HDACs expression, while LUSC presents high values for *HDAC1, 2* and *9*. TGCT differs with its up regulation for *HDAC1, 2, 3, 7, 8* and *9*, associated with down regulation of *HDAC4* and *11*. Figure 1 summarizes the results.

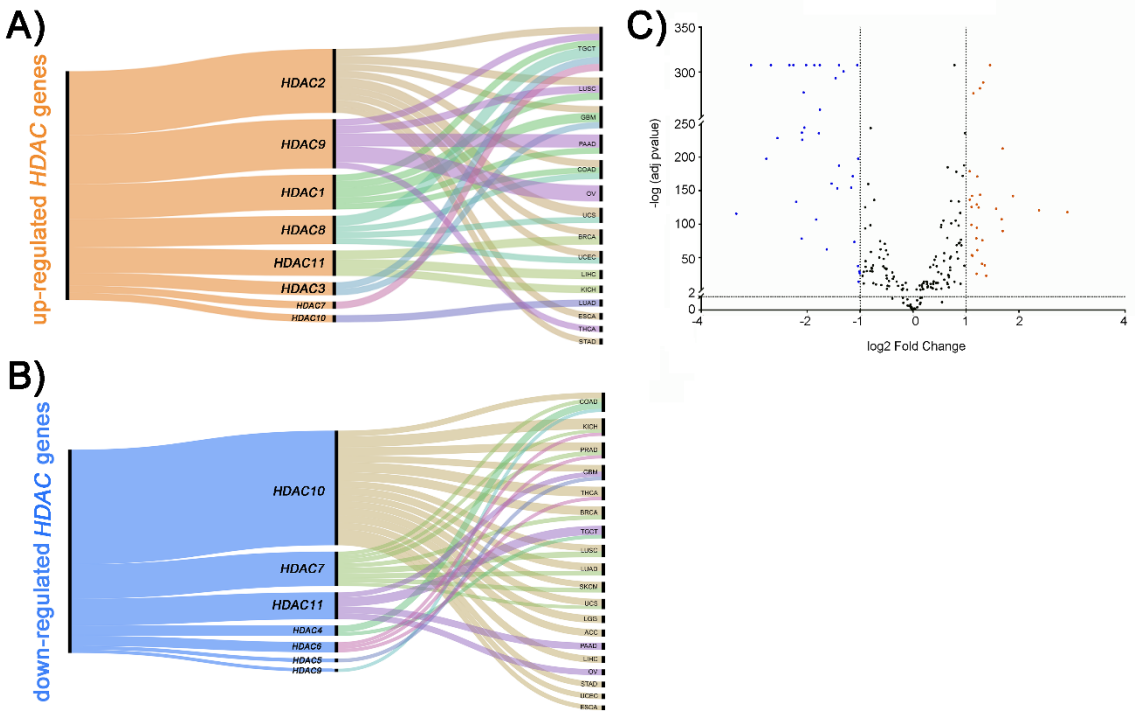

Figure 1. PANCAN expression of HDACs. **A)** The up-regulated and **B)** down-regulated HDACs distribution among the cancer types. The rawgraphs.io webserver was utilized to generate the alluvial diagrams. **C)** Volcano-plot representing the differentially expressed genes among the 19 cancer types, considering log2FoldChange > +1 or < –1, and adjusted p value of 0.01 for significance. Supplementary file S1 presents the specific values of log2FoldChange and p values.

3.2. The histone deacetylase domains are conserved in *Apis mellifera* and *Homo sapiens*

The current knowledge about the honeybee histone deacetylases (Hdacs) is limited to the three whole genome sequencing assemblies (Amel_4.0, its upgrade to 4.5 version, and the HAv3.1 de novo assembly) [31–33] and proteome analysis [35] that provide the databases with different evidence levels. We searched for consensus information at Ensembl, HymenopteraMine and NCBI websites. Interestingly, there are four histone deacetylase genes (*Am_Hdac1, Am_Hdac3, Am_Hdac5 and Am_Hdac6*) and we found records for subunits of histone deacetylase containing enzymatic complexes (SAP18, SAP30, SAP130-A and SDS3), as well as two sirtuin-type domain containing proteins. The classic histone deacetylase orthologs are summarized in Table 1 and the genomic context is illustrated in Figure 2A. Noteworthy, all records are provided by annotations that have not been manually curated, meaning that they can be modified with de advances of new studies and updates in the future.

Table 1. Search results for histone deacetylase orthologs in *Apis mellifera* at Ensembl, HymenopteraMine and NCBI databases.

