UNIVERSIDADE FEDERAL DA FRONTEIRA SUL
CAMPUS CHAPECÓ
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOMÉDICAS - PPGCB

GILNEI BRUNO DA SILVA

ESTUDO DO POTENCIAL ANTINEOPLÁSICO DO ÁCIDO ROSMARÍNICO EM CÉLULAS DE MELANOMA CUTÂNEO

CHAPECÓ – SC
2023

GILNEI BRUNO DA SILVA

ESTUDO DO POTENCIAL ANTINEOPLÁSICO DO ÁCIDO ROSMARÍNICO EM CÉLULAS DE MELANOMA CUTÂNEO

Dissertação apresentada ao programa de Pós-Graduação em Ciências Biomédicas da Universidade Federal da Fronteira Sul (UFFS), como requisito para obtenção do título de Mestre em Ciências Biomédicas.

Orientadora: Prof.ª Dra. Margarete Dulce Bagatini
Coorientador: Prof. Dr. Marcelo Moreno

CHAPECÓ – SC

2023

UNIVERSIDADE FEDERAL DA FRONTEIRA SUL

Av. Fernando Machado, 108 E
Centro, Chapecó, SC - Brasil
Caixa Postal 181
CEP 89802-112

Bibliotecas da Universidade Federal da Fronteira Sul - UFFS

Silva, Gilnei Bruno da
Estudo do Potencial Antineoplásico do Ácido Rosmarínico em Células de Melanoma Cutâneo / Gilnei Bruno da Silva. -- 2023.
131 f.:il.

Orientadora: Doutora Margarete Dulce Bagatini
Co-orientador: Doutor Marcelo Moreno
Dissertação (Mestrado) - Universidade Federal da Fronteira Sul, Programa de Pós-Graduação em Ciências Biomédicas, Chapecó,SC, 2023.

1. Melanoma Cutâneo. 2. Ácido Rosmarínico. 3. Composto Fenólico. 4. Efeito Antineoplásico. I. Bagatini, Margarete Dulce, orient. II. Moreno, Marcelo, co-orient. III. Universidade Federal da Fronteira Sul. IV. Título.

GILNEI BRUNO DA SILVA

ESTUDO DO POTENCIAL ANTINEOPLÁSICO DO ÁCIDO ROSMARÍNICO EM CÉLULAS DE MELANOMA CUTÂNEO

Dissertação apresentada ao programa de Pós-Graduação em Ciências Biomédicas da Universidade Federal da Fronteira Sul (UFFS), como requisito para obtenção do título de Mestre em Ciências Biomédicas.

Esta dissertação foi defendida e aprovada pela banca em: 31/01/2023.

BANCA EXAMINADORA

MARGARETE DULCE BAGATINI:00440641012
Assinado de forma digital por MARGARETE DULCE BAGATINI:00440641012
Dados: 2023.03.01 13:49:18 -03'00'

Prof.ª. Dra. Margarete Dulce Bagatini – UFFS

Orientadora

ames Dr. Marcelo Moreno Oncologia Oncológica
Assinado de forma digital por Marcelo Moreno
Dados: 2023.03.01 18:51:42 -03'00'

Prof. Dr. Marcelo Moreno – UFFS

Coorientador

Prof.ª. Dra. Zuleide Maria Ignácio – UFFS

Avaliadora

gov.br
Documento assinado digitalmente
ANIELA PINTO KEMPKA
Data: 01/03/2023 14:34:15-0300
Verifique em https://verificador.iti.br

Prof.ª. Dra. Aniela Pinto Kempka – UDESC

Avaliadora

AGRADECIMENTOS

Agradeço a Deus por sempre estar comigo e oportunizar as maravilhas da vida.

Agradeço a toda minha família pelo carinho e suporte ao longo do percurso formativo.

Agradeço aos meus colegas de mestrado pelas trocas de experiências e parcerias, em especial a minha parceira de pesquisa Daiane Manica.

Expresso minha gratidão a minha orientadora Dra. Margarete Dulce Bagatini, e ao meu coorientador Dr. Marcelo Moreno, por todos os ensinamentos e trocas de experiências.

Agradeço ao Fundo de Apoio à Manutenção e ao Desenvolvimento da Educação Superior de Santa Catarina (FUMDES) pelo suporte financeiro por meio de bolsa de mestrado.

Agradeço a Universidade da Fronteira Sul (UFFS).

MUITO OBRIGADO!

RESUMO

Introdução: o melanoma cutâneo é uma neoplasia maligna que possui prognóstico reservado nos estágios III e IV, devido ao insucesso do tratamento sistêmico. Com os avanços tecnológicos, diversos compostos bioativos de origem natural vêm sendo estudados e apresentado resultados promissores como forma de terapia adjuvante para neoplasias malignas. Um desses compostos que se destaca é o ácido rosmarínico (AR). Da mesma forma, o sistema purinérgico tem sido apontado por diversas pesquisas como um interessante alvo na terapêutica antineoplásica. Por outro lado, o estresse oxidativo também parece estar envolvido na fisiopatologia do câncer. **Objetivo:** analisar *in vitro* o potencial antineoplásico do ácido rosmarínico em células de melanoma cutâneo através dos parâmetros de estresse oxidativo e sinalização purinérgica. **Metodologia:** as células SK-MEL-28 foram cultivas em meio DMEM sob condições mínimas necessárias. No controle negativo as células neoplásicas foram tratadas somente com meio de cultura. Na sequência, as culturas foram tratadas com as concentrações de 50 µM, 100 µM, 200 µM, 400 µM e 800 µM de AR durante 24 horas. Foram também utilizadas as células mononucleares de sangue periférico para verificação da viabilidade celular e citotoxicidade em células não neoplásicas. Após os tratamentos, foram coletados o sobrenadante e as células neoplásicas para a avaliação da viabilidade celular e potencial citotóxico, parâmetros de estresse oxidativo e sistema purinérgico. Os parâmetros de estresse oxidativo foram avaliados através dos níveis das defesas antioxidantes PSH e NPSH, e pró-oxidantes EROs. Também foi avaliada a expressão gênica das caspases 8 e 3, inflamassoma (NLRP3), a atividade enzimática da caspase 3, e a formação de corpos apoptóticos. Por fim, em relação ao sistema purinérgico, foi verificada a expressão gênica e proteica da CD39 e CD73, atividade enzimática da CD39, CD73 e ADA, e quantificação dos níveis extracelulares de ATP. **Resultados:** Verificou-se que o AR reduziu significativamente a viabilidade das SK-MEL-28, enquanto não teve efeito citotóxico sobre as células não neoplásicas. Além disso, em altas concentrações também apresentou potencial antioxidante promovendo redução nos níveis de EROs, e aumento nos níveis de PSH e NPSH. O AR aumentou a expressão gênica das caspases e 8 e 3, e reduziu a expressão do NLRP3. A atividade enzimática da caspase 3 também foi aumentada. Foi possível detectar a formação de corpos apoptóticos nas concentrações de 400 µM e 800 µM. Quanto aos parâmetros purinérgicos, o AR em

altas concentrações modulou a expressão gênica e proteica da CD73. A atividade enzimática das ectonucleotidases CD39, CD73 e ADA também sofreu modulação. Não foi detectada alteração nos níveis extracelulares de ATP. **Conclusão:** O AR é um potente candidato a ser estudando em ensaios clínico para o tratamento do MC. Nesse estudo com células de MC da linhagem SK-MEL-28 o AR reduziu a viabilidade celular neoplásica, modulou a expressão gênica e proteica de mediadores apoptóticos, contribuiu para o equilíbrio redox, bem como modulou a sinalização purinérgica.

Palavras-chave: Melanoma. Ácido rosmarínico. Radicais livres. Sinalização purinérgica. Inflamação.

ABSTRACT

Introduction: cutaneous melanoma is a malignant neoplasm that has a poor prognosis in stages III and IV, due to the failure of systemic treatment. With technological advances, several bioactive compounds of natural origin have been studied and have shown promising results as a form of adjuvant therapy for malignant neoplasms. One such compound that stands out is rosmarinic acid (RA). Likewise, the purinergic system has been pointed out by several studies as an interesting target in antineoplastic therapy. On the other hand, oxidative stress also seems to be involved in the pathophysiology of cancer. **Objective:** to analyze *in vitro* the antineoplastic potential of rosmarinic acid in cutaneous melanoma cells through parameters of oxidative stress and purinergic signaling. **Methodology:** SK-MEL-28 cells were grown in DMEM medium under minimal conditions necessary. Neoplastic negative control cells were treated only with culture medium. Next, the cultures were treated with concentrations of 50 μM, 100 μM, 200 μM, 400 μM and 800 μM of AR for 24 hours. They were also used as peripheral blood mononuclear cells to verify cell viability and cytotoxicity in non-neoplastic cells. After treatment, the supernatant and neoplastic cells were collected for the evaluation of cell viability and cytotoxic potential, parameters of oxidative stress and purinergic system. Oxidative stress parameters were evaluated through levels of PSH and NPSH antioxidant defenses, and ROS pro-oxidants. The gene expression of caspase 8 and 3, inflammasome (NLRP3), the enzymatic activity of caspase 3, and the formation of apoptotic bodies were also evaluated. Finally, regarding the purinergic system, gene and protein expression of CD39 and CD73, enzymatic activity of CD39, CD73 and ADA, and quantification of extracellular levels of ATP were verified. **Results:** It was found that RA significantly reduced the viability of SK-MEL-28, while it had no cytotoxic effect on non-neoplastic cells. In addition, at high concentrations they also showed antioxidant potential, promoting a reduction in ROS levels and an increase in PSH and NPSH levels. AR increased the gene expression of caspases e 8 and 3, and reduced the expression of NLRP3. Caspase 3 enzymatic activity was also increased. It was possible to detect the formation of apoptotic bodies at concentrations of 400 μM and 800 μM. As for the purinergic parameters, the AR in high concentrations modulated the gene and protein expression of CD73. The enzymatic activity of the CD39, CD73 and ADA ectonucleotidases was also modulated. No change in extracellular ATP levels was

detected. **Conclusion:** RA is a powerful candidate to be study in clinical trials for the treatment of CM. In this study with CM cells of the SK-MEL-28 lineage, the RA showed antineoplastic effect on reduction of cell viability, modulated the gene and protein expression of apoptotic mediators, contributed to the redox balance, as well as modulated the purinergic signaling.

Keywords: Skin cancer. Adjuvant therapies. Free radicals. Purinergic signaling. Inflammation.

LISTA DE ILUSTRAÇÕES

INTRODUÇÃO

MANUSCRITO 1

MANUSCRITO 2

LISTA DE ABREVIATURAS E SIGLAS

ADA	Adenosina desaminase
Ado	Adenosina
ADP	Adenosina difosfato
AMP	Adenosina monofosfato
ANOVA	Análise de Variância
AR	Ácido rosmarínico
ATP	Adenosina trifosfato
C4H	Ácido cinâmico 4-hidroxilase
DNA	Ácido desoxirribonucleico
H_2PO_4	Ácido de ortofosfórico
TBA	Ácido tiobarbitúrico
TCA	Ácido tricloroacético
MTT	Brometo de 3-(4,5-dimetil-2-tiazolil)-2,5-difenil-2H-tetrazólio
BCRJ	*Banco de Células do Rio de Janeiro*
CCR	Câncer colorretal
CoA	Coenzima A
COX-2	Ciclooxigenase-2
CT	Controle negativo
DC's	Células dendríticas
CMSP	Células mononucleares de sangue periférico
VCl_3	Cloreto de vanádio
4CL4	Cumarato-CoA ligase
DCF	Diclorofluoresceína
DCFH-DA	Diclorofluoroscencina
DMASO	Dimetilsulfóxido
CO_2	Dióxido de carbono
DMF	Dimetilformamida
DNPH	Dinitrofenilhidrazina
DTNB	5,5'-Ditiobis(2-Ácido Nitrobenzoico)
5'-NT	Ecto-5'-nucleotidase ou CD73
DMEM	*Eagle Modificado por Dulbecco*
E-NPPS	Ectonucleotídeo pirofosfatase/fosfodiesterase

E-NTPDases	Ectonucleosídeo trifosfato difosfohidrolase ou CD39
ELISA	*Enzyme Linked-Immuno-Sorbent Assay*
EROs	Espécies reativas de oxigênio
FDA	*Food and Drug Administration*
KH_2PO_4	Fosfato de potássio monobásico
PAT	Fenilalanina amonialiase
TGF- β	Fator de crescimento transformador-beta
VEGF	Fator de crescimento endotelial vascular
GSH	Glutationa
HDAC	Histona deacetilases
HPPR	Hidroxifenilpiruvato redutase
3-H, 3'-H	3- e 3'-hidroxilase
OH	Hidroxila
α-MSH	Hormônio estimulador de melanócitos alfa
RAS	Hidroxifenillactato hidroxicinamoil transferase
NLRP3	Inflamassoma do tipo domínio de pirina NLR contendo proteína 3
TME	Microambiente tumoral
MC	Melanoma cutâneo
MDA	Malondialdeído
MMP-2	Metaloproteinases de matriz-2
MMP-9	Metaloproteinases de matriz-9
NK	*Natural killer*
NED	N-1-naftiletilenodiamino-bicloridrato
NOx	Óxido nítrico
BRAF	Oncogene inibidor de sarcoma viral murino homólogo B1 do v-Raf
NRAS	Oncogene homólogo viral de neuroblastoma
MCP-1	Proteína 1 quimio-atraente de monócitos
H_2O_2	Peróxido de hidrogênio
AMPK	Quinase ativada por adenosina monofosfato
MEK	Quinase ativada por mitogênio
FAK	Quinase de adesão focal
UV	Radiação ultravioleta
UVA	Radiação ultravioleta do tipo A
UVB	Radiação ultravioleta do tipo B

UVC	Radiação ultravioleta do tipo C
MC1R	Receptor de melanocortina-1
TLR4	Receptor *Toll-like* 4
RPMI	*Roswell Park Memorial Institute*
PBS	Solução salina tamponada com fosfato
TFK	Tampão fosfato de potássio
NPSH	Tióis não proteicos
PSH	Tióis proteicos
TAT	Tirosina aminotransferase
EMT	Transição epitelial-mesenquimal
TNM	Tumor, nódulos e metástases
IUPAC	União Internacional de Química Pura e Aplicada

SUMÁRIO

APRESENTAÇÃO

Os resultados desta dissertação estão apresentados inicialmente por uma seção denominada **“INTRODUÇÃO”**, que contém uma revisão de literatura dos tópicos mais relevantes sobre o tema de pesquisa, e os **“OBJETIVOS”**. As seções **“MATERIAL E MÉTODOS”**, **“RESULTADOS”**, **“DISCUSSÃO”**, e **“CONSIDERAÇÕES FINAIS”** encontram-se em cada um dos trabalhos acadêmicos produzidos ao longo do percurso formativo. Eles estão evidenciados na seção **“ARTIGO E MANUSCRITOS”**, no formato de um artigo publicado e de dois manuscritos.

Os itens **“DISCUSSÃO”** e “**CONCLUSÃO E CONSIDERAÇÕES FINAIS**”, encontrados ao final desta dissertação, contemplam interpretações gerais elucidadas a partir dos resultados obtidos do artigo e dos manuscritos. A seção “**REFERÊNCIAS**” é relativa às literaturas utilizadas na produção dos itens introdução e discussão gerais desta dissertação.

1 INTRODUÇÃO

1.1 A PELE HUMANA

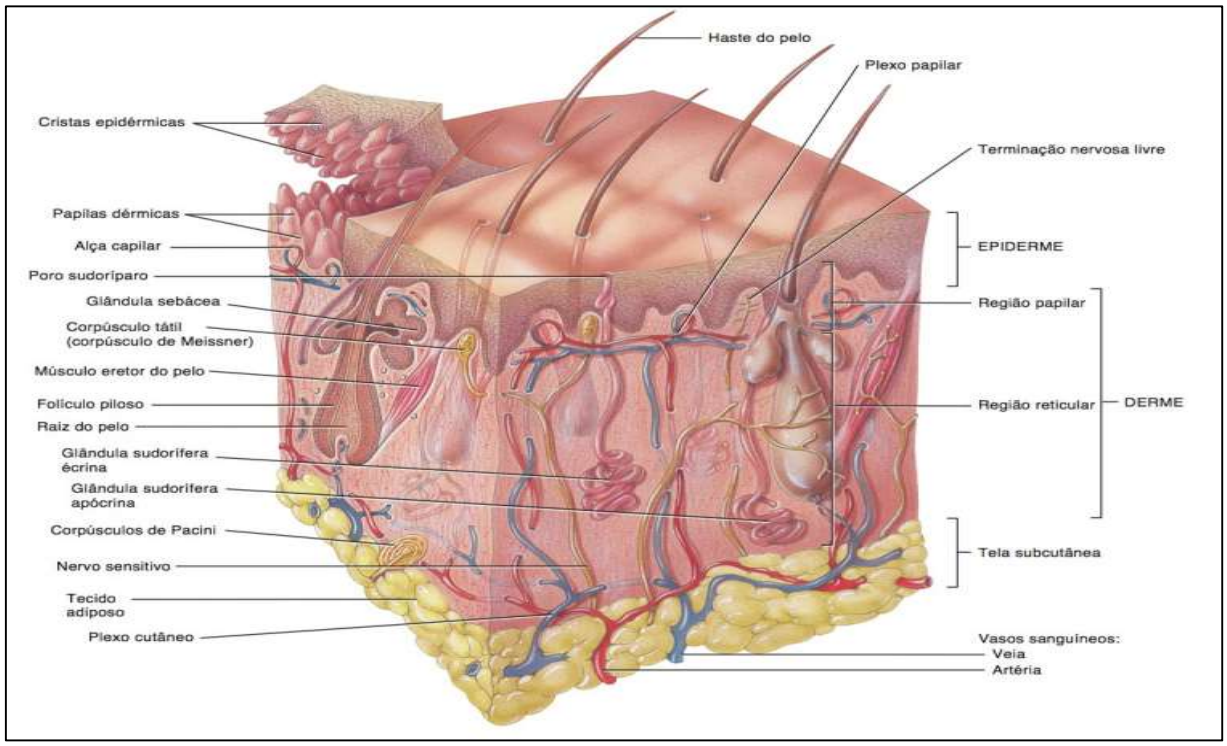
A pele é um complexo órgão e o maior do corpo humano, tanto em peso quanto em extensão, podendo corresponder a 7% do peso total de um indivíduo adulto. Ela recobre toda a superfície externa do organismo, e com isso, protege o indivíduo contra agentes tóxicos, fricção, injúrias, radiação. Além disso, é indispensável no controle da temperatura e perda de fluídos corporais, além de ser uma crucial barreira contra a invasão de patógenos (HSU et al., 2014; NGUYEN; SOULIKA, 2019; TORTORA et al., 2019).