Gene*	**NCBI ID**	**Amel_4.5 assembly**	Amel_HAv3.1 assembly	Protein**	Score***	Consensus****
Am_Hdac1	411503	chr9:10688165-10691404	**chr9:10901790-10906629**	**UniProtKB: A0A088AP92 (492aa)**	**3**	**Identical**
Am_Hdac3	412350	chr8:6625271-6626572	**chr8:6575527-6577323**	**UniProtKB: A0A087ZYB8 (433aa)**	**3**	**Identical**
Am_Hdac5	408331	chr11:13857048-13870975	chr11:16219663-16243418	UniProtKB: A0A087ZWJ2 (1121aa)	2	Almost identical
Am_Hdac6	725938	chr9:6536824-6544817	chr9:7890969-7898580	UniProtKB: A0A087ZVG5 (1119aa)	1	Almost identical

*Gene names according to the consensus of the databases, preceded by "*Am_"* nomenclature given herein to indicate that it is the *Apis mellifera* ortholog.
**Protein data from UniProtKB and the length of amino acids in brackets.
***Scores ranging from 1 to 5, with increasing levels of evidence. None of the presenting records has been curated by the authors on their databases.
****Consensus of the genes and proteins length when comparing the three databases: Ensembl, HymenopteraMine and NCBI. Almost identical are the ones which have small variations.
Linking Groups of *Apis mellifera* are referred to as chromosomes (chr).
Information from this table was updated according to available data on November 17th, 2020.

Given that the amino acid residues spanning the active site of histone deacetylases are remarkably conserved among different species [9], we evaluated whether the honeybee orthologs are similar to the human enzymes. The phylogenetic tree of the protein sequences (S3) demonstrates that class I Hdacs probably have a closer evolutionary relationship with the human enzymes than the class II ones do. The multiple protein sequence alignment highlighted the conserved domains at the active site main residues of histidine and aspartate. The main variance comes from analysis of the flanking region, where class I and II vary considerably. The details that suggest the human enzymes as models for the honeybee enzymes are demonstrated in the pairwise analysis (S3). The Hs_HDAC1 and Am_Hdac1 have 76.18% of identity and present the highest alignment score (20130.0). Interestingly, the Hs_HDAC2 is also a good model for Am_Hdac1, given the 76.02% identity and score of 20120.0. There are increased gaps and differences between Hs_HDAC3 and Am_Hdac3, reflected by the identity of 69.66% and 16710.0 score. The class II human enzymes are not good models to analyze the honeybee Hdacs because their similarities are restricted to the active site and do not reflect the enzymes topology as denoted by the 40.77% of identity for Hs_HDAC5 and Am_Hdac5 comparison and 36.16% for Hs_HDAC6 and

Am_Hdac6, respectively. Moreover, the Hs_HDAC1 and Hs_HDAC2 are good models to use in the investigation of the binding modes of possible HDACi from RJ. Although there are no available crystal structures of the *Apis mellifera* Hdacs on public databases, the Hdac1 and Hdac3 have higher evidence scores (3 out of 5 at UniProt database, as shown in Table 1) and are suitable for a simulation of tridimensional structure modelling. By using amino acid sequences, their tertiary structures were calculated on SWISS-MODEL webserver [50]. The best fits found were the crystallographic models of human HDACs, and particularly Am_Hdac3 (UniProtKB: A0A087ZYB8) had the best modelling fit, based on GMQE (Global Model Quality Estimation) and QMEAN (estimator of local and global quality) parameters, while Am_Hdac1 (UniProtKB: A0A088AP92) had slightly higher sequence identity and coverage (S6).