Estruturalmente, esse órgão é dividido em três camadas: a epiderme, a derme, e a hipoderme (Figura 1). A camada mais fina e externa, a epiderme, é avascular e composta por quatro tipos principais de células, que são os queratinócitos, os quais correspondem a 90%, os melanócitos, que representam aproximadamente 8%, e 2% é composto pelas células de *Langerhans* (macrófagos) e pelas estruturas neuroendócrinas conhecidas como células de *Merkel*. A derme, por sua vez, é vascularizada e composta por tecido conjuntivo que contém fibras de colágeno e elastina, bem como os anexos cutâneos como folículos pilosos, glândulas sebáceas e sudoríparas, nervos e células imunes. Por fim, a hipoderme, também conhecida por tecido celular subcutâneo, é a porção mais profunda composta principalmente por tecido adiposo, que funciona como isolante térmico e produtora de diversos mediadores celulares, como citocinas e fatores de crescimento (Figura 1) (CILDIR et al., 2013; HSU et al., 2014; TORTORA et al., 2019).

Quanto à origem embriológica, a pele é derivada de diferentes folhetos embrionários. A epiderme começa a ser formada a partir da ectoderme, por volta da 4ª semana após a fertilização. Por outro lado, a derme origina-se da mesoderme, sendo chamada inicialmente de mesênquima e, por volta da 11ª semana, começam a se formar as fibras de colágeno e elastina, além dos demais componentes. As células de *Langerhans* são provenientes da medula óssea vermelha e se instalam na pele posteriormente. Vale destacar que os melanócitos se desenvolvem a partir da crista neural do embrião, e durante a embriogênese, migram para a camada basal da epiderme. A quantidade de melanócitos em cada indivíduo depende da constituição genética que irá configurar o fototipo cutâneo. Após o nascimento, essas células

possuem a função de produzir melanina, que irá ser depositada sobre o núcleo dos queratinócitos toda a vez que o indivíduo entrar em contato com raios ultravioletas, e com isso, proteger o DNA dessas células contra potenciais danos causados por esse tipo de radiação. Esse processo resulta na pigmentação da pele humana (TORTORA et al., 2019).

Figura 1 – Micromorfologia da pele humana.



Fonte: Adaptado de Tortora et al. (2019).

1.2 CÂNCER

O câncer pode ser definido como o resultado da acumulação de diversas mutações impróprias no DNA, as quais levam a um fenótipo morfofisiológico celular anormal, havendo um desequilíbrio na homeostase. Dessa forma, as células que sofrem essas alterações podem apresentar um comportamento de crescimento celular descontrolado e promover o surgimento de grandes populações com características anormais de tecidos, ou seja, à formação do tumor. Esses tipos celulares possuem mecanismos que conseguem burlar as defesas do organismo para sobreviverem e multiplicarem-se (WEINBERG, 2008).

É importante esclarecer que as mutações são eventos essenciais para a evolução dos seres vivos e nem todas implicam em mudanças detectáveis ou

deletérias ao metabolismo celular (RIBEIRO et al., 2006). Contudo, pesquisas experimentais elucidaram que algumas mutações produzem oncogenes, os quais podem trazer ganhos funcionais que suprem tumores (HANAHAN; WEINBERG, 2000).

Entre os anos 2000 e 2011 foi elaborada a hipótese da existência de 10 *Hallmarks* do câncer, que compreendem marcas compartilhadas entre as células cancerígenas das quais células normais, de forma geral, não possuem. São elas: a autossuficiência da sinalização proliferativa, insensibilidade a sinais anti-crescimento, resistência à apoptose, potencial replicativo ilimitado, sustentação da angiogênese, invasão tissular e metástase, desregulação energética celular com a reprogramação do metabolismo, evasão do sistema imune, inflamação promotora tumoral e instabilidade genômica/mutação (HANAHAN; WEINBERG, 2000; HANAHAN; WEINBERG, 2011).

As referidas mutações oncogênicas dependem de diversos agentes desencadeantes e atualmente sabe-se que os fatores ambientais são os principais promotores que afetam os seres humanos, através de mecanismos epigenéticos. Assim, podem ser divididos principalmente em: fatores físicos, químicos e biológicos. Os fatores físicos são as ondas eletromagnéticas, radiação ionizante e ultravioleta. No caso dos químicos destacam-se o tabagismo, o consumo de álcool, hidrocarbonetos policíclicos aromáticos, pesticidas e as nitrosaminas. E por fim, os biológicos compreendem alguns vírus capazes de alterar o DNA, a dieta desequilibrada, falta de atividade física e a exposição à compostos químicos provenientes dos alimentos ultraprocessados (BELTRÃO-BRAGA et al., 2004; LEWANDOWSKA et al., 2019).

1.3 MELANOMA CUTÂNEO: CARACTERIZAÇÃO EPIDEMIOLÓGICA E CLÍNICA

O melanoma cutâneo (MC) é uma das patologias malignas que acomete a epiderme, caracterizando-se como um distúrbio citológico dos melanócitos, produtores de melanina, aumentando a capacidade proliferativa dessas células devido a uma série de alterações no ciclo celular e nos mecanismos de apoptose. Dessa forma, o MC é uma oncopatologia caracterizada pela alta invasividade das células tumorais e possui alta capacidade metastática, ocasionando um curto período de sobrevida, bem como altas taxas de mortalidade (PADDOCK et al., 2016; SCHADENDORF et al., 2015).

No contexto epidemiológico, um estudo publicado por Enninga et al. (2017) verificou que 201.719 diagnósticos de MC foram feitos entre 1992 e 2011 no mundo. Entre eles, a taxa de sobrevivência era melhor nas mulheres, e a hipótese era a diferença no comportamento e na biologia humana entre os gêneros. Porém, ao se analisar o estágio da doença, não se notou diferença entre os gêneros na taxa de mortalidade. Apesar disso, a sobrevida é melhor quando há diagnóstico precoce em ambos os sexos.

Até o ano de 2020, a incidência de MC no mundo, incluindo ambos os sexos e todas as idades, era de 324.635 novos casos e mais de 57.000 mortes, afetando principalmente a América do Norte, Europa e Oceania. No Brasil, esse tipo de câncer estava entre os 20 mais frequentes, com uma estimativa para 2020 de 4.200 casos novos para homens e 4.250 para mulheres, com maior frequência no Sul do país. No ano anterior (2019) foram registrados 8.624 novos casos (INCA, 2019). Olhando para o futuro, estima-se que para 2030 o aumento será de 15,8% de novos casos em todo o mundo (IARC, 2021). Apesar de MC não ser o tipo de câncer mais prevalente, é responsável por mais de 75% dos óbitos por câncer de pele, sendo considerado um grande problema de saúde pública (REBECCA et al., 2020).

Sabe-se que o principal fator de risco para o desenvolvimento do melanoma é a exposição à radiação ultravioleta, presente na luz solar. Além disso, existem três tipos de radiação UV: UVA, UVB e UVC, embora esta última seja bloqueada pela camada de ozônio, enquanto os UVA e UVB atingem a Terra, a exposição a esses tipos pode causar danos ao DNA e consequente o desenvolvimento de melanoma. Embora o UVA seja a radiação mais emitida (90% - 95%), o UVB é mais propenso a causar queimaduras e danos à pele (SAMPLE et al., 2018; VOLKOVOVA et al., 2012).

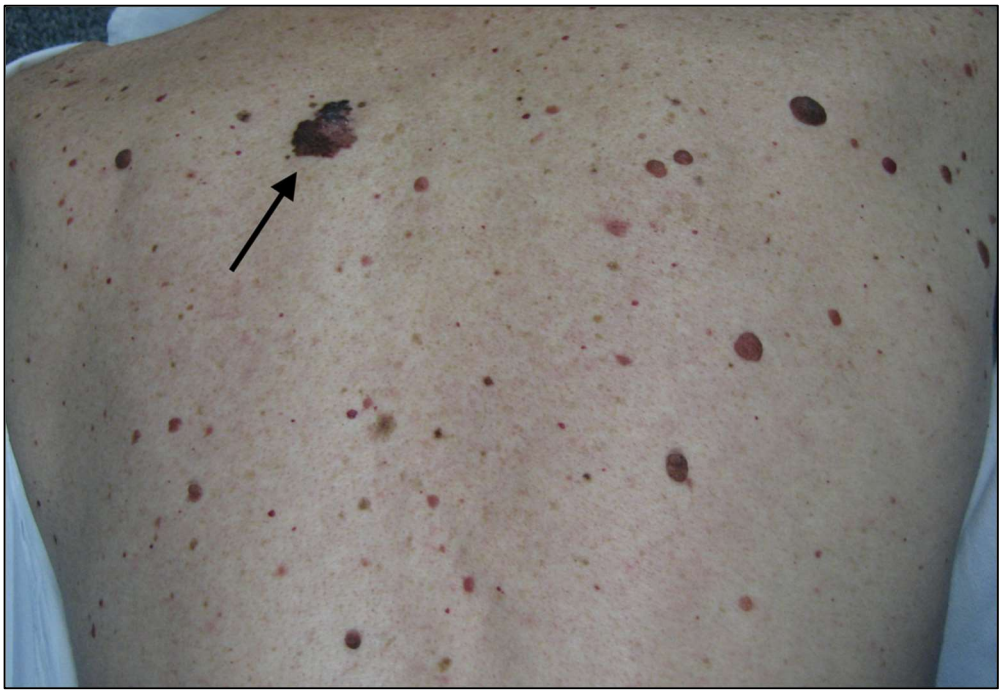
A cor da pele é um fator de risco importante na fisioplatologia do melanoma, pois, em peles e cabelos escuros, a eumelanina é mais abundante, um tipo de melanina que é sintetizada sob ação do hormônio estimulador de melanócitos alfa (α-MSH), sinalizando para um receptor de melanocortina 1 (MC1R), enquanto indivíduos com cabelos ruivos e sardas têm mais feomelanina devido à mutação de perda de função em MC1R que impede a produção de eumelanina. Assim, a eumelanina reduz os danos induzidos pelo UV, ao contrário da feomelanina, que contribui para a formação de radicais livres e, consequentemente, para o dano ao DNA (SAMPLE et al., 2019). Portanto, de acordo com Rastrelli et al. (2014), indivíduos com pele clara, sardas e cabelos ruivos são mais predispostos ao melanoma em aproximadamente

50%, assim como o número de nevos melanocíticos, história familiar e suscetibilidade genética.

Algumas evidências mostram que outros fatores de risco podem exercer uma influência sobre a incidência de melanoma. De forma geral, o risco é maior para as mulheres nas primeiras décadas de vida, enquanto os homens estão mais propensos quando atingem a vida adulta. Mesmo tendo mais diagnósticos femininos iniciais, o prognóstico é melhor, mas não é o mesmo com a idade (PAULSON et al., 2020; VOLKOVOVA et al., 2012).

Considerando a malignidade do MC, o diagnóstico precoce e correto é muito importante para o sucesso do tratamento. No entanto, isso pode ser difícil devido à sua variabilidade na citomorfologia e sua semelhança com lesões benignas (DAVIS et al., 2019; LIU et al., 2020) (Figura 2). A princípio, existem quatro tipos principais de MC, de acordo com suas características histológicas e de crescimento, a saber: disseminação superficial, melanoma nodular, lentigo maligno e lentiginoso acral. A maioria deles permanece na epiderme, crescendo horizontalmente, conhecida como fase de crescimento radial, enquanto na fase de crescimento vertical ocorre infiltração na derme, conferindo-lhe potencial metastático (LIU et al., 2020).

Figura 2 – Aspecto clínico de um melanoma cutâneo em comparação as demais lesões benignas (a seta indica um nevo neoplásico)



Fonte: Arquivo pessoal – Dr. Marcelo Moreno.

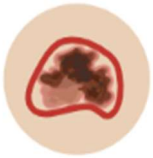
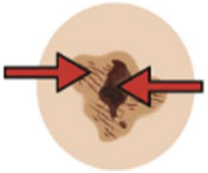
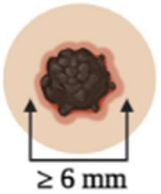
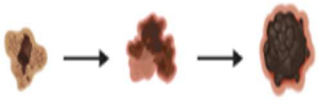
Como já mencionado, o diagnóstico deve ser cuidadoso, uma vez que o MC pode se manifestar de várias formas clínico-patológicas (CABRERA et al., 2018; DAVIS et al., 2019; LIU et al., 2020). No entanto, o padrão ouro para o diagnóstico é a análise histopatológica associada ao reconhecimento clínico. Neste último, é importante estar atento ao histórico do paciente e aos fatores de risco e realizar o exame de pele de todo o corpo, podendo contar com o auxílio da dermatoscopia (Figura 3). Posteriormente, a regra do ABCDE deve ser aplicada a cada lesão, além do sinal “patinho feio” para que um nevo diferente dos demais, no mesmo indivíduo, seja seguido com mais cuidado (Tabela 1). Além disso, as técnicas de imagem podem ser apoiadas para o diagnóstico precoce, como dermatoscopia e confirmação do diagnóstico, sendo que a biópsia de pele é a mais útil (ELDER et al., 2020; SWETTER et al., 2020).

Figura 3 – Dermatoscopia de um melanoma cutâneo.

Fonte: Arquivo pessoal - Dr. Marcelo Moreno.

Tabela 1 – Elucidação da regra do ABCDE no diagnóstico do melanoma cutâneo.

SÍMBOLO	SIGNIFICADO	REPRESENTAÇÃO

A - Assimetria	Áreas tangentes à lesão não são equivalentes.	
B – Borda Irregular	Borda irregular da lesão.	
C – Variação de Cor	Múltiplas cores.	
D – Diâmetro	Diâmetro igual ou superior a 6 mm.	
E – Evolução	Mudança na forma, cor e tamanho ao longo do tempo.	

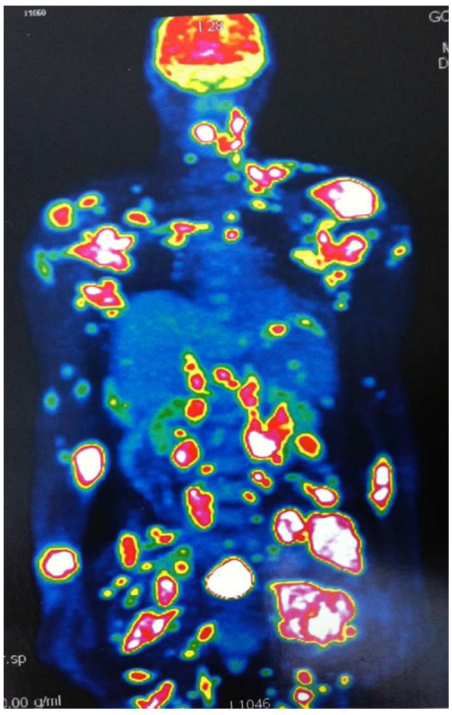
Fonte: Adaptado de Silva et al. (2022).

O MC é uma doença curável quando diagnosticado nos estágios iniciais da patologia (lesão primária), chegando a 95% de sobrevida após 5 anos do diagnóstico. Nesses casos, a ressecção cirúrgica é o tratamento usualmente empregado. Porém, quando as células neoplásicas invadem camadas inferiores da pele ou órgãos adjacentes, como no caso do melanoma metastático, as taxas caem para 65% de sobrevida, e caso atinjam linfonodos, ficam abaixo de 10%, e o tratamento cirúrgico é pouco efetivo (BALCH et al., 2009; SIEGEL et al., 2013, SOENGAS; LOWE, 2003). Por isso, o correto estadiamento do melanoma é de grande importância no diagnóstico médico.

Nesse sentido, o estadiamento do MC é baseado na classificação de tumor, linfonodos e metástase (TLM), onde “T” refere-se ao tamanho do tumor primário, “L” refere-se à invasão ou não dos linfonodos, e “M”' refere-se à presença de metástases à distância. Sumariamente, os estágios do melanoma vão de I a IV, sendo os estágios III e IV os mais críticos e difíceis de serem tratados, visto que as células malignas

podem invadir tecidos como os linfonodos (MICHIELIN et a., 2019) (Figura 4). Mesmo com o advento dos inibidores de *checkpoints* e imunoterapias, pesquisas clínicas que buscaram compreender a efetividade dessas terapias têm evidenciado uma remissão total ou parcial próxima a 31,7% em pacientes com melanoma metastático comparado a terapias quimioterápicas (WEBER et al., 2015). Isso mostra que os tratamentos para os estágios avançados do MC ainda são pouco efetivos.

Figura 4 – Tomografia por emissão de pósitrons (PET-CT) de um paciente com melanoma cutâneo estágio IV (áreas captantes de contraste estão representadas em tons claros).



Fonte: Arquivo pessoal – Dr. Marcelo Moreno.

1.3.1 Resposta imune e inflamação no melanoma cutâneo

O sistema imune humano é o conjunto de células, tecidos e moléculas que participam do mecanismo de defesa contra antígenos invasores, que tentam penetrar no organismo para replicação, o que acaba promovendo um desequilíbrio na homeostase. Este sistema atua com base nos mecanismos de defesa do hospedeiro que são compostos por imunidade inata (nativa), que é mais abrangente e responsável pela proteção inicial, e imunidade adquirida (adaptativa), que é mais específica e eficaz contra infecções. A imunidade inata é composta por macrófagos,

células dendríticas (DCs), *natural killer* (NK), mastócitos, eosinófilos, neutrófilos e outras estruturas não celulares como as citocinas. A imunidade adaptativa, por outro lado, é formada principalmente pelo conjunto de linfócitos T e B, além das citocinas, sendo estas últimas aquelas que representam a imunidade humoral (NICHOLSON, 2016; SATTLER, 2017; SHIBUTANI et al., 2015).

Resultante dos mecanismos efetores de proteção do sistema imune, ocorre a inflamação, que é denominada como o conjunto de complexas funções que as defesas do hospedeiro desempenham com o objetivo de proteger o organismo e manter a homeostase, a fim de eliminar antígenos estranhos e tecidos lesados. Para que essas funções ocorram, há o envolvimento de células, citocinas, vasos sanguíneos, entre outros elementos, os quais preconizam a eliminação da causa e promovem o reparo necessário (KUMAR; ABBAS, ASTER, 2013).

Como acima mencionado, decorrente de inúmeros tipos de danos, a inflamação é a resposta do organismo na tentativa de reparar lesões. Para isso, o sistema imune recruta células de importância, como os leucócitos, linfócitos e os mastócitos teciduais, o qual acarreta nas mudanças celulares e respostas imunológicas que promovem a proliferação celular no tecido lesado e, quando ativada por muito tempo, cria um ambiente propício para a gênese do melanoma (COUSSENS; WERB, 2002).

No contexto tumoral, a literatura tem evidenciado quais são os principais fenótipos de linfócitos que se infiltram no TME, e as suas respectivas funções. A destacar, os linfócitos T citotóxicos (CD8+) exercem a função direta de indução da morte das células tumorais e produção de interferon gama (IFN-y). No caso dos linfócitos T auxiliares (CD4+), eles promovem a produção de citocinas pró-inflamatórias e medeiam a citotoxicidade direta em células tumorais. Os linfócitos T reguladores são responsáveis pela supressão direta e indireta de linfócitos efetores e inibição das funções das células apresentadores de antígenos (APCs). Por fim, os linfócitos B (CD20+), participam ativamente na síntese de anticorpos antitumorais e medeiam a apresentação de antígenos (MAIBACH et al., 2020).