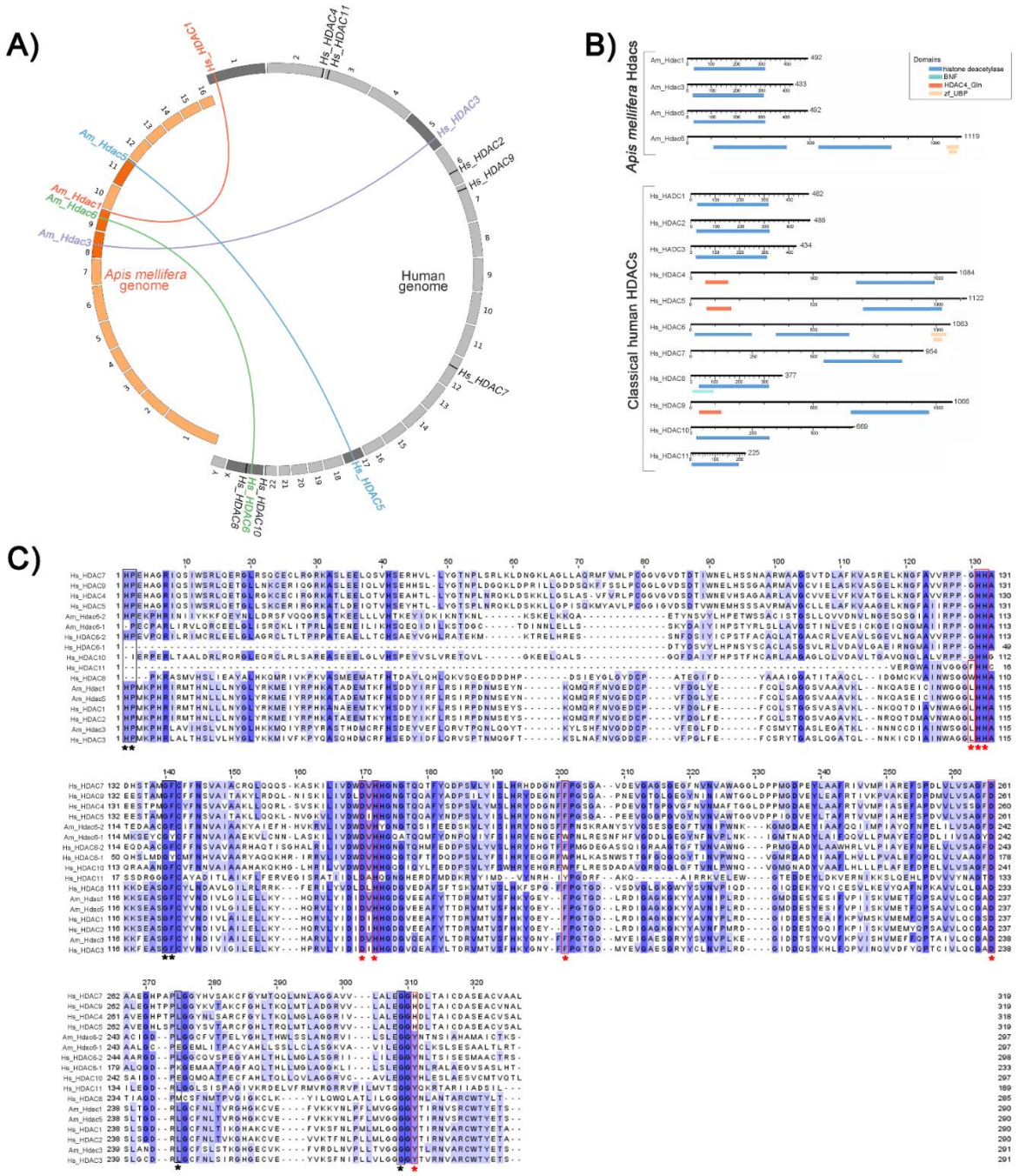

Figure 2. A) Schematic Circular representation of human *(Homo sapiens*, Hs) and Honeybee (*Apis mellifera*, Am) genomes in a proportion 1:9. The human genome is represented according to hg38 assembly, light gray indicating the ideograms of chromosomes that do not include histone deacetylase genes and dark grey, those that do so. The honeybee genome is represented according to Amel_4.5 assembly, light orange indicating the ideograms of the chromosomes (or linking groups) that include histone deacetylase genes and dark orange, those that do so. The links represent orthologs found between the two species, as detailed in Table 1. Figure generated using Circos 0.69-9 [38]. **B)** Deacetylase domains of human and honeybee proteins have very similar lengths and positions. **C)** Multiple protein sequence alignment performed on Clustal Omega [39] and visualized with Jalview 2.11.1.3 [41] including the 11 human HDACs and the 4 honeybee orthologs. The figure illustrates the deacetylase domain of the active site of these proteins (according to PFAM) [61]. The intensity of the shades of blue illustrates de conservation of the residues, the conservation status, quality consensus and occupancy are shown below the sequences. The residues highlighted in red are those involved in the catalytic reaction of deacetylation, thus are the critical ones. The residues highlighted in black are those important for the pocket structural function to interact with the substrate/inhibitor.

3.3. Chemical diversity of royal jelly derived fatty acids

We performed a non-systematic review on the PubMed database (pubmed.ncbi.nlm.nih.gov) to list the commonly fatty acids described in the royal jelly composition (specifically those named by the authors as a unique entity). The given names were used for structure search on PubChem [44], then their compound identification number (CID) was manually curated to guarantee their identities (resulting in the CID list available at Supplementary File S4). The fatty acids list (**Table 2**) was uploaded on ChemMine Tools Web Server [45] to estimate the chemical diversity of the dataset.

Table 2. Fatty acids from royal jelly

Compound name*	CID	Reference
3,11-dihydroxydodecanoic acid	11535975	[62]
3,9-Dihydroxydecanoic acid	24990605	[62]
3-hydroxydecanoic acid	26612	[62]
4,10-dihydroxy-2-decenoic acid	n/a	[62]
4,9-dihydroxy-2-decenoic acid	n/a	[62]
9,10-dihydroxy-2-decenoic acid	25141159	[62]
9-oxo-2-decenoic acid	1713086	[62]
(10R,11R)-dihydroxydodecanoic acid	91394357	[63]
(11S),12-dihydroxydodecanoic acid	11564887	[63]
(11S)-hydroxydodecanoic acid	11637116	[63]
10-Acetyloxydecanoic acid	13497206	[63]
10-hydroxydecanoic acid	74300	[63]
11-Oxododecanoic acid	547024	[63]
3,10-Dihydroxydecanoic Acid	9859090	[63]
3,11-dihydroxydodecanoic acid	11535975	[63]
3-Hydroxydodecanedioic acid	16663321	[63]
4-Hydroxyacetophenone	7469	[63]
6-Propyloxan-2-one	12777	[63]
8-Hydroxyoctanoic acid	69820	[63]
9-Hydroxy2-nonanone	537408	[63]
Acetovanillone	2214	[63]
delta-Decalactone	12810	[63]
Dihydroferulic acid	14340	[63]
Methyl 3-hydroxydecanoate	521747	[63]
Methylparaben	7456	[63]
Octanoic acid	379	[63]
Sebacic acid	5192	[63]
Trans-10-acetoxydec-2-enoic acid	86301641	[63]
2-decene-1,10-dioic acid	181528	[64]
2-octene-1,8-dioic acid	6079438	[64]
7-Hydroxyoctanoic acid	167627	[64]
9-Hydroxy-2-decenoic acid	5312739	[64]
9-Hydroxydecanoic acid	4575239	[64]
10-hydroxy-2-decenoic acid	5312738	[65]
Phenylbutyrate	20354	[14]

**Names shown according to their references, for alternative names, please check their respective PubChem entries.*

From total weight of RJ, 3-8% are lipid, and from these, 80%–85% are free fatty acids [66]. Our data corroborates the literature, as the majority of fatty acids are medium-chain fatty acids, normally 8–12 carbon atoms, some hydroxylated in terminal or internal position, as mono-hydroxyl fatty acids or dicarboxylic acids, and saturated or unsaturated at the 2-position [67], meaning that there is no great heterogeneity. Most molecular structures were similar and had a close relationship when grouped for their structure (Figure 2A), except for those small aromatic fatty acids. The physicochemical characteristics (OpenBabel Descriptors [48]) reflected the same difference for the short-chain fatty acids when compared to the others among the dataset (Figure 2B). The firs and small clusters include molecules with lower molecular weight, distinct patterns of number of atoms, bonds, single bonds, double bonds, hydrogen bonds donors and acceptors, molecular refractivity, and topological polar surface area. Five out of 32 fatty acids had aromatic bonds, and none of the molecules has fluorine atoms nor triple bonds, so we excluded these items in the analysis.

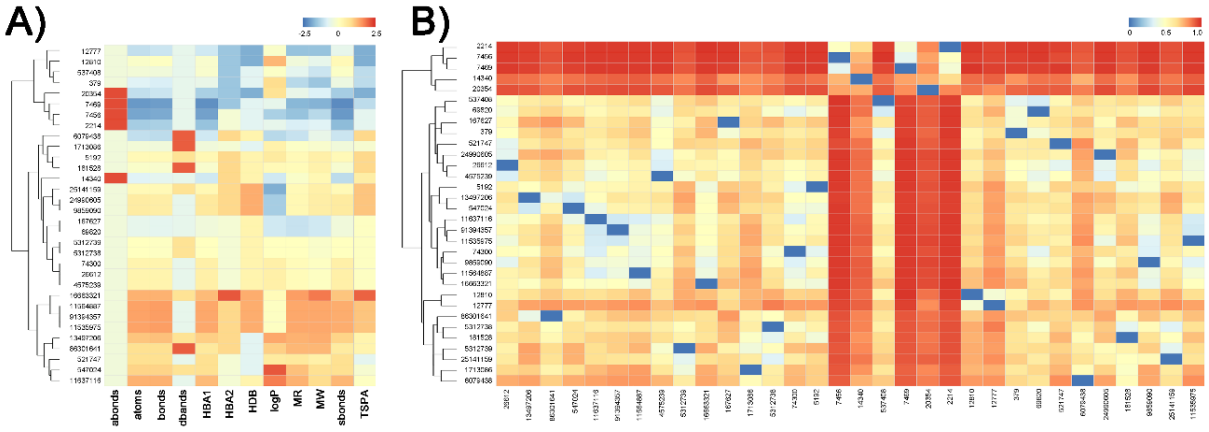

Figure 3. Heatmap showing the hierarchical clusterization of royal jelly derived fatty acids. The PubChem Compound Identification (CID) is shown as label for the molecules. Values shown as z-scores and variables named according to OpenBabel Descriptors (https://open-babel.readthedocs.io/en/latest/Descriptors/ descriptors.html). Figure generated using ChemMine Tools web server [45].