Em contrapartida, essas células inflamatórias, podem produzir grandes quantidades de espécies reativas que reagem com o DNA causando mutações (SINGH, 2019). De igual maneira, a cronificação da inflamação é fortemente associada à gênese do melanoma, uma vez que estudos confirmam a eficácia da terapia anti-inflamatória na prevenção e tratamento do câncer. Além disso, as células inflamatórias estão presentes nos tumores, mas também em doenças que envolvem

uma inflamação crônica, como a doença de Crohn e a pancreatite crônica, que possuem um alto risco de evolução cancerígena. À vista disso, a inflamação crônica está envolvida em diversas etapas da tumorigênese, entre elas a transformação celular, sobrevivência, proliferação, angiogênese e até na metástase (GONDA, TU e WANG, 2009; REUTER et al., 2010; SINGH, 2019).

Embora a imunidade exerça um papel importante na morte das células tumorais, o MC possui alta capacidade de evadir-se da resposta imunológica ou favorecer-se dos mediadores, o que dificulta o tratamento e culmina na progressão tumoral. Nesse sentido, é sabido que naturalmente os melanócitos exibem funções diretamente relacionadas ao sistema imunológico, como na fagocitose e apresentação de antígenos, além de sintetizar e secretar algumas citocinas (GASQUE; BANDJEE, 2015; LE POOLE et al., 1993). Por serem derivadas dos melanócitos, as próprias células de melanoma produzem citocinas espontaneamente ou induzidas (MICKSCHE, 1994), como comprovado por um estudo no qual células de melanoma metastático são capazes sintetizar e secretar IL-6, tendo a histamina como moduladora (MOLNÁR et al., 2000). Também, foram encontradas altas concentrações de interleucina 1 alfa (IL-1α), interleucina 1 beta (IL-1β), interleucina 6 (IL-6), interleucina 8 (IL-8), TNF-α, e fator de crescimento transformante beta (TGF-β) no TME primário de melanoma (GRIVENNIKOV; GRETEN; KARIN, 2011).

Recentemente, Manica et al. (2017), comprovou que mesmo após a ressecção cirúrgica das lesões causadas pelo MC, algumas interleucinas continuam aumentadas nos pacientes acometidos, como IL-2, IL-4, IL-6, IL-10, e TNF. Os autores desse estudo atribuíram essa desregulação no perfil inflamatório às altas concentrações de ATP no TME, em decorrência da inibição das ectonucleotidases que hidrolisam essa molécula na sinalização purinérgica, o que pode proporcionar mecanismos de que sustentem as recidivas e metástases. Apesar de a IL-10 ter função anti-inflamatória, interessantemente, os achados de Manica et al. corroboraram uma hipótese previamente proposta por Itakura et al. (2011), de que a IL-10, em conjunto com outros mediadores inflamatórios, pode levar à metastização.

Acrescido ao que foi elucidado acima, achados indicam que as células tumorais estão constantemente se adaptando às defesas do hospedeiro (EDDY; CHEN, 2020). Nesse sentido, a interação entre as células cancerígenas e as células imunes, como linfócitos T citotóxicos e macrófagos, que ocorre através da proteína de morte celular programada 1 (PD-1) e o ligante de morte programada 1 (PD-L1) no TME, é utilizada

como uma estratégia de evasão, visto que resulta na supressão da resposta imune por exaustão dessas células de defesa e impossibilidade de detecção das células tumorais (BARBER et al., 2006; BAUMEISTER et al., 2016). Especificamente, o MC é um dos tipos de câncer que utiliza esse mecanismo, haja vista que é um dos tipos de tumores sólidos com altos níveis de expressão de PD-L1 (KAUNITZ et al., 2017).

Além do eixo PD-1/PD-L1, as células tumorais também utilizam de outros artifícios do próprio hospedeiro para escapar da resposta imune. O antígeno 4 associado a linfócitos T citotóxicos (CTLA-4), é uma proteína expressa nas células T e desempenha a função de supressão imune por inibir a ativação dos linfócitos T citotóxicos, como nos casos da autotolerância. Com isso, há um bloqueio no reconhecimento de antígenos, processo chamado de anergia das células T (SANSOM, 2000). Ocorre que muitos antígenos apresentados pelas células cancerígenas, apesar das vastas possibilidades de mutações antigênicas na tumorigênese, são reconhecidos como próprios, não resultando na destruição do câncer (BOS et al., 2012). Com isso, as células tumorais se beneficiam da autotolerância do sistema imunológico e ocorre a progressão da patologia.

Outro mecanismo envolvido na evasão da resposta imune e suporte da progressão tumoral são os exossomas, que são nanovesículas formadas por fragmentação da membrana e citoplasma de células. Essas estruturas contêm no seu interior os mesmos componentes da célula que o originou, como DNA, RNA, proteínas e lipídeos. Por serem liberados em maiores quantidades pelas células tumorais que as células normais e apresentarem em seu interior um nicho favorável molecularmente, os exossomas, podem suprimir as respostas efetoras antineoplásicas adjacentes e do sistema imune, o que favorece o processo de metástase (ISOLA; EDDY; CHEN, 2016).

1.3.2 Estresse oxidativo e o melanoma cutâneo

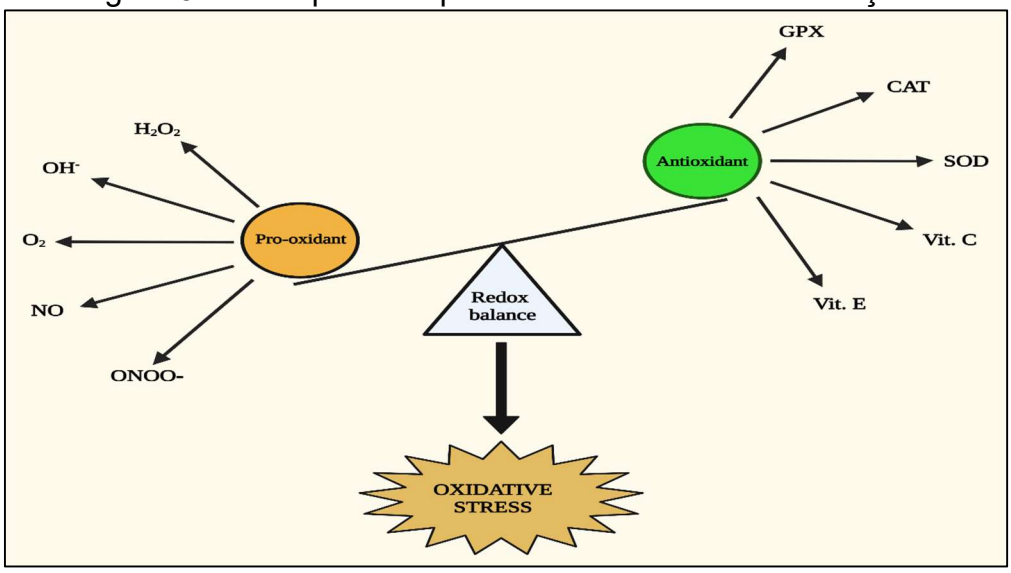
O estresse oxidativo é um processo resultante do desequilíbrio entre a produção de radicais livres e as reações metabólicas mediadas pela ação dos antioxidantes, cuja função é inibir ou reduzir os prejuízos do excesso desses radicais. Dessa maneira, em quantidades adequadas, esses radicais são necessários para funções fisiológicas do corpo humano, entre elas, a produção de ATP pela cadeia transportadora de elétrons (BARBOSA et al., 2010) e, quando em excesso, pode

prejudicar a homeostase, alterar o metabolismo de diversos tecidos e ainda, estimular a iniciação do câncer ao desencadear mutações no DNA (SAHA et al., 2017).

De forma geral, as espécies reativas de oxigênio (EROs) e espécies reativas de nitrogênio (ERNs) são produzidas continuamente através do metabolismo oxidativo, da bioenergética mitocondrial e do sistema imunológico. As principais EROs formadas são peróxido de hidrogênio (H_2O_2) e oxigênio (O_2), que se convertem nas espécies reativas radical hidroxila (OH^-) e o ânion superóxido (O_2^-). As ERNs podem se apresentar como óxido nítrico (NOx) ou peroxinitrito ($ONOO^-$) (BAGATINI *et al.*, 2018; BIRBEN *et al.*, 2012). Nesse contexto, o processo de geração de EROs é um dos mecanismos imunológicos efetores contra as infecções capazes de destruir partículas invasoras (JACOB et al., 2013; OCHIENG et al., 2015).

O organismo humano, com o objetivo de manter o equilíbrio e proteger os sistemas da toxicidade dos radicais livres, se beneficia de moléculas antioxidantes endógenas e exógenas, divididas em enzimáticas e não enzimáticas. As enzimas são representadas principalmente pelas enzimas superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPX). Entre os antioxidantes não enzimáticos, destacam-se as vitaminas C e E, antioxidantes fenólicos, glutationa (GSH) e carotenoides, que podem ser encontrados naturalmente em produtos alimentícios (BIRBEN *et al.*, 2012) (Figura 2).

Figura 5 – Principais componentes envolvidos no balanço redox.



Fonte: Adaptado de Silva (2022).

Acerca dos processos inflamatórios crônicos, é sabido que as EROs estão presentes em grandes quantidades nesse microambiente, além de células inflamatórias e fatores de proliferação celular, e por conta disso, há um aumento descontrolado na quantidade de radicais livres, o que leva ao estado de estresse oxidativo (SCHMATZ, 2011). Algumas evidências clínicas sugerem que o estresse oxidativo representa um importante elemento no desenvolvimento de diversas doenças inflamatórias crônicas (HUSSAIN et al., 2016).

Vale ressaltar que os raios UV são importantes dentre os fatores que levam ao desenvolvimento de tumores de pele, como o MC, pois a pele é um sítio propício às exposições solares e, além da luz natural, a irradiação ionizante e a própria irradiação UV parecem aumentar os níveis de EROs. Ainda, esse órgão está em constante contato com agentes pró-oxidantes endógenos e ambientais, o que pode gerar um estado de estresse oxidativo (AZZAM; JAY-GERIN; PAIN, 2012; REUTER et al., 2010).

Dados da literatura corroboram que o estresse oxidativo está envolvido no processo de carcinogênese de diversas formas, como por alteração do metabolismo energético, desequilíbrio entre antioxidantes e oxidantes, ativação crônica inespecífica do sistema imune com produção excessiva de citocinas pró-inflamatórias, desencadeando assim no aumento das EROs ou pelo efeito de medicamentos usados no tratamento do câncer (CANNAVÒ et al., 2019).

Falhas no sistema antioxidante levam ao acúmulo de EROs, o que contribui para que as células de melanoma fiquem mais agressivas, possibilitando a auto-renovação, além de impedir a ação das antiproteases e sofrerem metástases, ou seja, tornam-se resistente (OBRADOR et al., 2019). Achados experimentais de Piskounova et al. (2015) já comprovavam que células circulantes e nódulos metastáticos de melanoma enxertados em camundongos apresentavam maiores níveis de espécies reativas do que tumores subcutâneos primários.

Opostamente, em algumas células cancerígenas, as EROs podem limitar a iniciação e progressão tumoral através dos efeitos causados pelo estresse oxidativo, o que indica certa dependência a vários mecanismos para suprimir essas espécies reativas e lidar com o estresse ocasionado por elas. Isso sugere que o câncer pode ser melhor tratado com substâncias pró-oxidantes, as quais irão elevar o estresse oxidativo no ambiente neoplásico ou prevenir contra as adaptações celulares de resistência celular (GILL; PISKOUNOVA; MORRISON, 2016).

Uma vez que o DNA é alterado, o estágio inicial do câncer é atingido. A segunda etapa, denominada como “promoção” refere-se aos múltiplos promotores que induzem a proliferação celular, aumentam as espécies reativas de oxigênio e diminuem os reparos do DNA. Isso implica na replicação de células que perderam o controle do seu crescimento saudável e a um ciclo vicioso de estresse oxidativo, levando ao desenvolvimento do câncer (COUSSENS; WERB, 2002; MURATA, 2018).

Dessa forma, alguns parâmetros de estresse oxidativo e/ou atividade antioxidante, como a quantificação do H_2O_2, peroxidação lipídica, níveis de GSH, atividade da SOD e da CAT, podem ser aferidos a fim de se estabelecer os danos provocados pelo processo de oxidação (OBRADOR et al., 2019; PISOSCHI; POP, 2015).

1.3.3 Principais estratégias terapêuticas no tratamento do melanoma cutâneo

MCs são tratados cirurgicamente, pela inclusão da lesão primária e margem de segurança, de acordo com a profundidade de invasão dos melanócitos neoplásicos. Lesões *in situ* são ressecadas com margem de 0,5 cm, enquanto as lesões com até 1 mm de invasão são ressecadas com margem de 1 cm. As demais as margens devem ser de 2 cm. Além disso, pacientes com lesões que possuem invasão na derme acima de 1 mm, devem ser submetidos à biópsia do linfonodo sentinela para verificar comprometimento loco regional da doença. Pacientes com MC em estágio I e II, possuem sobrevida, em 5 anos, maior que 80%. Apesar disso, em estágios avançados (estágio III e IV), possuem índices de sobrevida baixos (aproximadamente 20% a 30%), pois somente recentemente é possível oferecer a esses grupos de pacientes, alternativas terapêuticas sistêmicas com algum grau de resposta (SCHADENDORF et al., 2018). No entanto, a taxa de sobrevida de pacientes com melanoma metastático ainda é muito baixa (REBECCA et al., 2020), e 15% a 20% dos tumores são resistentes ao tratamento convencional em sua primeira tentativa (CZARNECKA et al., 2020).

Isso ocorre porque as células de melanoma têm várias características que aumentam sua resistência, incluindo alta instabilidade genética que continuamente promove mutações transitórias, incluindo mutações na proteína quinase ativada por mitogênio (MEK), oncogene homólogo viral de neuroblastoma (NRAS) e que refletem no oncogene inibidor de sarcoma viral murino homólogo B1

do v-Raf (BRAF). Além disso, a rede de transdução é altamente eficaz na reconfiguração de sinais para prevenir a morte celular. Da mesma forma, as células do melanoma mimetizam o estroma, promovendo o desenvolvimento de fatores de crescimento e citocinas, entre outros mecanismos que favorecem o TME (REBECCA et al., 2020).

Até 2011, o tratamento convencional baseava-se no uso de quimioterápicos que causavam danos ao DNA celular e levavam à apoptose, como a dacarbazina, altas doses de IL-2 e o uso de Interferon-α2b (IFN-α2b) (JENKINS; FISHER, 2021; LO; FISHER, 2014). No entanto, a eficácia do medicamento em aumentar a taxa de sobrevivência não foi significativa e a busca por novas alternativas encontrou uma nova saída na imunoterapia e terapias alvo. Nesse contexto, anticorpos monoclonais e inibidores de BRAF e MEK têm se tornado alvos de diversos estudos, com o objetivo de controlar as respostas imunes (MOREIRA et al., 2020; PASQUALI et al., 2018). A terapia direcionada com o inibidor BRAF se deve ao fato desse gene estar mutado em mais de 50% dos casos de MC, resultando em uma alteração na proteína BRAF que auxilia no crescimento acelerado das células cancerosas (HARTMAN et al., 2019; LUKE et al., 2017).

Na mesma via de sinalização, os inibidores do gene MEK também reduzem as mutações do gene BRAF e atualmente constituem o padrão de tratamento em combinação com os inibidores de BRAF. Isso se deve a sobrevida livre de progressão por meio do uso isolado de vemurafenibe (inibidor BRAF), permite o desenvolvimento de resistência ao melanoma, enquanto a associação de outro inibidor verifica maior eficácia. O uso dessas terapias combinadas é aprovado pelo FDA e inclui: vemurafenib/cobimetinibe; dabrafenib/tramatenib; e encorafenibe/binimetinibe, o primeiro sendo inibidores de BRAF e o segundo sendo inibidores de MEK (CURTI et al., 2021; HARTMAN et al., 2019).

Embora essa combinação prolongue a sobrevida e seja a primeira escolha para pacientes sintomáticos com alta carga tumoral, a aquisição de resistência ainda é um fator limitante para o desenvolvimento de uma terapia eficaz (CURTI et al., 2021). Outra limitação é o fato desse tratamento não abranger 100% dos MCs, pois não é eficaz para aqueles que não apresentam mutação no gene BRAF (HARTMAN et al., 2019). Quando comparada à imunoterapia, a terapia direcionada oferece uma resposta mais rápida e, clinicamente, a combinação de ambas acaba sendo uma opção de tratamento (CURTI et al., 2021). Ipilimumabe,

um anticorpo anti-CTLA-4, e nivolumabe e pembrolizumabe, dois anticorpos anti-PD-1 e PD-L1, que atuam como inibidores de *checkpoint* imunológico e que possuem maior eficácia quando comparado ao tratamento com quimioterapia, IL-2, IFN-α2b ou inibidores de BRAF e MEK. Com isso, atualmente são drogas utilizadas em primeira linha de escolha para o tratamento de pacientes com MC em estágio III e IV, tanto na forma adjuvante como tratamento exclusivo em recidivas sistêmicas. Podem ser usados como monoterapia (nivolumabe ou pembrolizumabe) ou em associação (MOREIRA et al., 2020; NAMIKAWA et al., 2019).

Monoterapia com ipilimumabe a taxa de resposta é de aproximadamente 11% e tem diversos efeitos colaterais imunológicos em órgãos, pois promove exacerbação de células T citotóxicas normais e tumorais (RAMOS-CASALS et al., 2020); motivos que direcionaram a revisão dessa forma de tratamento. Entretanto, a associação de anticorpos monoclonais anti-PD-1 e PD-L1 resultam em uma resposta imune antitumoral mais satisfatória em 30% a 40% dos pacientes. Ainda não está claro o grupo de biomarcadores relacionados a presença ou ausência de reposta (EDDY et al., 2020).

Além de todas as possibilidades supracitadas, muito recentemente outra interessante terapia antitumoral tem ganhado destaque na literatura e revolucionado o campo de estudo no tratamento do câncer (KRUG et al., 2015). Conhecida como terapia de células T com receptor de antígeno quimérico (CAR-T), originada a partir da terapia celular adotiva (ACT), é uma técnica imunoterápica que consiste na aplicação de linfócitos T modificados do próprio hospedeiro ou de um doador saudável, redirecionando-as contra as células tumorais (ROSENBERG, RESTIFO, 2015; TRAN et al., 2008). Essa técnica tem sido indicada como uma importante e eficaz terapia no tratamento do melanoma e com horizontes promissores na pesquisa científica antitumoral (RAZAVI et al., 2021; RONCATI et al., 2020).

1.4 SISTEMA PURINÉRGICO: UMA BREVE CARACTERIZAÇÃO

O sistema purinérgico é uma via de sinalização celular presente basicamente em todos os tecidos do corpo, composta por moléculas extracelulares, os nucleotídeos e nucleosídeos, receptores e enzimas (ectoenzimas), que participam de mecanismos

fisiológicos e patológicos do organismo humano, como proliferação, diferenciação celular e morte, bem como imunomodulação (ATKINSON et al., 2006; BURNSTOCK et al., 2004; BURNSTOCK et al., 2012; VISOVATTI, 2012).