3.4. Royal jelly derived fatty acids might interact with HDAC2 domain

We validated our *in silico* screening strategy testing the smallest RMSD value for self-docking of SAHA into HDAC2 active site (chain A). Figure 3 represents the self-docking (Figure 3B, left panel) and the clusters generated by chemical space analysis: cluster M14, cluster M1 and cluster M53 (Figure 3B, right panel). The ligands share common interactions with PHE155, PHE210, LEU276, TYR308, ASP104, ASP181, ASP269, GLY154, GLY306, HIS145, HIS146, HIS183, the zinc ion (401), and the water molecules HOH606 and HOH791. However, the nature of this interactions can vary from Pi-Alkyl and Pi-pi for PHE210 and PHE155, from van der Waals to conventional hydrogen bond for ASP104, TYR308, and HIST145.

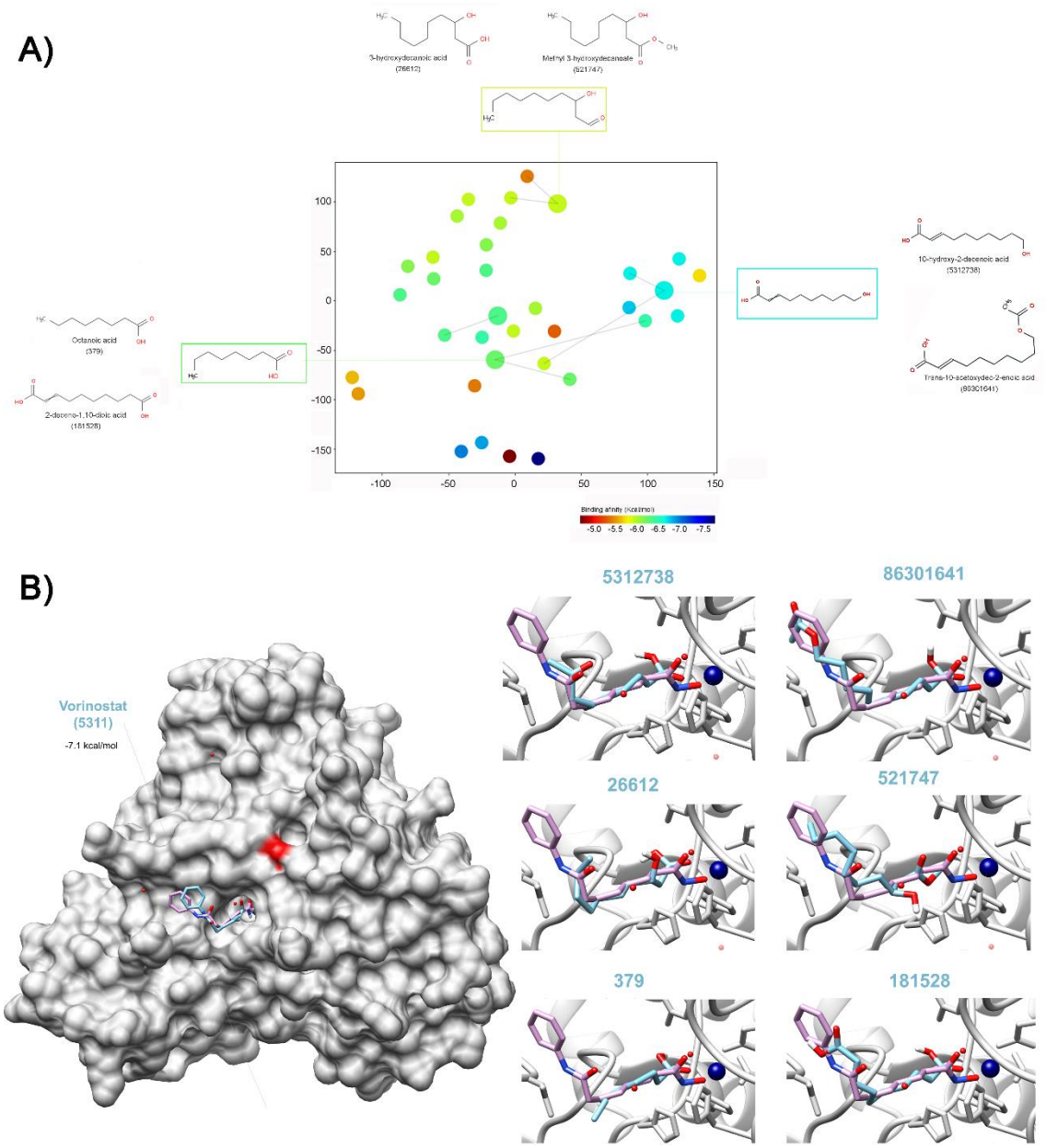

Figure 4. A). Chemical space with the cores for each cluster calculated. The clusters that demonstrated significative connections were M1 (color of core molecule in green lime), M14 (color of the core molecule in light blue) and M53 (color of core molecule in green). The scale of distribution of colors that rages from red to blue are proportional to the binding energy values in kcal/mol. Interestingly, the molecules with most favorable values did not present significative connections in this analysis. **B)** In the left panel, it is shown the crystallographic model of HDAC2 (chain A) in light gray (surface on) with SAHA co-located at the active site of the enzyme (lilac), close to the zinc ion (dark blue). In the right panel, the docking solutions of the best positioning of compounds highlighted in Figure 3A. Water molecules represented as red spheres, Nitrogen atoms represented in blue, Oxygen in red and Hydrogen in White. Image generated using UCSF Chimera 1.14 software [54].

Interestingly, the DHFA was ranked as the best docking solution, followed by SAHA but with close free binding energy values, but it did not connect with 10-HDA in chemical space analysis, given their structural differences. The 10-HDA and 9-OXO showed remarkably similar binding modes, however, 10-HDA presented higher energy values than other similar 10-carbon-long carboxylic acids. Also, phenyl butyrate had unexpectedly high energy values. Apocynin, methylparaben and DHFA presented similar poses inside the "enzymatic pocket", which could explain their ranking despite the energy values. Among the docking solutions with