Inicialmente, quando foi publicado um estudo de revisão sobre a sinalização purinérgica por Geoffrey Burnstock, atualmente considerado o pai do sistema purinérgico, a hipótese não foi bem aceita no meio acadêmico, possivelmente por conta da teoria consolidada envolvendo moléculas de ATP na bioquímica energética em células. Porém, agora está claro que esta molécula, assim como outros nucleotídeos, também atua como mensageiro extracelular em diversos efeitos biológicos (BURNSTOCK, 2006; BURNSTOCK, 2007).

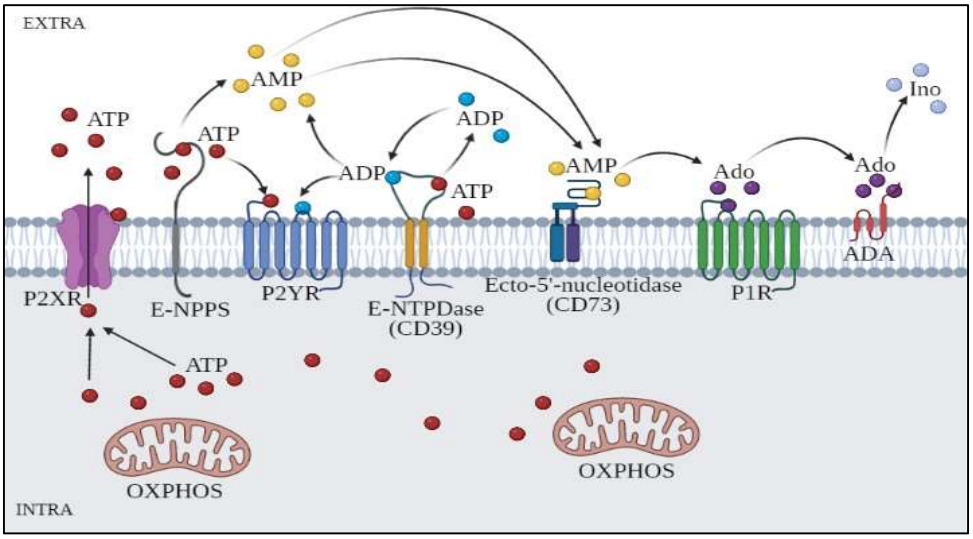
Dentre os compostos do sistema purinérgico, destacam-se os nucleotídeos ATP, ADP e AMP, além do nucleosídeo Ado, que desempenham papel de moléculas sinalizadoras. O ATP merece destaque e é foco de várias pesquisas, devido às diversas evidências que indicam que essa biomolécula atua em diversos processos do metabolismo celular, como indutor da síntese de DNA em timócitos, supressão de NKs, quimiotaxia e destruição tumoral (BURNSTOCK et al., 2012; LEAL et al., 2005).

Assim, os receptores celulares para as moléculas citadas são classificados como purinoceptores P1, que é mediado por Ado, e como purinoceptores P2, seletivo para ATP e/ou ADP. Os receptores P1 são separados em quatro subtipos: A1, A2A, A2B e A3, e o P2 em duas subfamílias: P2Y, acoplado à proteína G e P2X, que são canais iônicos (ver Figura 2). Atualmente, oito tipos de receptores P2Y foram identificados (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 e P2Y14), bem como sete tipos de receptores P2X (P2X1, P2X2, P2X3, P2X4, P2X5, P2X6 e P2X7) (BURNSTOCK, 2013; DI VIRGILIO, 2012; PFAFFENZELLER et al., 2020).

Além disso, na sinalização purinérgica existem estruturas enzimáticas extracelulares envolvidas, as quais são acopladas na membrana, conhecidas como ectonucleotidases, sendo elas ectonucleosídeo trifosfato difosfohidrolase (E-NTPDases-CD39), ectonucleotídeo pirofosfatase/fosfodiesterase (E-NPPS), fosfatase alcalina, 5'-nucleotidase (5'-NT-CD73) e adenosina desaminase (ADA). Todas essas enzimas desempenham um papel na quebra do ATP (ver Figura 1), que foi previamente descrito, e podem gerar seus derivados procedendo o início da cascata com a ação E-NTPDase, que catalisa a hidrólise de ATP e ADP a AMP. Posteriormente, a ecto-5'-nucleotidase catalisa a transformação do AMP em adenosina e, por fim, é desaminada pelo ADA, resultando na inosina. Também pode ser possível que o E-NPPS promova

a hidrólise do ATP diretamente para o AMP e continue a cascata até o final (YEGUTKIN, 2008; ZIMMERMANN et al., 2007).

Figura 6 – Estruturas e funcionamento do sistema purinérgico.



Fonte: Adaptado de Silva et al. (2022).

Assim, esse sistema de sinalização parece estar envolvido em processos patológicos e fisiológicos, como em diferentes tumores, e o papel desta via celular tem sido amplamente estudado (BAGATINI et al., 2018). Sabendo disso, em comparação com as células normais, as células tumorais possuem grande quantidade de ATP, sendo a depleção desse nucleotídeo uma estratégia para ativar as vias anticâncer. Além disso, os receptores P2 são observados em diversos tipos de câncer e podem apresentar efeitos inibitórios impedindo a proliferação celular, interferindo no ciclo celular e promovendo a morte celular. No entanto, isso depende do subtipo de receptor, pois as células cancerosas podem ter mais sensibilidade ou resistência à morte (BURNSTOCK et al., 2013).

Como já apontado, cada receptor do sistema purinérgico tem uma ação específica e diferenciada que depende de diversos fatores, como o tipo celular. Portanto, no contexto dos receptores de ATP, apesar de estarem frequentemente associados a efeitos anticâncer, no que diz respeito a P2Y1 e P2Y2, existem hipóteses demonstrando que no TME eles suportam o crescimento e a proliferação celular (BURNSTOCK et al., 2013; XIE et al., 2014). Isso pode ser corroborado com o artigo de Joo et al. (2014), em que observaram a ação desses receptores promovendo a formação de nicho metastático no câncer de mama.

Vale ressaltar que a formação da adenosina extracelular pode ocorrer principalmente a partir da atividade das enzimas CD39 e CD73, tanto em tecidos saudáveis quanto neoplásicos, embora outras vias estejam envolvidas, como a ação do E-NPP a partir da hidrólise do AMP, sendo o CD73 um dos principais gerador desta molécula. Alguns estudos mostraram um aumento na expressão dessas enzimas em diferentes contextos de câncer (ALIAGAS et al., 2014; CAPPELLARI et al. 2012; DI VIRGILIO et al., 2017; LONGHI et al., 2013).

Para fins de confirmação, muitas pesquisas têm relatado que a sinalização purinérgica está relacionada a neoplasias, como em câncer de pulmão (ZANINI et al., 2013; ZANINI et al., 2019), leucemia (LEDDEROSE et al., 2016), MC (BAGATINI et al., 2018; MÂNICA et al., 2019), câncer pancreático (HU et al., 2019), osteossarcoma (ZHANG et al., 2019) e câncer gástrico (HEVIA et al., 2019).

É evidente que ainda há muito a ser elucidado sobre o sistema purinérgico, desde sua descoberta até os dias atuais. Além disso, esse sistema de sinalização celular está mais consolidado como ubíquo no organismo humano (BURNSTOCK, 2011), o que pode ser indicado como um alvo terapêutico. No que se refere ao câncer, o interesse pela pesquisa dessa via de sinalização está crescendo devido a considerável possibilidade do seu envolvimento na fisiopatologia (BURNSTOCK et al., 2014).

1.4.1 Sinalização purinérgica no melanoma cutâneo

Como se sabe, o sistema purinérgico está envolvido na dinâmica do câncer, podendo participar, de acordo com os diversos fatores, como promotor ou inibidor tumoral. Isso ocorre por meio da ação de moléculas sinalizadoras, principalmente ATP, ADP, AMP e Ado, sobre os receptores celulares e pela atividade das ectonucleotidases na TME. É importante ressaltar que essa via celular tem estreita relação com a resposta imune no contexto do câncer, como em outras situações e, considerando toda a cadeia purinérgica, os níveis das moléculas purinérgicas são controlados por uma complexa rede de nucleotídeo/nucleosídeo-ectonucleotidases, expressas na superfície das células. Além disso, o ATP é uma molécula central que influencia amplamente as respostas imunes nos tecidos periféricos e centrais (BURNSTOCK, 2015; BURNSTOCK, 2016; CEKIC; LINDEN, 2016; STAGG; SMYTH, 2010), pode ser liberado das células inflamatórias e tumorais por meio de diferentes

mecanismos, como exocitose, canais da membrana plasmática ou lise, e pode ser acumulado no TME (DI VIRGILIO; ADINOLFI, 2017, GHIRINGHELLI et al., 2009).

Em células de melanoma, a alta concentração de ATP pode ter um efeito anticâncer porque ativa os receptores do tipo P2X7, levando à morte celular (BIAN et al., 2013). Apesar disso, quando o ATP é hidrolisado a ADP, pode apresentar efeito imunossupressor sobre o MC (MÂNICA et al., 2019). Por outro lado, o Ado, produto da hidrólise de ATP que medeia a resposta protetora, como efeitos imunossupressores e anti-inflamatórios em tecidos saudáveis, parece ter uma propriedade pró-carcinogênica, como estímulo de crescimento tumoral e angiogênese em MC afetados celular, além de maior mobilidade celular e metástase, ou seja, ao interagir com os receptores P1, uma alta concentração de Ado atua efetivamente na progressão tumoral (ANTONIOLI et al., 2014; DI VIRGILIO et al., 2018; PASQUALI et al., 2018; WHITTON et al., 2018).

Mânica et al. (2018) encontraram dados significativos sobre o papel do ATP em pacientes com melanoma, indicando que um aumento do processo inflamatório pelo ATP extracelular leva a um perfil imunossupressor mesmo após a remoção cirúrgica do MC e o sistema purinérgico pode desenvolver um microambiente inflamatório crônico que pode exercer influência direta na recidiva ou metástase. Este efeito envolve a desregulação dos níveis de nucleotídeos e nucleosídeos no sangue periférico.

Outros receptores purinérgicos importantes envolvidos no melanoma são os do tipo P2Y, como P2Y1, P2Y2 e P2Y12. Os receptores P2Y1 parecem reduzir a proliferação celular e os P2Y2R geralmente parecem aumentar o número de células (WHITE et al., 2005), enquanto os P2Y12R têm sido relacionados à metástase tumoral pela ativação de plaquetas em células de melanoma (GEBREMESKEL et al., 2015).

Curiosamente, um dos fatores que leva ao câncer de pele, a irradiação UV-B, parece ter uma relação com a sinalização purinérgica. Efeitos graves têm sido associados a esse tipo de irradiação e mostram reduzir a quantidade de receptores P2X1 e P2Y2, bem como destruir os receptores P2X7, podendo contribuir para a transformação maligna dos queratinócitos (RUZSNAVSZKY et al., 2011).

Ainda em relação à modulação dos receptores no contexto do melanoma, a estimulação das células tumorais A2AR e A3AR pode potencializar a proliferação desencadeando a morte das células do melanoma. A deleção de A2ARs em células mieloides demonstrou reverter a imunossupressão em camundongos com melanoma

B16 via potenciação de NK e respostas de linfócitos T citotóxicos (CEKIC et al., 2014; FISHMAN et al., 2002; MERIGHI et al., 2002). Um estudo in vivo para compreender o efeito da sinalização A1R, A2AR e A3R no melanoma B16 em camundongos descobriu que os receptores contribuem para TME modulando a angiogênese, neovascularização e infiltração de macrófagos imunossupressores associados a tumores (TAMs) (KOSZAŁKA et al., 2016).

Além dos receptores, as ectonucleotidases, por sua importância na cadeia purinérgica, têm se mostrado envolvidas no câncer. Assim, no contexto do melanoma, a inibição do CD39 pode levar ao acúmulo de ATP extracelular, reforçando a estreita relação entre essa doença neoplásica e a atividade de enzimas que hidrolisam os nucleotídeos de adenina. Além disso, sugere-se que altos níveis de ATP no microambiente pós-cirúrgico do MC sejam a causa de alterações deletérias (MÂNICA et al., 2018).

A CD73 também parece estar envolvido no melanoma, uma vez que os estudos indicam que a alta expressão do CD73 está sugestivamente presente na doença (JIANG et al., 2018; SADEJ; SPYCHALA; SKLADANOWSKI, 2006). No tipo de melanoma primário, o estado mutacional não mostrou qualquer associação com a expressão de CD73 em lesões metastáticas. No entanto, os pacientes com casos avançados tendem a ter mais expressão de CD73 em suas metástases (MONTEIRO et al., 2018). Por meio de um modelo de melanoma em camundongo, um inibidor de CD73 melhorou a imunidade antitumoral mediada por células T e B e reduziu o crescimento do tumor (FORTE et al. 2012).

Além do conhecimento já estabelecido sobre a capacidade da CD73 na hidrólise de nucleotídeos, ela está envolvida na invasão de células cancerosas. Foi mostrado que uma ação não enzimática da CD73 é promover a migração celular no ECM por meio da ativação da quinase de adesão focal (FAK) em células de melanoma (SADEJ; SKLADANOWSKI, 2012).

1.5 POTENCIAL TERAPÊUTICO DO ÁCIDO ROSMARÍNICO

Compostos naturais são substâncias químicas derivadas de organismos vivos que, com o avanço da tecnologia, têm sido alvo de diversas pesquisas envolvendo a saúde humana, especialmente quanto à ação anticâncer. Podem ser encontrados em toda a natureza e em diversas fontes. Vários estudos demonstraram que os

compostos naturais, no geral, podem não só promover a ação anticarcinogênica, como também, promover a apoptose e a interrupção do ciclo celular (AUNG et al., 2017; NOEL et al., 2020; OUYANG et al., 2014).

Nesse sentido, os compostos fenólicos, presentes em quase todos os alimentos, são identificados por possuírem pelo menos um anel aromático com um ou mais grupos hidroxila. Eles podem ser encontrados em frutas, verduras, legumes, cereais, cerveja, café, vinho e especiarias, e são divididos em classes denominadas flavonóides, ácidos fenólicos, álcool fenólico, estilbenos e ligninas, de acordo com suas características (MULLER et al., 2019).

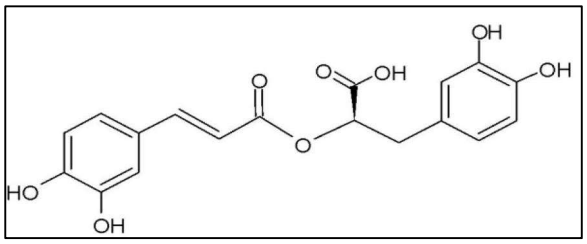
Dentre os ácidos fenólicos, pode-se citar o AR, que é um derivado do ácido cafeico e do ácido 3,4-diidroxifenillático, presente em plantas herbáceas, principalmente das famílias Boraginaceae e Lamiaceae, como o alecrim *(Rosmarinus officinalis L.)*, manjericão *(Ocimum basilicum)*, orégano *(Origanum vulgare)*, sálvia *(Salvia officinalis)* e a melissa *(Melissa officinalis)*. Possui alta capacidade farmacológica desejada no tratamento do cancer, como efeitos antioxidantes, anti-inflamatórios e antitumorais. Portanto, muitos tipos de pesquisa procurando esses efeitos em várias linhagens celulares e tipos de câncer, foram desenvolvidas, e os resultados parecem ser promissores (LUO et al., 2020; NUNES et al., 2017; RADZIEJEWSKA; SUPRUNIUK; BIELAWSKA, 2021).

Além disso, quanto ao potencial do AR e considerando a necessidade urgente de identificar novos tratamentos para o melanoma devido às caras terapias conservadoras (DAVIS; SHALIN; TACKETT, 2019; PRAMANIK et al., 2013), reforça-se que existem artigos que vêm apresentando resultados importantes em outros contextos de câncer (KARTHIKKUMAR et al., 2015; YANG et al., 2020). A partir disso, o AR parece ser promissor como terapia adjuvante no tratamento do MC.

No que diz respeito às características químicas, a nomenclatura da União Internacional de Química Pura e Aplicada (IUPAC) para o AR é (2R)-3-(3,4-dihidroxifenil)-2-[(E)-3-(3,4-dihidroxifenil) ácido prop-2-enoil]oxipropanoico, sendo sólido cristalino vermelho-alaranjado, com fórmula molecular $C_{18}H_{16}O_8$, peso molecular de 360,3 g/mol, ponto de fusão de 171-175ºC, bem solúvel na maioria dos solventes orgânicos, como o etanol, dimetilsulfóxido (DMSO) ou dimetilformamida (DMF), mas pouco solúvel em água. Em sua estrutura química (Figura 3), existem dois anéis fenólicos e dois grupos de posição orto OH em cada anel, que promovem sua atividade antioxidante através da concessão de H, além de uma dupla ligação

insaturada, um grupo carbonila e um grupo ácido carboxílico (BREWER, 2011; ELUFIOYE; HABTEMARIAM, 2019; PUBCHEM, 2021).

Figura 7 – Fórmula estrutural do ácido rosmarínico.



Fonte: Adaptado de Silva et al. (2022).

A biossíntese do AR ocorre em vias duplas paralelas que se unem para formar efetivamente a molécula de ácido fenólico. Uma das vias começa com a transformação do aminoácido precursor L-fenilalanina pela fenilalanina amonialiase (PAT) em ácido T-cinâmico. Em seguida, o ácido T-cinâmico é convertido em ácido 4-cumárico pelo ácido cinâmico 4-hidroxilase (C4H) com adição de um grupo OH na posição 4 do anel aromático e posteriormente transformado em 4-cumaril-CoA por 4-cumarato- CoA ligase (4CL). Outra via é a transformação do aminoácido L-tirosina, envolvendo a enzima tirosina aminotransferase (TAT), em ácido 4-hidroxifenilpirúvico e, em seguida, convertido em ácido 4-hidroxifenillático pela hidroxifenilpiruvato redutase (HPPR). Na etapa pré-final, ambas as vias são unidas, ou seja, o 4-cumaril-CoA proveniente da via da L-fenilalanina e o ácido 4-hidroxifenillático, da L-tirosina, são incorporados pela hidroxicinamoil-CoA hidroxifenillactato hidroxicinamoil transferase (RAS), e a coenzima A (CoA), presente no 4-coumaril-CoA, é liberada. Finalmente, AR via 3- e 3'-hidroxilase (3-H, 3'-H) é sintetizado com a introdução de grupos OH nas posições 3' e 3 dos anéis aromáticos (FIALOVÁ et al., 2019; PETERSEN, 2013).

Considerando a estrutura molecular, vários estudos foram realizados para entender a farmacocinética para aplicação deste composto em seres humanos e há a indicação de que pode ser administrado por via tópica, pulmonar, intranasal e por infusão intravenosa, sendo a via perioral a principal forma de ingestão. Quanto à metabolização, há a tendência de ocorrer pela microflora intestinal, onde é degradado a compostos fenólicos simples por microrganismos, enquanto a distribuição ocorre pelas albuminas plasmáticas e excretada principalmente pelo sistema renal. Além

disso, as evidências mostraram que os remédios fitoterápicos contendo AR não relataram efeitos colaterais graves e apresentaram resultados positivos (HITLE et al., 2020).

Apesar do mecanismo de ação deste composto não estar de fato elucidado, muitos estudos *in vitro* e *in vivo* foram realizados para comprovar seu efeito anti-inflamatório (LUO et al., 2020), por exemplo, descreveram o potencial terapêutico do AR em doenças inflamatórias como colite em reduzir a infiltração de células inflamatórias, além de inibir a indução da ciclooxigenase-2 (COX-2) e a síntese de IL-1β, IL-6 e IL-22. Uma revisão realizada por Yahfoufi et al. (2018) comprovaram o papel imunomodulador dos polifenóis in vivo e in vitro, devido à interferência na regulação das células imunes em diversas vias ao inativar o NF-κB, modulando as vias do ácido araquidônico responsáveis pela síntese de COX-2 e inibindo algumas enzimas envolvidas na produção de EROs.

Jang, Hwang e Choi (2018) também provaram tal eficácia ao analisar a parada do ciclo celular e apoptose através da modulação da expressão gênica relacionada à iniciação do câncer de próstata pela histona desacetilase (HDAC). Da mesma forma, no estudo de Luo et al. (2020) foi analisado o efeito da AR no câncer bucal e verificou-se que houve inibição da proliferação celular devido ao modo seletivo de apoptose, uma vez que o efeito inibitório estava menos presente em células normais. Também é importante mencionar que o AR tem a capacidade de prevenir a metástase do câncer colorretal via fosforilação da proteína quinase ativada por adenosina monofosfato (AMPK), uma enzima que preserva a homeostase celular (HAN; KEE; HONG, 2018).

Da mesma forma, o AR mostrou-se eficiente como antioxidante por reduzir os radicais livres resultantes de lesões de isquemia/reperfusão renal, foco do estudo de Ozturk et al. (2014). Assim, este ácido fenólico atua aumentando a expressão de genes citoprotetores, reduzindo a citotoxicidade induzida pela xantina oxidase e peróxido de hidrogênio (H_2O_2) e aumentando a produção de prostaglandina E2 e reduzindo a as taxas de leucotrieno B4, IL-6, IL-1-beta e TNF-alfa, além de inibir a ativação do sistema complementar. Em paralelo, esse ácido fenólico tem a capacidade de reduzir os radicais livres através do grupo hidroxila apresentando alta eficiência, em torno de 98,92%, de atividade antioxidante total (SEVGI; TEPE; SARIKURKCU, 2015).

Como pode-se constatar, inúmeras pesquisas têm evidenciado o AR no

tratamento de patologias oncológicas, mas essas hipóteses de ação são variadas. Uma delas é a ação anti-inflamatória, visto que o processo inflamatório está intimamente relacionado com a gênese tumoral. Essa ação é comprovada por modelos in vivo, como realizado por Swarup et al. (2007), que notou a redução da mortalidade e alterações nos níveis de citocinas pró-inflamatórias em camundongos infectados com o vírus da encefalite japonesa. Esses pesquisadores encontraram redução nos níveis de IL-12, TNF-α, IFN-γ, MCP-1 e IL-6 quando comparados aos infectados sem tratamento. Da mesma forma, os efeitos anti-inflamatórios em uma lesão neuroinflamatória foram comprovados não apenas em um modelo in vitro, mas também in vivo, ao reduzir a expressão do receptor Toll-like 4 (TLR4) e CD14, receptores transmembrana que ativam o NF-kβ, além de suprimir a ativação do inflamassoma NLRP3, responsável pela maturação das citocinas pró-inflamatórias IL-1β e IL-18 (WEI et al., 2018).

Completando a ideia anterior, alguns trabalhos indicam o potencial imunomodulador do AR, como o trabalho publicado por Lembo et al. (2014), que mostra o aumento de parâmetros imunológicos como atividade fagocitária, bem como concentrações de algumas características bioquímicas do sangue, como colesterol total e suas frações de lipoproteínas e triglicerídeos no tratamento de um modelo animal de frango com concentração de 100 ou 200 mg em óleo kg^{-1}. Para reforçar esses dados, este composto fenólico também apresentou efeito anti-inflamatório contra inflamação local e sistêmica em ratos (ROCHA et al., 2015), diminuição dos níveis de TNF-α, IFN-γ, IL-6 e IL-12 (FRIEDMAN, 2015) e redução significativa de mieloperoxidase (MPO), atividade e níveis de TNF-α em camundongos (MARINHO et al., 2021).

Da mesma forma, o efeito antiproliferativo também está associado às propriedades anticancerígenas do AR. Foi testado em células das linhagens Panc-1 e SW1990 que são células tumorais de pâncreas. Nesse estudo, não apenas a capacidade antiproliferativa ficou clara, mas também a redução da capacidade de invasão e migração celular que promove a apoptose e suprime a transição epitelial-mesenquimal (EMT) nas células do câncer pancreático (HAN et al., 2019).

Por outro lado, no carcinoma hepatocelular, a via do AR envolve inflamação e angiogênese, segundo Cao et al. (2016). Por ensaios imunoenzimáticos (ELISA), os níveis de fatores inflamatórios TNF-α, IL-6 e IL-1β, bem como de fatores angiogênicos (VEGF e TGF-β), foram reduzidos quando esse composto fenólico foi administrado

em camundongos. Além disso, por Western blot, verificou-se que houve redução na expressão de NF-kβ p65, que regula os fatores angiogênicos durante o desenvolvimento do CHC e, consequentemente, resultando em efeito antiangiogênico. Ainda, os resultados mostraram que os efeitos toxicológicos do AR foram insignificantes, além de obter uma taxa de sobrevivência de 100% nos camundongos.

Atualmente, sabe-se que a produção de EROs pode desencadear a formação de novos tumores e, por esta razão, o efeito antioxidante é um fator positivo para a ação antitumoral. Os ácidos fenólicos, por meio do grupo hidroxila, são conhecidos pelo seu potencial antioxidante, o que impede sua reação com a molécula de DNA, resultando em uma mutação. Dentre os ácidos fenólicos, o AR foi o mais eficaz nesse quesito (98,92% de atividade), além de ter o maior efeito quelante, seguido do ácido cafeico, na proteção do DNA contra substâncias tóxicas como UV e H_2O_2 (SEVGI; TEPE; SARIKURKCU, 2015). Segundo Oğuz et al. (2020), o AR é um agente hepatoprotetor eficaz e aumenta a capacidade antioxidante ao reduzir o estresse oxidativo.

A administração do AR em células de melanoma da linhagem B16F10 radiorresistentes teve efeito redutor sobre a glutationa (GSH) após exposição à irradiação radioativa, quando comparada às células epiteliais da próstata PNT2, consideradas radiossensíveis. Esse efeito possivelmente se deve à associação com as vias de síntese da eumelanina, consumo intracelular de GSH e redução dos mecanismos protetores contra o estresse oxidativo, que indicam um dano potencial significativo às células radiorresistentes durante a radioterapia. Observou-se que, ao mesmo tempo, o AR poderia apresentar uma ação protetora nas células saudáveis durante a terapia (OLIVARES et al., 2020).

Ao analisar o AR como um composto promissor para o tratamento do MC, é relevante que ele previna a metástase e, por isso, essa ação é importante a ser destacada. Partindo-se dessa ideia, dados da literatura provam que o AR é promissor nesse quesito, como observado em um estudo publicado em 2010 por Xu et al. (2010), que comprovaram a inibição da metástase no câncer colorretal (CCR) pela via da quinase regulada por sinal extracelular. Mais recentemente, Han, Kee e Hong (2018) relataram que tal composto inibe a proliferação de células CRC, bem como dificulta a metástase por fosforilação de AMPK.

No CCR avançado, os principais órgãos invadidos são o fígado e os pulmões,

e o uso do AR reduziu drasticamente o número de nódulos tumorais metastáticos nesses órgãos. Isso porque, para que ocorra a metástase, as células devem invadir a matriz extracelular e depois migrar para outro sítio. Durante esse processo, entretanto, ocorre invasão, migração e adesão celular, cujos processos são induzidos por metaloproteinases de matriz (MMP-2 e MMP-9). A ação do AR consiste na ativação da AMPK, que reduz a expressão e a atividade dessas pró-enzimas e, consequentemente, dificulta a disseminação das células cancerosas e a promoção de metástases (HAN; KEE; HONG, 2018).

Por fim, em relação à agregação plaquetária, há estudos que buscaram a influência do AR nesse processo, como Zou et al. (2018), que por meio do extrato de Danshen e tendo o AR como molécula ativa, mostraram um potente efeito inibitório sobre as plaquetas de ratos, agregação induzida pelo ácido araquidônico, e que o docking molecular da enzima tiol oxidorredutase (ERp57) é importante para esse processo. Da mesma forma, o extrato de *Salvia yunnanensis*, que contém AR em sua composição, inibiu a agregação plaquetária de coelhos de forma criptotansinona *ADP-like* na ligação com P2Y12R, sendo o AR fundamental nesse desfecho, podendo ser aplicado com antitrombóticos (LI et al., 2020).

Com isso, é possível perceber que há poucos trabalhos na literatura que evidenciem o potencial da AR no tratamento da MC, considerando os diversos desfechos apresentados anteriormente e, quando se trata de ensaios clínicos, esse número é ainda menor, assim como aqueles sobre o sistema purinérgico. Essas verificações reforçam a necessidade de melhor pesquisar e compreender os mecanismos nesse tema.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Analisar *in vitro* o potencial antineoplásico do ácido rosmarínico em células de melanoma cutâneo através dos parâmetros de estresse oxidativo e sinalização purinérgica.

2.2 OBJETIVOS ESPECÍFICOS

a) Elaborar um artigo de revisão de literatura sobre o tema de pesquisa;
b) Investigar o efeito citotóxico e viabilidade celular;
c) Verificar o potencial de migração celular;
d) Mensurar as concentrações dos marcadores antioxidantes PSH e NPSH, e dos marcadores pró-oxidantes EROs e NOx;
e) Analisar a expressão gênica da caspase 8 e inflamassoma NLRP3;
f) Analisar a expressão gênica e atividade enzimática da caspase 3;
g) Analisar a expressão gênica e proteica das ectonucleotidases CD39 e CD73;
h) Verificar a atividade enzimática das ectonucleotidades CD39, CD73 e ADA;
i) Pesquisar os níveis extracelulares de ATP.

3 ARTIGO E MANUSCRITOS

3.1 ARTIGO

Novel possibility for cutaneous melanoma treatment by means of rosmarinic acid action on purinergic signaling

Gilnei Bruno da Silva, Milena Ayumi Yamauchi, Daniela Zanini & Margarete Dulce Bagatini

Pulicado na Revista *Purinergic Signalling*.

Purinergic Signalling
https://doi.org/10.1007/s11302-021-09821-7

REVIEW ARTICLE

Novel possibility for cutaneous melanoma treatment by means of rosmarinic acid action on purinergic signaling

Gilnei Bruno da Silva[1] · Milena Ayumi Yamauchi[1] · Daniela Zanini[1] · Margarete Dulce Bagatini[1]

Received: 6 May 2021 / Accepted: 12 October 2021

Abstract

Cancer cases have increased significantly in Brazil and worldwide, with cutaneous melanoma (CM) being responsible for nearly 57,000 deaths in the world. Thus, this review article aims at exploring and proposed hypotheses with respect to the possibility that RA can be a promising and alternative compound to be used as an adjuvant in melanoma treatment, acting on purinergic signaling. The scarcity of articles evidencing the action of this compound in this signaling pathway requires further studies. Considering diverse evidence found in the literature, we hypothesize that RA can be an effective candidate for the treatment of CM acting as a modulating molecule of purinergic cellular pathway through P2X7 blocking, mitigating the Warburg effect, and as antagonic molecule of the P2Y12 receptor, reducing the formation of adhesive molecules that prevent adherence in tumor cells. In this way, our proposals for CM treatment based on targeting purinergic signaling permeate the integral practice, going from intracell to extracell. Undoubtedly, much is still to be discovered and elucidated about this promising compound, this paper being an interesting work baseline to support more research studies.

Keywords Cancer · Purinergic system · Phenolic compound · Melanoma. Rosmarinic acid · Chemotherapy

Introduction

Globally, 1 out of 5 individuals develops cancer during their lifetime, which suggests that more than 50 million people are living 5 years after a cancer diagnosis, the cutaneous melanoma (CM) type being responsible for nearly 57,000 deaths in the world [1]. In addition, this is one of the most aggressive types among the cutaneous tumors due to its highly metastatic and low survival rates [2].

In Brazil, skin cancer has presented expressive growth in the last years, corresponding to 27% of all the malignant tumors, and CM is responsible for 8400 new cases per year, with more incidence in the South of the country [3, 4]. This increased number of cases has been linked to excessive sunbathing, due to harms by ultraviolet (UV) radiation [5].

To treat this disease, drugs that lead to unwanted side effects are used; in addition, none shows efficient mechanisms to avoid lethal progression of the pathology [6]. Biochemical therapy has been indicated as a promising adjuvant strategic management [7], but the development of new options is crucial so that there is a rise in patients' survival [8].

Thus, many research science teams are looking for the best melanoma treatment; in other words, one with few or no side effects, in addition to low pharmacologic resistance during the therapeutic path. An interesting alternative to this would be to use natural substances, such as phenolic compounds that can have anticancer effects, either associated with conventional pharmacology or in isolation, as long as there is scientific support.

Highlights
- Novel agents need to be researched in melanoma treatment as alternatives to conservative therapies;
- This review sought to find relationships between RA and its properties on CM;
- Diverse evidence suggests that RA can be a promising phenolic compound adjuvant to treat melanoma through cell signaling pathway modulation;
- Hypothetically, RA can modulate the purinergic system by blocking P2X7R and acts as an ADP-inducing antagonist in P2Y12R;
- It seems that this compound has the ability to induce apoptosis on the melanoma cell type.

✉ Margarete Dulce Bagatini
margaretebagatini@yahoo.com.br

[1] Graduate Program in Biomedical Sciences, Universidade Federal da Fronteira Sul, Fronteira Sul, Chapecó, SC 89815-899, Brazil

Published online: 05 November 2021

An essential signaling pathway recently related to tumor cell progression is the purinergic system, which interferes with mechanisms such as disordered cell proliferation, promotion of angiogenesis, and failure of mechanisms controlling apoptosis. This is due to an imbalance in the concentrations of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) nucleotides and of adenosine (Ado), and nucleoside present in the tumor environment (TME), as well as overexpression or, in some cases, low expression of P2 receptors and ectonucleotidases [9–11]

To corroborate the aforementioned conjectures, a large number of in vitro and *in vivo* studies have indicated the potential of phenolic compounds, especially caffeic acid (CA) and rosmarinic acid (RA), as antioxidant, antiproliferative, anticancerogenic, and antitumor agents, as well as modulators of cell pathways and biochemical cascades [12–17]. Furthermore, some studies have suggested the ability of RA to modulate the purinergic system [18].

It is important to highlight that, up to the present day, few works involving RA with CM can be found in the scientific literature, and the same happens with the purinergic system, which reinforces the need for research on this theme. Therefore, this review article aims at exploring and proposed hypotheses with respect to the possibility that RA can be a promising and alternative compound to be used as an adjuvant in melanoma treatment, acting on purinergic signaling, since it seems to have antitumor, antiangiogenic, and antiproliferative effects, as well as involved in cell pathway signaling. To such end, based on other research studies with different cancer contexts where RA, chemical characteristics of phenolic compounds and purinergic signaling modulatory molecule effects, we hypothesized two possible mechanisms through which the purinergic system can be the RA target in the pharmacological therapeutic perspective for CM.

Cutaneous melanoma

The skin is the largest organ of the human body and covers its entire external surface, where it has an important function in the protection against toxic agents, friction, injuries, and radiation. In addition to that, it is divided into the epidermis, the outermost layer, and the dermis, the deeper stratum, constituted of conjunctive tissue. On the epidermis, there are keratinocytes, melanocytes, Langerhans cells, and Merkel cells [19]. CM is one of the malignant pathologies that affects the epidermis, being characterized as a cytological disorder that affects melanocytes, which produce melanin, increasing the proliferative ability of these cells due to a series of changes in the cell cycle and in the apoptosis mechanisms. CM is an oncological pathology characterized by the high invasiveness of tumor cells, and has a high metastatic capacity, causing a short survival period and high mortality rates [2, 8].

In the epidemiologic context of this disease, in a study published by Enninga et al. [20], 201,719 diagnoses of cutaneous melanoma were made between 1992 and 2011 in the world. Among those, survival rate was better in women, and the hypothesis was the difference in behavior and human biology. However, when analyzing the disease stage, there is no difference between the genders in the mortality rate, revealing that the female advantage is restricted to a localized and regional disease. Despite this, the survival rate is better when there is an early diagnosis in both genders.

By the year 2020, the incidence of CM in the world, including both genders and all ages, was 324,635 new cases and more than 57,000 deaths, mostly affecting North America, Europe, and Oceania. In Brazil, this type of cancer, which recorded 8624 new cases last year, is the 20th most frequent in the country. Looking at the future, the estimate for 2030 is a 15.8% increase of new cases worldwide [1]. Estimates for 2020 in Brazil were 4200 new cases for men and 4250 for women, more frequently in the South of the country [21], and, despite CM is not the most prevalent type of cancer, it is responsible for more than 75% of the deaths due to skin cancer, being considered a major public health problem [22].

As in other solid tumors, the staging of CM is based on the tumor, nodes, and metastasis (TNM) classification, where "T" refers to the size of the primary tumor, "N" refers to the invasion or not of the lymph nodes, and "M"' refers to the presence of distant metastases. Below, we present Table 1 referring to the staging of CM, as well as to fundamental criteria to define the therapeutic of action that will be chosen for the treatment of this malignant pathology [23].

Considering the malignancy of CM, early and correct diagnosis is very important for a successful treatment. However, this can be difficult because of its variability in cytomorphology and its similarity to benign lesions [24, 25]. At first, there are four main types of CM, according to their histological and growth characteristics, namely: superficial spreading, nodular melanoma, lentigo maligna, and acral lentiginous. Most of them remain in the epidermis, growing horizontally, known as the radial growth phase, while in the vertical growth phase, infiltration occurs in the dermis, conferring metastatic potential to it [25].

As already mentioned, diagnosis must be careful, since CM can manifest in various clinicopathologic forms [24–26]. Nevertheless, the gold standard for diagnosis is histopathological analysis associated with clinical recognition. In the latter, it is important to pay attention to the patient's history and risk factors and to perform a skin examination of the entire body being able to count on the assistance of dermoscopy. Subsequently, the ABCDE rule must be applied to each lesion, in addition to the "ugly

Table 1 Melanoma TNM classification

Tumor (T)	Breslow thickness (mm)	Ulceration/mitotic rate
Tis	Not applicable	Not applicable
T1	≤1.0 mm	a: no ulceration/ < 1 mm^2
		b: ulceration/ ≥ 1 mm^2
T2	1.01–2.0 mm	a: no ulceration
		b: ulceration
T3	2.01–4.0 mm	a: no ulceration
		b: ulceration
T4	> 4.0 mm	a: no ulceration
		b: ulceration
Node (N)	**Number of metastatic nodes**	**Nodal metastatic mass**
N0	0	Not applicable
N1	1	a: micrometastasis
		b: macrometastasis
N2	2–3	a: micrometastasis
		b: macrometastasis
		c: in-transit metastasis/satellites and no nodes
N3	≥4 metastatic nodes or in-transit metastases/satellite(s)	
	with metastatic node(s)	
Metastasis (M)	**Site**	**Lactate dehydrogenase value**
M0	No distant metastases	Not applicable
M1a	Distant skin, subcutaneous or nodal metastases	Normal
M1b	Lung metastases	Normal
M1c	All other visceral metastases	Normal
	Any distant metastasis	Elevated

Source: Adapted from Gershenwald and Scolyer [174]

duckling" sign so that a nevus different from the others, in the same individual, is followed more carefully (Table 2). Furthermore, imaging techniques can be supported for early diagnosis, such as dermoscopy and diagnosis confirmation; skin biopsy is the most useful [27, 28].