unfavorable performance, phenyl butyrate, delta-decalactone, delta-octalactone and methyl 3-hydroxydecanoate were closely related with each other, all locating "far" from zin ion when compared to better-ranked solutions. Table 3 summarizes all docking solutions with their respective energy values. The 10-HDA achieved minimum free binding energy of -6.1 kcal/mol, an unexpected result, which we believe is due to the ligand ionization state. When the 10-HDA is deprotonated at Oxygen 2, the energy value reaches -7.1, the same as SAHA. The variation of this energy values is beyond the scope of molecular docking, but can be investigated using other strategies, such as molecular dynamics and QSAR. The overall docking solutions included at least one torsion similar to the one visualized for SAHA - an almost perpendicular plane, projected outside the active site. Our results suggest that (in theory) 10-HDA and similar compounds could interact with the HDAC active site.

Table 3. Ranking of docking results from AutoDock Vina

Rank	CID	Compound Name	Energy (kcal/mol)*
1	14340	Dihydroferulic acid (DHFA)	-7.6
2	5311	Vorinostat (SAHA)	-7.1
3	7456	Methylparaben or Methyl 4-hydroxybenzoate	-6.8
4	7469	4'-Hydroxyacetophenone (Piceol)	-6.7
5	2214	Acetovanillone (Apocynin)	-4.6
6	6079438	2-Octenedioic acid	-6.6
7	1713086	9-oxo-2-decenoic acid (9-OXO)	-6.5
8	5312739	(E)-9-Hydroxy-2-decenoic acid	-6.5
9	86301641	(E)-10-Acetoxy-2-decenoic acid	-6.5
10	181528	(E)-2-Decene-1,10-dioic Acid	-6.3
11	13497206	10-acetyloxydecanoic acid	-6.2
12	25141159	(E)-9,10-Dihydroxy-2-decenoic acid	-5.6
13	5192	Sebacic acid (SEA)	-6.3
14	16663321	3-hydroxydodecanedioic acid	-6
15	547024	11-Oxododecanoic acid	-5.9
16	91394357	(10R,11R)-10,11-Dihydroxydodecaneoic acid	-6.2
17	11637116	(S)-11-Hydroxydodecanoic acid	-6
18	167627	7-Hydroxyoctanoic acid	-5.8
18	11564887	(11S)-11,12-Dihydroxydodecanoic acid	-6.2
19	69820	8-Hydroxyoctanoic acid	-5.8
20	74300	10-Hydroxydecanoic acid	-6.1
21	4575239	9-hydroxydecanoic acid	-6.1
22	5312738	(E)-10-hydroxydec-2-enoic acid (10-HDA)	-5.8
23	379	Caprylic acid	-6.1
24	11535975	3,11-dihydroxydodecanoic acid	-5.9
25	24990605	3,9-dihydroxydecanoic acid	-5.8
26	9859090	3,10-dihydroxydecanoic acid	-5.9
27	537408	9-hydroxynonan-2-one	-5.2
28	20354	Phenyl butyrate	-5.3
29	26612	3-hydroxydecanoic acid	-5.8
30	12810	Delta-decalactone	-5.5
31	12777	Delta-octalactone	-5.4
32	521747	Methyl 3-hydroxydecanoate	-5.3