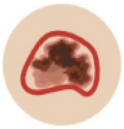
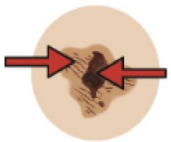
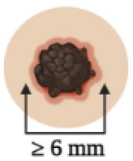
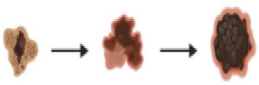
It is known that the main risk factor for melanoma development is exposure to UV radiation, which is present in sunlight. In addition, there are three types of UV radiation: UVA, UVB, and UVC, although the latter is blocked by the ozone layer, while UVA and UVB reach the Earth; and exposure to these types can cause DNA damage and consequent development of melanoma. Although UVA is the most emitted radiation (90–95%), UVB is more prone to cause burns and skin harms [29, 30].

Ultimately, some research evidence shows that other risk factors can exert an influence on the incidence of melanoma. At first, risk is greater for women in the first decades of life, while men are more prone when they reach adult life. In addition, even if having more female diagnoses at the beginning, prognosis is better, but it is not the same as people age [29, 31].

Another associated risk factor is skin color because, in dark and hair, eumelanin is most abundant, a type of melanin which is synthesized under the α-melanocyte-stimulating hormone (α-MSH) signaling to a melanocortin-1 receptor (MC1R), while individuals with red hair and freckles have more pheomelanin due to loss-of-function mutation in MC1R that prevents eumelanin production. Thus, eumelanin reduces the UV-induced harms, unlike pheomelanin, which contributes to the formation of free radicals and, consequently, to DNA damage [30]. Therefore, according to Rastrelli et al. [32], individuals with fair skin, freckles, and red hair are more predisposed to melanoma by approximately 50%, as is the number of melanocytic nevi, family history, and genetic susceptibility.

Among the possible pathological factors of cancer, oxidative stress connected with inflammation and the purinergic system is frequently cited. Oxidative stress is a process caused by an imbalance between the production of free radicals and metabolic reactions mediated by the action of antioxidants, whose function is to inhibit or reduce the lesion from an excess of these reactive products. Reactive oxygen species (ROS) are

Table 2 Explanation of the ABCDE rule on CM diagnosis. Source: Adapted from Michielin et al. [23]

SYMBOL	MEANING	REPRESENTATION
A - Asymmetry	Peripherals outside of the lesion are not equal.	
B - Border irregularity	Uneven border of the lesion.	
C - Color variation	Multiple colors throughout the lesion.	
D - Diameter	Diameter larger than 6 mm.	
E - Evolution	Change in shape, color and size throughout time.	

free radical products mainly generated by the mitochondria, during cellular respiration resulting in a reduction of O_2, and are in abundance in the inflammatory process, in addition to other cells of the immune system and cell proliferation factors, and, for this reason, there is an uncontrolled increase in the amount of free radicals, which results in an oxidative stress field [33–36].

A literature review corroborates that oxidative stress is involved in the initiation of carcinogenesis in many ways, such as modification in energy metabolism, imbalance between antioxidants and oxidants, and nonspecific chronic inflammation activation with excessive production of pro-inflammatory cytokines, triggering ROS increase [37]. Oxidative stress can also modify the DNA structure and could contribute to tumor onset through environmental pollutants, as UV radiation does to CM [36].

Hallmarks of cancer: the Warburg effect

Around the 2000s, researchers Douglas Hanahan and Robert A. Weinberg published the article entitled "Hallmarks of cancer" in the renowned *Cell* Journal, bringing several impacts in the Oncology science worldwide. Attribution of the word "hallmarks" to cancer means that there are 6 characteristics common to tumors which are different from normal cells. Later, in 2011, these researches amplified the hallmarks and re-published updating in relation to it in the same journal the article entitled "Hallmarks of cancer: The next generation," now complementing the previous 6 with 4 new hallmarks. This conceptualization continues to be important nowadays [38, 39].

Therefore, the first hallmarks proposed were sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. The last 4 included deregulation cellular energetics (reprogramming cellular metabolism), immune evasion, tumor promoting-inflammation, and genome instability and mutation [38, 39].

Among the aforementioned, one of the new hallmarks, reprogramming cellular metabolism, curiously deserves to be highlighted due to its interference ability in other tumor parameters and hallmarks of cancer. In uncontrolled proliferation of tumors, there is a reprogrammed metabolic profile, with dramatically increased rate of glucose uptake and lactate production even in the presence of oxygen and fully functioning mitochondria, a phenomenon known as the Warburg effect (also called aerobic glycolysis) [38, 40, 41].

Most of the tumors present metabolic dependence on the Warburg effect and this condition is related with the promotion of cancer cell invasiveness, aggressiveness, and drug resistance [42–44]. Specifically on CM, although it has been associated with the glycolytic phenotype, recent studies have indicated that the metabolic phenotype nature of this pathology seems to go further, having dynamic plasticity [45–48].

Diverse evidence suggests that the immune cells' response in the TME contributes to cancer cell survival and growth [49]. Interestingly, this abnormal metabolic profile has been pointed out as one of the factors which support immune evasion to tumor cells and promote tumor progression [50–53]. What justified this fact is that, as cytoplasmic is produced, lactate is released in the extracellular microenvironment by monocarboxylate transporters (MCTs), leading to pH reduction and to TME acidification [54, 55]. Lactate is associated with inhibition of T-cell proliferation and alters cytokine production, as well as induces apoptosis of natural killer (NK) cells [56, 57]. Lactate also promotes cell suppression, both from innate and adaptive immunity, preventing maturation of dendritic cells (DCs) and favoring differentiation of regulatory T cells [58].

In addition to acting in immune mechanisms, the Warburg effect can interfere in gene expression and in respiratory function, such as increased expression of glucose transporter, hexokinase, and pyruvate kinase muscle (PKM2), modification in the expression of phosphoglycerate mutase (PGAM) that allows pyruvate production without ATP generation, increase in the pyruvate dehydrogenase kinase (PDK) levels, expression of specific transcription factors, mainly myelocytomatosis viral oncogene (MYC), hypoxia-inducible factor 1 alpha (HIF-1α), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and organic cation transporter 1 (OCT1) that sustain this effect [59].

Taking into consideration the strong evidence suggesting that the Warburg effect interferes in tumoral dynamics, such as supporting immune evasion, angiogenesis, or another mechanism, the inhibition of this tumor cell pathway seems to be one of the cancer targets in therapeutic perspectives, even against CM.

Main current therapeutic strategies for melanoma treatment

The early stages of melanoma can be surgically treated by tissue resection. Despite that, in advanced stages such as metastatic cases, it used to be considered untreatable, given its high therapeutic resistance rate. With therapeutic strategies aimed at the immune system and with relative efficacy approved by the Food and Drug Administration (FDA), this value has significantly improved. However, the 5-year survival rate of patients with metastatic melanoma is still very low, approximately 23% [22], and 15 to 20% of the tumors are resistant to conventional treatment in their first attempt [60].

This is because melanoma cells have several striking characteristics that boost their resistance, including high genetic instability that continuously promotes transient mutations, including mutations in mitogen-activated protein kinase (MEK), neuroblastoma viral oncogene homolog (NRAS), and that circumvent murine viral sarcoma inhibitor v-Raf oncogene homolog B1 (BRAF). Furthermore, its transduction network is highly effective in reconfiguring signals to prevent cell death. Similarly, melanoma cells mimic the stroma, promoting the development of growth factors and cytokines, among other mechanisms that favor TME [22].

Until 2011, conventional treatment was based on the use of chemotherapeutic agents that caused damage to cellular DNA, such as dacarbazine, leading to apoptosis. However, the drug's effectiveness in increasing the survival rate was not significant and the search for new alternatives found a new way out in immunotherapy and targeted therapies. In this context, monoclonal antibodies and BRAF and MEK inhibitors have become targets of several studies, with the aim of controlling immune responses [61, 62].

Ipilimumab, an anti-CTLA-4 antibody, and nivolumab and pembrolizumab, two anti-PD-1 and PD-L1 antibodies, were the immunological checkpoint inhibitors used in a literature review study by Namikawa and Yamazaki in 2019 [63], who showed greater efficacy when compared to the conventional treatment. They therefore concluded that the first line of choice for the treatment of the most advanced melanomas currently approved consists of three options, namely: nivolumab or pembrolizumab monotherapy or association of nivolumab with ipilimumab, and the choice is due to the melanoma subtypes [62, 63].

However, the use of ipilimumab is limited, as its response rate is approximately 11% and it has several

immunological side effects in organs, as it promotes exacerbation of normal and tumor cytotoxic T cells [64]. Furthermore, although anti-PD-1 and PD-L1 monoclonal antibodies exert a more satisfactory antitumor immune response (between 30 and 40%), not all patients are sensitive to them, which requires another treatment alternative [65].

Targeted therapy with the BRAF inhibitor is due to the fact that this gene is mutated in more than 50% of the cutaneous melanoma cases, the result of which is an alteration in the BRAF protein that assists in the accelerated growth of cancer cells [66, 67]. In the same signaling pathway, MEK gene inhibitors also reduce BRAF gene mutations and currently constitute the care standard in combination with BRAF inhibitors. This is because the progression-free survival of the isolated use of vemurafenib (inhibitor BRAF) allows for the development of melanoma resistance, while the association of another inhibitor verifies greater efficacy. The use of these combination therapies is approved by the FDA and includes vemurafenib/cobimetinib; dabrafenib/tramatenib; and encorafenib/binimetinib, the first being BRAF inhibitors and the second being MEK inhibitors [67, 68].

Although this combination prolongs survival and is the first choice for symptomatic patients with high tumor burden, acquisition of resistance is still a limiting factor for the development of an effective therapy [68]. Another limitation is the fact that this treatment does not cover 100% of the cutaneous melanomas, as it is not effective for those that do not present any BRAF gene mutation [67]. When compared to immunotherapy, targeted therapy offers a faster response and, clinically, the combination of both ends up being a treatment option [68].

Due to all these limitations pointed out, it is necessary to search for new therapeutic strategies in order to fully approach the patient and improve the prognosis. Furthermore, resistance to treatment is one of the main challenges to be overcome, as well as the side effects observed in immunotherapy [69]. In the medical, biomedical, and pre-clinical research studies, the cell lines are extremely important, such as animal cell culturing, since they provide almost all the genetic properties of cancer. In addition, apart from these cells derived from an in vivo sample, the environmental differences between the microenvironments such as the oxygen percentage and the tumor's interactions with other cells can change the results of the studies. However, the cell line, handled in an in vitro model, allows for the study of pathological features and for the application of new theories that cannot be carried out *in vivo*, especially as a preclinical model of a therapeutic view and, for this reason, widely used in cancer and drug research studies [70, 71].

Purinergic system: a brief characterization

The purinergic system is a signaling cell pathway present in basically all body tissues, composed by extracell molecules of nucleotides and nucleosides, receptors and enzymes (ectoenzymes), which participate in physiologic and pathologic mechanisms of the human organism, such as cell proliferation, differentiation, and death, as well as immunomodulation [72–75]. A recent study hypothesized on the potential role of the purinergic system on therapy in cardiovascular diseases mediated by SARS-CoV-2 [76].

Initially, when a review study about purinergic signaling was published by Geoffrey Burnstock, currently considered the father of the purinergic system, the hypothesis was not well accepted in the academic world, possibly because of the consolidated theory involving ATP molecules in energetic biochemistry into cells. However, now it is clear that this molecule, as well as other nucleotides, also acts as an extracell messenger in diverse biological effects [77, 78].

Among the purinergic system compounds, ATP, ADP, and AMP nucleotides stand out, as well as Ado nucleoside, which performs the role of signaling molecules. ATP deserves to be highlighted and is the focus of several research studies, due to diverse evidence indicating that this biomolecule acts in the cell metabolism process, as inducement of DNA synthesis in timocitus, suppression of NKs, chemotaxis, and tumor destruction [79, 80].

Thus, the cell receptors for the aforementioned molecules are classified as purinoceptor P1, which is mediated by Ado, and as purinoceptor P2, selective for ATP and/or ADP. The P1 receptors are separated into four subtypes: A1, A2A, A2B, and A3, and the P2 into two subfamilies: P2Y, G-protein-coupled, and P2X, which are ligand-gated ion channels (see Fig. 1). Currently, eight types of P2Y receptors have been identified (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14), as well as seven types of P2X receptors (P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, and P2X7) [81–83].

In addition, in purinergic signaling, there are enzyme structures involved that are coupled extracells in membranes, known as ectonucleotidases, them being ectonucleoside triphosphate diphosphohydrolase (E-NTPDases-CD39), ectonucleotide pyrophosphatase/phosphodiesterase (E-NPPS), alkaline phosphatase, ecto-5′-nucleotidase (5′-NT-CD73), and adenosine deaminase (ADA). All these enzymes play a role in the breakdown of ATP (see Fig. 1), which was previously described, and can generate their derivatives proceeding the initiation of the cascade with E-NTPDase action, which catalyzes the hydrolysis of ATP and ADP to AMP. Subsequently,

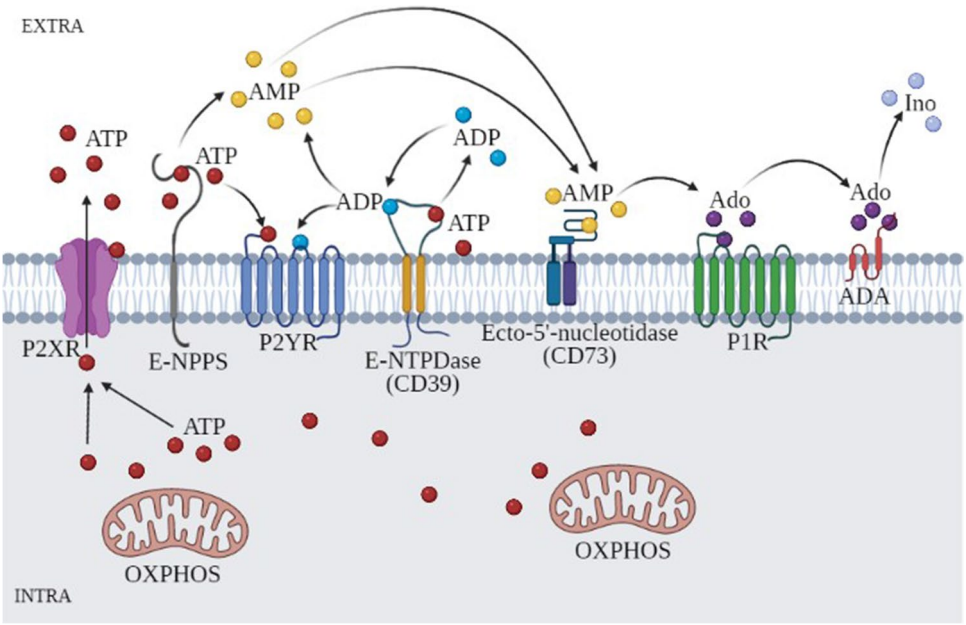


Fig. 1 Structures and functioning of the purinergic system. Initially, adenosine triphosphate (ATP), a key molecule of the purinergic system, is mainly generated intracellularly via oxidative phosphorylation (OXPHOS) in the mitochondria and can be released to the extracellular microenvironment by P2X type receptors (P2XR), such as P2X7. Once outside, it is available to act as cell signaling in P2XR and P2YR or to be hydrolyzed by ectonucleotidases to other nucleotides, such as adenosine monophosphate (AMP), adenosine diphosphate (ADP), and nucleosides, such as Adenosine (Ado). Only P1 type receptors (P1R) have affinity to Ado. Pyrophosphatase/phosphodiesterase (E-NPPS) has affinity to break down ATP straight to AMP, whereas ectonucleoside triphosphate diphosphohydrolase (E-NTPDase-CD39) can breakdown ATP to ADP or ADP to AMP. The only ectoenzyme capable of hydrolyzing AMP to Ado is ecto-5′-nucleotidase (E-5′-NT-CD73). Finally, adenosine deaminase (ADA), the finisher of the purinergic cascade, can break down Ado to inosine (Ino)

ecto-5′-nucleotidase catalyzes the transformation of AMP into adenosine and, finally, is deaminated through ADA, resulting in inosine. It can also be possible that E-NPPS promotes hydrolysis of ATP directly to AMP and continue the cascade until the end [84, 85].

Thus, the purinergic signaling system seems to be involved with the health and disease process, such as in different tumors, and the role of this cell pathway has been extensively studied [86]. Knowing this, in comparison with normal cells, tumor cells have a large amount of ATP, depletion of this nucleotide being a strategy to activate anticancer pathways. In addition, P2 receptors are observed in many cancer types and can present inhibitory effects preventing cell proliferation, interfering in cell cycle, and promoting cell death. However, this depends on the subtype of receptor, as cancer cells can have more death sensitivity or resistance [87].

As already pointed out, each receptor of the purinergic system has a specific and different action that depends on several factors, such as cell type. Therefore, in the context of ATP receptors, despite being frequently associated with anticancer effects, as far as P2Y1 and P2Y2 are concerned, there are hypotheses shown on the TME that they support cell growth and proliferation [87, 88]. This can be corroborated with the paper by Joo et al. [89], in which they observed the action of these receptors promoting niche metastatic formation on breast cancer.

It is worth highlighting that the formation of extracell adenosine can mostly occur from the activity of the CD39 and CD73 enzymes, both in healthy and neoplastic tissues, although other pathways are involved, such as E-NPP action from AMP hydrolysis, CD73 being a major generator of this molecule. Some studies have shown an increase in the expression of those enzymes in different cancer contexts [90–93].

For confirmation purposes, many researches work to report that purinergic signaling is related to neoplasms, such as in lung cancer [94, 95], leukemia [96], CM [86, 97], pancreatic cancer [98], osteosarcoma [99], and gastric cancer [100].

It is evident that much remains to be elucidated about the purinergic system, considering from its discovery to the present day; in addition, this cell signaling system is more consolidated as widespread on the human organism [101], which can be indicated as a therapeutic target. In reference to cancer, the interest in research is growing, only because of that considerable possibility [87].

Purinergic signaling in melanoma

As is well known, the purinergic system is involved in cancer dynamics, with the possibility of participating, according to the many factors, as a tumor promoter or inhibitor. This occurs by means of signaling molecules' action, mainly ATP, ADP, AMP, and Ado, on cell-receptors and through the ectonucleotidases' activity in TME. It is important to emphasize that this cell pathway has a close relationship with the immune response in the cancer context, as in other situations, and, considering the entire purinergic chain, the levels of purinergic molecules are controlled by a complex network of nucleotide/nucleoside-ectonucleotidases, expressed on the cells' surface. In addition to that, ATP is a pivotal molecule which largely influences immune responses in peripheral and central tissues [102–105], can be released from the inflammatory and tumor cells via different mechanisms, such as exocytosis, plasma membrane channels, or lysis, and can be accumulated in TME [92, 106].

In melanoma cells, a high concentration of ATP can have an anticancer effect because it activates the P2X7 type receptors, leading to cell death [107]. Despite that, when ATP is hydrolyzed to ADP, it can present an immunosuppression effect on CM [97]. On the other hand, Ado, a product of ATP hydrolysis that mediates the protective response, such as immunosuppressive and anti-inflammatory effects on healthy tissues, seems to have a pro-carcinogenic property, such as stimulus of tumor growth and angiogenesis on CM-affected cell, in addition to higher cell mobility and metastasis; in other words, when interacting with P1 receptors, a high concentration of Ado acts effectively in tumor progression [11, 50, 61, 108].

Mânica et al. [109] found significant data about the role of ATP in melanoma patients, indicating that an increased inflammatory process by extracellular ATP leads to an immunosuppressive profile even after CM surgical removal; and the purinergic system can develop a chronic inflammatory microenvironment which can exert a direct influence on relapse or metastasis. This effect involves deregulation of nucleotide and nucleoside levels in peripheral blood.

Other outstanding purinergic receptors involved in melanoma are those of the P2Y types, such as P2Y1, P2Y2, and P2Y12. P2Y1 receptors seem to reduce cell proliferation and P2Y2s usually appear to increase cell numbers [110], whereas P2Y12s have been related to tumor metastasis by platelet activation in melanoma cells [111].

Interestingly, one of the factors which leads to skin cancer, UV-B irradiation, seems to have a relationship with purinergic signaling. Severe effects have been associated with this irradiation type and shown to reduce the amount of P2X1 and P2Y2 receptors, as well as to destroy P2X7 receptors, with the possibility of contributing to malignant transformation of keratinocytes [112].

Also regarding modulation receptors in the melanoma context, stimulation of A2AR and A3AR tumor cells may enhance proliferation triggering melanoma cells' death. Deletion of A2ARs in myeloid cells was shown to revert immunosuppression in B16-melanoma-bearing mice via potentiation of NK and cytotoxic T lymphocytes responses [113–115]. A study in vivo to understand the effect of A1R, A2AR, and A3R signaling in B16 melanoma in mice found that the receptors contribute to TME by modulating angiogenesis, neovascularization, and infiltration of immunosuppressive tumor-associated macrophages (TAMs) [116].

In addition to the receptors, ectonucleotidases, given their importance in the purinergic chain, have been shown as involved in cancer. Thus, in the melanoma context, inhibition of CD39 can lead to extracellular ATP accumulation, reinforcing the close relationship between this neoplastic disease and the activity of enzymes that hydrolyze adenine nucleotides. In addition, high ATP levels in the post-surgery CM microenvironment are suggested to be the cause of deleterious changes [109].

CD73 also seems to be involved in melanoma, since the studies indicate that high CD73 expression is suggestively present in the disease [117, 118]. In the primary melanoma type, mutational status did not show any association with CD73 expression in metastatic lesions; however, patients with advanced cases tended to have more CD73 expression in their metastases [119]. Through a mouse model of melanoma, a CD73 inhibitor improved T and B cell–mediated anti-tumor immunity and reduced tumor growth [120].

Curiously, in addition to already established knowledge about CD73 capacity in the hydrolysis of nucleotides, it is involved in the invasion of cancer cells; it is also known that the nonenzymatic action of CD73 is to promote cell migration on ECM through activation of focal adhesion kinase (FAK) in melanoma cells [121].

Natural compounds: the promising rosmarinic acid

In the last years, due to technological advances, natural compounds, derivatives of living organisms, have been the target of several research studies involving human health, especially when it comes to anticancer action. They can be found in the entire nature and distributed in a wide range of world regions. Many papers have shown that, in general, these compounds can not only promote anticancer action, but also do apoptosis and arrest cell cycles [122–124].

Therefore, the phenolic compounds, present in almost all foods, are identified for having at least one aromatic ring with one or more hydroxyl groups. They can be found in fruits, vegetables, legumes, cereals, beer, coffee, wine, and spices, and are divided into classes called flavonoids, phenolic acids, phenolic alcohol, stilbenes, and lignins, according to their characteristics [125].

Among the phenolic acids, RA can be mentioned, which is a caffeic acid and a 3,4-dihydroxyphenyllactic acid derivative, present in herbal plants, especially in those from the *Boraginaceae* and *Lamiaceae* families, such as rosemary (*Rosmarinus officinalis L.),* basil *(Ocimum basilicum),* oregano *(Origanum vulgare),* sage *(Salvia officinalis)*, and *Melissa officinalis*, with high desired pharmacological capacity in cancer treatment, namely: its antioxidant, anti-inflammatory, and antitumor effects. Therefore, many kinds of research looking for these effects, in various cell lines and cancer types, were developed, and the findings appear to be promising [126–128].

In addition, regarding RA potential and considering the urgent need to identify novel treatments for melanoma due to the expensive conservative therapies [24, 129], it is reinforced that there are articles which have been presenting promising outcomes in other cancer contexts [130, 131], but which can also be indicated as a promising compound as an adjuvant therapy on the treatment for this skin neoplasia, in spite of few works existing.

Concerning the chemical characteristics, the International Union of Pure and Applied Chemistry (IUPAC) nomenclature for RA is (2R)-3-(3,4-dihydroxyphenyl)-2-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxypropanoic acid, to present it as crystalline solid red–orange, with molecular formula $C_{18}H_{16}O_8$, molecular weight of 360.3 g/mol, melting point of 171–175 °C, well-soluble in most organic solvents, such as ethanol, dimethyl sulfoxide (DMSO), or dimethyl formamide (DMF), but little soluble in water. In its chemical structure (Fig. 2), there are two phenolic rings and two OH ortho-position groups in each ring, which promote its antioxidant activity through H grant, in addition to an unsaturated double bond, a carbonyl group, and a carboxylic acid group [132–134].

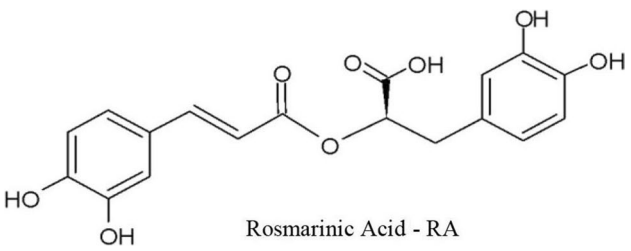


Fig. 2 Structural formula of RA. Chemical structure of phenolic compound rosmarinic acid ($C_{18}H_{16}O_8$)

The vegetable genesis of RA depends, in summary, on two pathways: the caffeoyl part is formed by L-phenylalanine action through cinnamic and 4-coumaric acid, and, at the same time, 3,4-dihydroxyphenyllactic acid is modulated by the action of L-tyrosine in 4-hydroxyphenylpyruvic acid [135, 136]. Each phase can be detailed as seen in Fig. 3, as well as the many enzymes involved in this mechanism.

Several studies were conducted to understand the pharmacokinetics to apply this compound on human beings and the indication that can be administered topically, pulmonary, intranasally, and via intravenous infusion, with the perioral route being the main form for intakes. Regarding metabolization, there is the tendency for it to occur by the intestinal microflora, where it is degraded to simple phenolic compounds by microorganisms, whereas distribution occurs by plasma albumins and excreted mainly through the renal system. Moreover, evidence has shown that herbal remedies containing RA reported no serious side effects and presented positive outcomes [137].

Despite the action mechanism of this compound not being in fact elucidated, many *in vitro* and *in vivo* studies were carried out to prove its anti-inflammatory effect [138], for example, describing the therapeutic potential of RA in inflammatory diseases such as colitis by reducing inflammatory cell infiltration, in addition to inhibiting induction of cyclooxygenase-2 (COX-2) and synthesis of IL-1β, IL-6, and IL-22. Furthermore, a bibliographic review conducted by Yahfoufi et al. [139] proved the immunomodulatory role of in vivo and in vitro polyphenols, due to interference with immune cell regulation in miscellaneous pathways by inactivating NF-κB, modulating arachidonic acid pathways responsible for COX-2 synthesis, and inhibiting some enzymes involved in the production of ROS.

Jang et al. [140] also proved such efficacy to see cell arrest cycle and apoptosis through modulation of gene expression related to prostate cancer initiation, histone deacetylases (HDAC). In the same manner, in the study by Luo et al. [138], the effect of RA on oral cancer was analyzed and it was verified that there was inhibition of cell proliferation due to the selective apoptosis mode, since the inhibitory effect was less present in normal cells. It is also important to mention that RA also has the capacity to prevent metastasis of the colorectal cancer phosphorylation adenosine monophosphate–activated protein kinase (AMPK) pathway, an enzyme that preserves cell homeostasis [141].

In conformity, RA proved to be efficient as an antioxidant by reducing the free radicals resulting from renal ischemia/reperfusion lesions, the focus of the study by Ozturk et al. [142]. In doing so, this phenolic acid acts increasing the expression of cytoprotective genes, reducing the cytotoxicity induced by xanthine oxidase and hydrogen peroxide (H_2O_2) and increasing the production

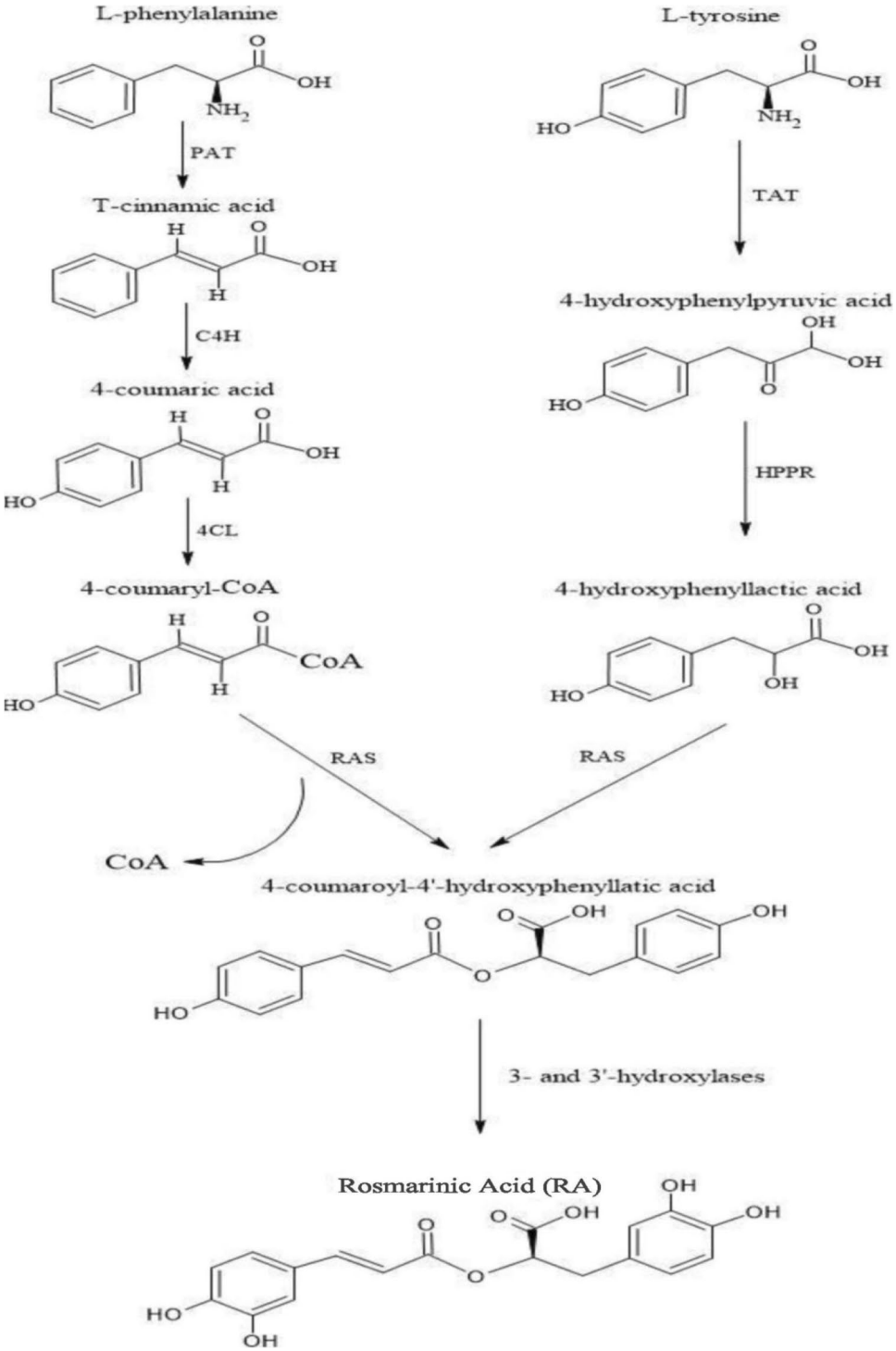


Fig. 3 Possible hypothesis of RA biosynthesis. Initially, RA biosynthesis occurs in double parallel vias which unite to effectively form the phenolic acid molecule. One of the vias begins with precursor amino acid L-phenylalanine transformation by phenylalanine ammonialyase (PAT) to T-cinammic acid. Afterwards, T-cinnamic acid is converted to 4-coumaric acid by cinnamic acid 4-hydroxylase (C4H) with addition of one OH-group in position 4 of the aromatic ring and subsequently transformed to 4-coumaryl-CoA by 4-coumarate-CoA ligase (4CL). Another via from amino acid L-tyrosine transformation, involving the tyrosine aminotransferase (TAT) enzyme, to 4-hydroxyphenylpyruvic acid and then, converted to 4-hydroxyphenyllactic acid by hydroxyphenylpyruvate reductase (HPPR). In the pre-final step, both vias are united; that is, the 4-coumaryl-CoA arising from the L-phenylalanine via and 4-hydroxyphenyllactic acid, from L-tyrosine, are incorporated by hydroxycinnamoyl-CoA hydroxyphenyllactate hydroxycinnamoyl transferase (RAS), and coenzyme A (CoA), present in 4-coumaryl-CoA, is released. Finally, RA via 3- and 3′-hydroxylase (3-H, 3′-H) is synthetized with introduction of OH-groups in positions 3′ and 3 of the aromatic rings

of prostaglandin E2 and reducing that of leukotriene B4, interleukin-6, interleukin-1-beta, and tumor necrosis factor-alpha, in addition to inhibiting the activation of the complementary system. Jointly, phenolic acid has the ability to reduce the free radicals through the hydroxyl group, and RA showed more efficiency, with 98.92% of the total antioxidant activity [143].

Anticancer and antitumor action of rosmarinic acid

Concerning the scientific research studies relating cancer and the potential of RA in the human body, scarcity of data is observed, although studies conducted using *in vitro* assays and in vivo animal models are easily found in the literature.

Table 3 presents some studies in different human cancer contexts where RA was used *in vitro* and *in vivo*, with their respective outcomes.

Countless research has evidenced RA in the treatment of cancer pathologies, but these action hypotheses are varied. One of them is the anti-inflammatory action, whereas the inflammatory process is closely related to tumor genesis, as previously mentioned. This action is proven by in vivo models, carried out by Swarup et al. [144] when reducing the mortality and pro-inflammatory cytokine levels in mice infected with the Japanese encephalitis virus. These researchers found a reduction in the IL-12, TNF-α, IFN-γ, MCP-1, and IL-6 levels when compared to those infected without treatment. Similarly, the anti-inflammatory effects in a neuroinflammatory lesion were proved not only in an in vitro model but also in an in vivo one, when reducing the expression of Toll-like receptor 4 (TLR4) and CD14, transmembrane receptors that activate the NF-kβ pathway, in addition to suppressing the activation of NLRP3 inflammasome, responsible for the maturation of pro-inflammatory cytokines IL-1β and IL-18 [145].

Completing the previous idea, some papers indicate the potential of RA for immune effect, such as the one published by Lembo et al. [146], which shows the increase of immune parameters like phagocytic activity, as well as concentrations of some biochemical blood characteristics, such as total cholesterol and its fractions of lipoprotein, and triglycerides in treating an animal chicken model with a concentration of 100 or 200 mg oil kg^{-1}. To reinforce this data, this phenolic compound also presented an anti-inflammatory effect against local and systemic inflammation in rats [147], decreased TNF-α, interferon-γ, IL-6, and IL-12 [148] levels, and significantly reduced MPO activity and TNF-α levels in mice [149].

As suggested by all these articles cited in Table 3, the apoptosis related to cell cycle arrest or alteration of apoptosis genes has strongly influenced the antitumor effect of RA. Both in the studies by Messeha et al. [17] and by Jang et al. [140], the use of this compound in breast and prostate cancer, respectively, revealed the apoptotic effect. At first, two breast cancer cell lines were analyzed, whose cell arrest occurred in the G0/G1 phase in one and in the S-phase in the other. In addition, the apoptotic gene expression, among the TNF, increased approximately 8.5-fold. In the second article, RA also proved its potential in prostate cancer when inhibiting HDAC, an enzyme highly expressed in cell cancer and that negatively regulates the expression of p53, a tumor suppressor gene.

In the same way, the antiproliferative effect is also associated with the anticancer properties of RA. Videlicet was tested in cells of Panc-1 and SW1990 cell lines, in pancreatic cancer, since it reduced the number of colonies when compared to the control group, by increasing the expression of MiR-506, a tumor suppressor miRNA. In addition, in that study, not only was the antiproliferative capacity clear but also the reduction in the cell invasion and migration capacity that promotes apoptosis and suppresses epithelial-mesenchymal transition (EMT) in the pancreatic cancer cells [150].

Conversely, in hepatocellular carcinoma, the RA pathway involves inflammation and angiogenesis, according to Cao et al. [151]. By enzyme-linked immunosorbent assays (ELISAs), the levels of inflammatory factors TNF-α, IL-6, and IL-1β, as well as of angiogenic factors (vascular endothelial growth factor, VEGF, and transforming growth factor-β, TGF-beta), were reduced when that phenolic compound was administered in mice. Furthermore, by Western blot, it was found that there was a reduction in the expression of NF-kβ p65, which regulates the angiogenic factors during CHC development, and consequently resulting in an antiangiogenic effect. Also in that study, the results showed that the toxicological effects of RA were insignificant, in addition to obtaining a 100% survival rate in the mice.

Regarding the present time, the production of ROS can trigger the formation of new tumors and, for this reason, the antioxidant effect is a positive factor for antitumor action. The phenolic acids, through the hydroxyl group, are known for their antioxidant potential, which prevents their reaction with the DNA molecule, resulting in a mutation. Among the phenolic acids, RA was the most effective in this regard (98.92% of activity) in addition to having the greatest chelating effect, followed by caffeic acid, and a protective effect in DNA against toxic substances like UV and H_2O_2 [143]. At last, according to Oguz et al. [152], it is an effective hepatoprotective agent and increases antioxidant capacity by reducing oxidative stress.