**Energy values are respective to the best conformation chosen.*

Interestingly, when the molecular characteristics of the fatty acids are clustered, there is no clear correlation with the physicochemical properties of the fatty acids and the molecular docking results. Conversely, the top 4 docking results present remarkable similarity, though when top 10 results are analyzed, no pattern in structure is observed. Moreover, both hierarchical and binning clustering locate the 10-HDA in a dense cluster that diverges from cluster the top 5 docking results. This indicates that this dataset is slightly diverse, and it reflects the docking results raking.

4. Discussion

The conservation of the HDACs proteins in different organisms suggests that each class has a non-redundant role in basic cellular processes, although HDACs often appear to have similar or overlapping substrates [68]. Our findings support the possibility to use the human enzymes as models to understand other organisms and vice versa, because the conservation of their amino acid sequence and overall function [9] has been improved the extrapolation of the molecular knowledge between model organisms. Similar to human enzymes, the Am_Hdac5 has a histidine instead of a tyrosin (Am_Hdac5 residue H1006, *versus* Hs_HDAC2 residue Y308) and for class I and IIb, the tyrosine residue is conserved, as confirmed by other study that additionally highlighted that residues P942, E973 and H976 of Hs_HDAC4 are conserved among class IIa, whereas class I had arginine, glycine and tyrosine residues at these positions, respectively [69]. The conservation of the key amino acids involved in the catalytic reaction indicates that the *Apis mellifera* Hdacs probably have similar biochemical features with the human orthologs. This is supported by evidence that known (human) HDACi have biological effects on honeybees [70,71].

From total weight of RJ, 3-8% are lipid, and from these, 80%–85% are free fatty acids, 4–10% phenols, 5–6% waxes, 3–4% steroids, and 0.4–0.8% phospholipids [66,72]. Based on the scientific literature review of RJ chemical composition, the majority of fatty acids are medium-chain, normally 8–12 carbon atoms, some hydroxylated in terminal or internal position, as mono-hydroxyl fatty acids or dicarboxylic acids, and saturated or unsaturated at the 2-position [67], meaning that there is slight heterogeneity. The fatty acids composition in other bee products has been reported to be different than the royal jelly. Recently, 578 components isolated from both honeybee propolis and stingless bee propolis were analyzed and the chemical space and chemical diversity of these compounds was characterized [73]. Phenolics and terpenoids were the two compound classes that were most often found in propolis, and the main fatty acids identified were isocupressic acid, mangiferonic acid, benzoic acid and moronic acid [73]. In honey, caffeic acid, isoferulic acid, p-coumaric acid, gluconic acid and citric acid, among others are the most abundant – a slight overlap of its composition with propolis [74,75]. In beebread, the fatty acids occurring are hexadecenoic acid, octadecanoic acid, icosanoic acid and omega 3, 6, and 9 family of compounds [76], as well as caffeic and ferulic acids [77,78]. In beeswax, the free fatty acids fraction (12%–14%) is represented by long-chain compounds (24–32 carbons long) [79,80] and in bee venom there are no organic acids reported [81,82]. It

is suggested that a small fraction of some of the cited phenolic compounds is commonly shared between propolis, royal jelly and honey, such as ferulic acid, quercetin, kaempherol, galangin, pinocembrin, naringin, apigenin, acacetin, and chrysin [83]. Notwithstanding, the aforementioned data suggests that there are different lipidic profiles for each bee product, which reinforces that their different composition could explain the wide variety of their effects *in vitro* and *in vivo*, and the relevance of natural products databases for understanding their biological roles, and also, to provide new drug candidates to treat human diseases [84–87].

To our best knowledge, this is the first study about the docking poses of royal jelly derived fatty acids inside the active site of HDACs. Fatty acids are a subset of HDACi; however the known molecules are the short-chain fatty acids: valproic acid, 4-phenylbutyrate [88,89] and butyric acid [90]. The inhibition mechanism is commonly the interaction with Zinc ion in the active site, that is shaped like an approximately 11 Å-long channel made up of hydrophobic and aromatic residues. At the bottom of this channel, the key catalytic residues are triad a histidine and two aspartic acid residues [91], as highlighted in Figure 1C, with 100% of residue conservation. The putative interaction of royal jelly derived fatty acids with these residues implies that they could possibly act as weak pan-HDACi for both human and honeybee enzymes.

Data about the association of honeybee products with their bioactivity via molecular docking approach is scarce in the literature. Most studies about the *in silico* assessment of honeybee products with enzymes (of biomedical interest) has been directed to SARS-CoV-2 therapeutic targets [92]. Propolis and honey derived compounds ellagic acid, hesperetin, kaempferol, artepillin C, hesperetin and quercetin were tested against SARS-CoV-2 proteins [93]. The propolis derived flavonoids rutin, myricetin, caffeic acid phenethyl ester (CAPE), hesperetin and pinocembrin were docked against angiotensin-converting enzyme (ACE)-related carboxypeptidase, ACE-II [94]. Additionally, chrysin was effectively docked into spike glycoprotein fragment with human ACE2 receptor, as non-competitive molecule and impart their anti-viral activity by destabilizing spike protein binding with human host ACE2 receptor [95]. Other propolis constituents (such as (+)-Sesamin, Isonymphaeol B, C and D, and β-Amyrin acetate) have been docked into *Mycobacterium tuberculosis* proteins to become possible new anti-tuberculosis drugs [96]. Honey constituents 3-phenyllactic acid, CAPE, lumichrome, galangin, chrysin, and caffeic acid were also tested against SARS-CoV-2 protein [97]. Furthermore, CAPE might interact with human neutrophil elastase (HNE) [98], and *p*-coumaric acid and EGCG might interact with the MTAse domain of the human DNMT1 and

directly compete with its intrinsic inhibitor *S*-Adenosyl-l-homocysteine (SAH) [23]. For royal jelly, some authors have been studying the major royal jelly proteins and their uses for SARS-CoV-2 or Angiotensin-converting enzyme (ACE) [99,100].

The literature about HDACs gene expression in cancer is wide and heterogenous, depending on the technics used, sampling, source of information (patient derived-tissues, cell lines, animal models), analysis type and study design. Since 2009, when it was questioned "What are the cancer relevant targets?", several lines of evidence [101–103] indicate that HDACs are overexpressed in a variety of tumors [104], although clinical trials have not yet found the definitive answer of the "key" HDAC to each cancer type. Here, we contribute to advance the identification of differentially expressed HDAC genes in human cancers, as possible targets for epigenetic therapy of cancer.

HDAC1 was reported overexpressed and associated with poor prognosis in lung [105] and breast cancer [106]. Similarly, *HDAC1* was up regulated in LUSC dataset, but in BRCA it did not achieve significant log2FoldChange (it was approximately 0,888). *HDAC1* was reported up regulated in colorectal cancer patients [107], as well as in pancreatic adenocarcinoma [108] and prostate adenocarcinoma [109], but no other previous studies were found with GBM and TGCT tumor types. Our data corroborates *HDAC2* overexpression in breast cancer seen by other methods using similar approaches and TCGA data analysis [110] and this gene was correlated with poor prognosis [111]. Additionally, *HDAC2* expression seems to be higher in less differentiated tumors [112], multidrug resistance protein, high histological grade and shorter survival in patients who received chemotherapy containing anthracyclines [113] and positive lymph node status, and DNA-damage response genes [111]. *HDAC2* is believed to be overexpressed in colorectal cancer [114], esophageal squamous cell carcinoma [115] and gastric cancer [116,117]. *HDAC9* is thought to be highly expressed and associated with tumor progression and poor prognosis in pancreatic adenocarcinoma [118].

The reduced expression of *HDAC10* draws attention because it is shared between various cancer types. Reduced expression levels of *HDAC10* has been associated with worse prognosis and lower survival NSCLC in patients [119]. As for gastric cancer patients, its expression was an independent prognostic factor, indeed correlated with poor prognosis and lower survival [120]. High cytoplasmic expression of HDAC10 in colon cancer tissues predicts good prognosis, while cytoplasmic HDAC10 expression in para-carcinoma tissues is associated with poor outcome of patients [121].

Study Limitations:

Herein, no molecular dynamics or pharmacophore analysis were performed to restate the putative interactions detected using molecular docking. The effects of HDACs in tumors are not limited to their mRNA expression levels, given the interactions with other epigenetic machinery enzymes and the absence of proteome data from TCGA to prove their transcription to be translated into proteins. Nonetheless, altogether our results indicate that HDACs are good targets for epigenetic therapy of cancer and that HDAC2, besides being overexpressed in some cancer types, could also interact with 10-HDA and other royal jelly derived fatty acids.

5. Conclusions

Royal jelly is a promising source of bioactive molecules for biomedical interest. Specifically, its fatty acids such as the 10-HDA and other similar molecules may be useful for new histone deacetylase inhibitors design. Our *in silico* data suggests that 10-HDA could interact with human HDAC2 and that it is a potential target for human carcinomas since it is up-regulated in various tumor types.

6. References

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