Moreover, the administration of RA in radio-resistant B16F10 lineage melanoma cells had a reducing effect on glutathione (GSH) after exposure to radioactivity irradiation, when compared to PNT2 prostate epithelial cells, considered radiosensitive. This effect is possibly due to the association with eumelanin synthesis pathways, intracellular consumption of GSH, and reduction of protective mechanisms against oxidative stress, which indicate a significant potential damage to radio-resistant cells during radiotherapy. It was observed that, at the same time, RA could present a protective action on healthy cells during therapy [153].

When analyzing RA as a promising compound for CM treatment, it is important that it prevents metastasis and, for this reason, that action was also to be searched and found in the literature. In a research study published in 2010 by Xu et al. [154], the antimetastatic action of this compound was already being studied, and, through experiments in animals, it has been proved that there is inhibition of metastasis in colorectal cancer (CRC) via the extracellular signal-regulated kinase pathway. More recently, Han et al. [141] report that such compound inhibits the proliferation of CRC cells, as well as hinders metastasis by AMPK phosphorylation.

Table 3 Some studies with pure RA in different human cancer pathological contexts over the past three years. IC_{50} (half maximum inhibitory concentration), MARK4 (microtubule affinity regulating kinase 4), TNFRSF25 (TNF receptor superfamily 25), TNFSF10 (TNF superfamily member 10), TNFRSF11B (TNF receptor superfamily 11B), BNIP3 (BCL-2 interacting protein 3), BIRC5 (baculoviral IAP repeat-containing 5), GADD45A (growth arrest and DNA damage-inducible 45 alpha), H_2O_2 (hydrogen peroxide), EGFR (epidermal growth factor receptor), NOX (NAD(P)H oxidase), PI3K/AKT/mTOR (phosphatidylinositol 3-kinase/serine-threonine protein kinase/mammalian target of rapamycin), MG132 (carbobenzoxy-L-z-L-leucyl-L-leucinal), AMPK (AMP-activated protein kinase), Bax (Bcl-2-associated X protein), HDAC2 (histone deacetylases 2), PARP-1 (poly(ADP-ribose)polymerase 1), and MALAT-1 (metastasis-associated lung adenocarcinoma transcript 1)

Study type	Pathological context	Dose/time	Main findings	References
In vitro A549 and MDA-MB-231 cells	Basal alveolar adenocarcinoma and breast cancer	IC_{50} and $2 \times IC_{50}$ (72 h) $IC_{50} = 6.204$ µM	↑apoptosis ↓MARK4 protein ↓cell proliferation	[15]
In vitro Hep-G2 cells	Liver carcinoma	0, 2.5, 5, 10, 20, 40, 80, 160, and 320 µM to cell viability assay (12 h) 0, 7, 14, 28 µM to apoptosis assay (24 h) $IC_{50} = 14$ µM	↑apoptosis (caspase-3 and caspase-9) ↓cell proliferation - cytotoxic effect against liver carcinoma cells	[16]
In vitro TNBC cell lines: MDA-MB-231 and MDAMB-468	Triple-negative breast cancer	0–500 µM (48–96 h) $IC_{50} = 350$ µM	↑apoptosis (TNFRSF25, TNFSF10, TNFRSF11B, BNIP3, BIRC5 and GADD45A) ↓cell proliferation - cycle arrest MDAMB-468 (S phase) and MDA-MB-231 (G0/G1 phase)	[17]
In vitro HNSCC cell lines: UM-SCC-1, UM-SCC-6 and OSC2	Head and neck carcinoma	80 µg/mL single and combined with blue light (24–96 h)	↑apoptosis ↓cell proliferation ↓H2O2 (EGFR increases NOX signaling)	[175]
In vitro SMMC 7721 cells	Liver carcinoma	0, 5, 10, 20, 50, 100, 200, 300, 400, and 500 µg/mL (24 h, 48 h, and 72 h)	↑apoptosis (PI3K/AKT/mTOR) ↓cell proliferation	[14]
In vitro Hep-G2 cells	Liver carcinoma	0, 10, 100, and 1.000 µM (24 h)	↑apoptosis - increased MG132-induced cytotoxicity, proteasome inhibition, autophagy, cellular stresses (1000 µM only)	[176]
In vitro HCT116 cells	Colorectal cancer	0, 50, 100, and 200 µM (24 h, 48 h, 72 h, and 96 h)	↑apoptosis ↓cell proliferation ↓metastatic (AMPK phosphorylation)	[141]
In vitro PC-3 and DU145 cells	Prostate cancer	25, 50, 100, 200, 250, and 300 µM (48 h–2 weeks)	↑apoptosis ↓cell proliferation ↓colony formation ↓HDAC2 (enzyme involved in tumor formation) - modulated Bax, caspase-3 and PARP-1	[140]
In vitro OVCAR-3 cells	Ovarian cancer	0, 5, 10, 20, 40, 80, and 160 µM (48 h and 72 h) $IC_{50} = 34.6$ and 25.1 µM/time respectively	↑apoptosis (MALAT-1) ↓cell proliferation - morphological alterations cells (shrinkage and rounding)	[12]
In vivo nude mice	Liver carcinoma	0, 5, 10, and 20 mg/kg (for 5 days)	↓colony formation ↓decreased volume and weight of tumor (inhibited growth of xenografts)	[14]
In vivo BALB/c female mice	Colorectal cancer	100 mg/kg/day (14 days)	↓metastatic (AMPK phosphorylation) ↓procaspase-9 and Bax - cycle arrest of the G0/G1 phase	[141]

In advanced CRC, the main invaded organs are the liver and the lungs, and the use of RA in this study drastically reduces the number of metastatic tumor nodules in the latter organs. That is because, for metastasis to occur, the cells must invade the extracellular matrix and then migrate to another site. During this process, however, cell invasion, migration, and adhesion occur, whose processes are induced by matrix metalloproteinases (MMP-2 and MMP-9). The action of RA consists of AMPK activation, which reduces the expression and activity of these proenzymes and, consequently, makes it difficult for the cancer cells to spread and promote metastasis [141].

Finally, regarding platelet aggregation, there are studies that searched for the influence of RA on this process, such as Zou et al. [155], who, through Danshen extract and having RA as active molecule, showed a potent inhibitory effect on the rats' platelets, aggregation induced by arachidonic acid, and that molecular docking thiol oxidoreductase enzyme (ERp57) is important for that process. Similarly, *Salvia yunnanensis* extract, which contains RA in its composition, inhibited ADP-induced rabbit platelet aggregation by binding rosmarinic acid with P2Y12R, cryptotanshinone, RA being fundamental in this outcome, which could be applied with antithrombotics [18].

With that, it is possible to notice that there are few works in the literature evidencing the potential of RA in CM treatment, considering the several outcomes presented previously and, when it comes to clinical trials, that number is even smaller, as well as those concerning the purinergic system. Thus, these verifications reinforce the need to better research and understand the mechanisms in that theme.

The purinergic system can be the target of rosmarinic acid in a pharmacological therapeutic perspective for cutaneous melanoma

In normal cells, hemostasis control occurs tightly through the production and release of growth factors, which regulate cell growth and proliferation and preserve normal tissue architecture. When these mechanisms are deregulated, homeostasis is also disrupted, leading to decreased apoptotic capacities, hyperproduction of growth factors, and, consequently, exacerbated and uncontrolled cell proliferation, as well as alterations in receptor molecules, which results in changes in the signaling cell pathway, and TME can increase [39].

In this aspect, the role of cell signaling seems to be involved in various mechanisms that can lead to cancer development, such as the microtubule affinity regulating kinase (MARK4) pathway that is associated with cancer progression and metastasis [156] and many other signaling pathways, such as NF-κB [157] and mTOR [14]. As shown before in another section of this paper, several studies have indicated the pharmaceutical potential of RA in modulating cell pathways in different cancer contexts; therefore, it can be a promising compound to be used in targeted therapies for molecules and receptors that participate in these processes.

Currently, interest in researching purinergic signaling, which is a cell pathway multistep coordinated cascade where ATP, ADP, and Ado act, has been growing because of evidence that confirms its important role in inflammatory processes, immune response, and cancer progression in different cell types [11, 102, 158]. These nucleotides and corresponding nucleoside involved in purinergic signaling have specific effects, depending on cell type and agonist/antagonist receptor-ligand [87].

The dynamics of the biochemical and cellular composition of TME are very important for the regulation of tumor cell metabolism, proliferation, motility, and dissemination, and can promote a protective effect or facilitate tumor progression [159, 160]. In this sense, ATP and Ado have recently been detected in high concentrations in TME [11, 92], ATP being frequently associated with the pro-inflammatory response, which plays a significant role in promoting antitumor effects, whereas Ado, a product of the hydrolysis of these nucleotides, presents immunosuppressive effects as negative-feedback, which prevents inflammation and tissue damage [161].

Another factor present in TME and that can be connected with purinergic signaling is lactate, arising from a phenomenon known as the Warburg effect, which occurs in most cancer cells. This phenomenon happens before the blood vessels are formed, where they nourish through the change in metabolic pathways and acquire energy via the glycolytic cascade under aerobics conditions [162]. Data indicates that cancer cells synthesize ATP via the Warburg effect, which leads to lactate formation and TME acidification [163], and, even in lower glucose concentrations, these cells can grow, differently from normal cells [39].

Thus, a plausible mechanism to this is that cancer cells expressing P2X7 receptors and activated by ATP, via the PI3K-AKT pathway, cause a moderate increase in the concentration of mitochondrial Ca^{2+}, stimulation of oxidative phosphorylation (OXPHOS), and increased ATP generation. Activation of this receptor upregulates the expression of the glucose transporter 1 (GLUT1) plasma membrane glucose transporter and the expression of several enzymes of the glycolytic cascade. This leads to increased lactate generation, which is extruded from cancer cells by monocarboxylate transporter 4 (MCT4), causing acidification of the tumor microenvironment, but is also taken up via monocarboxylate transporter 1 (MCT1) and used as a metabolite for energy-generating reactions in tumor cells. Lactate has been associated with inhibition of maturation (DCs) and with promoters

of macrophage differentiation, which can contribute to cancer cell proliferation, migration, and invasion [11].

Curiously, although other receptors with anticancer potential are described in the literature, P2X7, expressed by cancer cells as in CM, is the most associated with tumor cell killing via ATP [82, 164, 165]. On the other hand, P2X7 is overexpressed in some tumors and leukemias, which is associated with cancer progression and low survival prognosis [166], which could be explained from the hypotheses described above. It is to be highlighted that no specific agonist to this receptor has been described up to the present day [167].

In this context, the reduction of the Warburg effect seems to be an interesting strategy for CM treatment in the initial stage, because lactate can prevent tumor death in the microenvironment, as already shown, but there is little evidence about the modulation capacity of compounds that promote this mechanism. A study published in 2015 showed that RA has the ability to inhibit lactate generation through the suppression of HIF-1α; in this way, it presented a strong capacity to inhibit the Warburg effect in gastric cancer [168]. Regarding this theme, later papers have not been easily found in literature, and, considering these promising results, this phenolic acid could be a novel agent possibility arising to treat melanoma.

Nevertheless, following the reverse of what was proposed by Di Virgilio et al. [11], with the use of RA in CM treatment, this molecule could be a blocker of the P2X7R target and an inhibitor of the PI3K-AKT pathway, thereby suppressing HIF-1α, increase of Ca^{2+}, and, consequently, reduction of ATP in OXPHOS generation, as well as ATP release for this receptor. Furthermore, without PI3K-AKT activation, the GLUT1 transporter does not have the capacity to promote glucose intake, and lactate generation would be harmed. Finally, with less lactate release, the pH of TME can be altered and other mechanisms involved with tumor killing can be restored, such as immune responses (Fig. 4).

From another perspective, ATP signaling acts as immunosuppression in CM, as it is degraded to ADP and AMP in TME, promoting proliferation of regulatory T cells [97]. ADP is the main platelet recruiter, acting in the P2Y1 and P2Y12 G-protein-coupled receptors. While the first initiates platelet activation by morphological changes and has a weak response to ADP, the second has an effective response when promoting recruitment of these thrombocytes. For this reason, the use of P2Y12 inhibitors has been a therapeutic target for many pathologies, cancer among them [169].

The P2Y12 receptor is an important tumor growth modulator, since it promotes platelet activation by cancer cells. Once activated, it releases TGF-β and increases tumor cell adhesion to the endothelium, metastasis, angiogenesis, and drug resistance. This fact is verified by in vitro models which predict its in vivo aggressiveness when this event occurs. Consequently, by preventing the action of this receptor, the tumor would stop growing and this was observed in some types of cancer, such as ovarian and colorectal [170, 171].

With this purpose, data from the literature prove that *Salvia yunnanensis* (SY) is an herb rich in several compounds, including RA, which acts by blocking platelet aggregation induced by ADP and arachidonic acid through the P2Y12 receptor. Although the P2Y1 and P2Y12 receptors act in the formation of platelets, only P2Y12 is related to thrombin and other platelet agonists and, therefore, is the target of another hypothesis of this study. They are located in platelets and in the central nervous system, especially in microglia [18].

Thus, when analyzing the effects of the SY components, RA proved to be one of the main antiplatelet constituents in two ways: the first is the inhibitory effect of ERp57, a thiol oxidoreductase enzyme from the family of protein disulfide isomerase (PDI), which has an influence on platelet aggregation induced by arachidonic acid [155]. In addition, using molecular docking at the P2Y12 receptor, a high affinity between them and an ADP antagonistic effect was observed, which resulted in the inhibition of platelet aggregation [18].

Given the aforementioned, Mânica et al. [97] suggest that platelets are activated in patients with CM and that it can be a key to metastasis in this disease. This supports the use of RA as a potential tumor progression switch by purinergic signaling in P2Y12, since the immunosuppression caused by the ATP levels and which acts on this process can be inhibited by modulating these nucleotide and nucleoside degradation enzymes. Consequently, it is known that most of the cellular mutations are controlled by the immune system, which first tries to correct them by specialized intracellular proteins and, when that does not occur, the cell undergoes apoptosis. However, immunosuppression does not allow this control and, therefore, is closely related to the development, progression, and recurrence of the tumor. Therefore, by suppressing immunosuppression, the action of the immune cells can be resumed [172].

Knowing this, another possibility to treat CM based on the modulation of purinergic signaling by RA could be targeting P2Y12, leveraging the opportunity that the antagonist capacity of this molecule has on this receptor. Thereby, grounded on Ballerini et al. [173] and on Mânica et al. [97], since the action of RA on the P2Y12 receptor exerts an antagonist effect, it prevents ADP-ligand and inhibits adenylate cyclase (AC) and subsequent cascades, such as cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA), which finally inhibits phosphorylation of vasodilator-stimulated phosphoprotein (VASP), in turn harming the expression of adhesive proteins in the membrane surface of CM cells and adhesion between cells. This mechanism promotes decreased tumor mass and, in turn, constrains tumor progression and metastasis (Fig. 5).

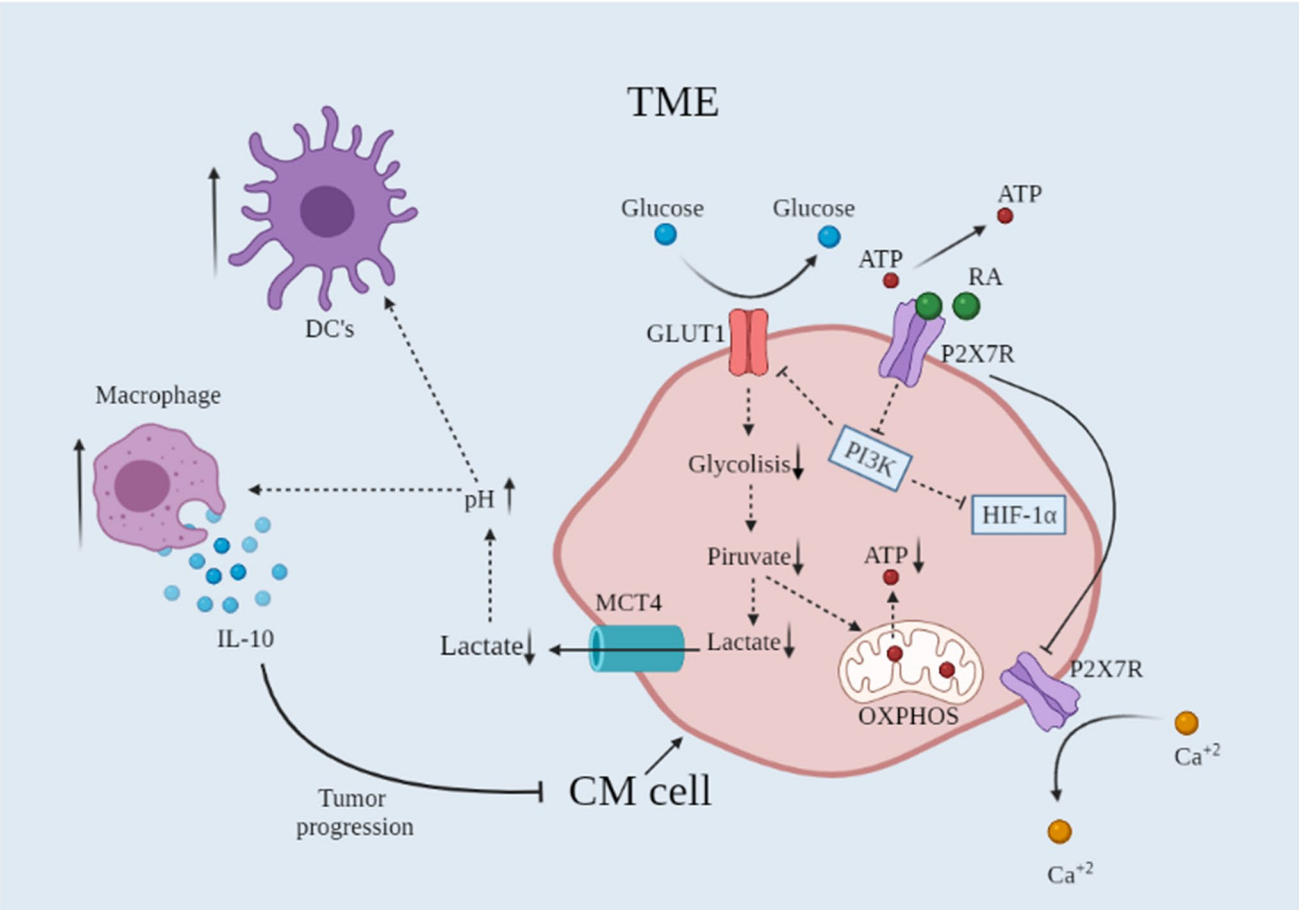


Fig. 4 Possible mechanism to block the P2X7 receptor and inhibit the Warburg effect through RA action. Since ionotropic purine receptor P2X type 7 (P2X7) is prevented from ATP-ligand by RA blocking, uptake of calcium ion (Ca^{2+}) is decreased and oxidative phosphorylation (OXPHOS) in the mitochondria downregulates ATP generation and release. In addition, the blocked P2X7 inhibits the phosphoinositide-3-kinase (PI3K–AKT) pathway, which downregulates the expression of glucose transporter 1 (GLUT1) in the plasma membrane with a reduction of glucose intake and mitigates pH acidification in TME due to lactate release by monocarboxylate transporter 4 (MCT4) from the aerobic glycolysis cascade. With pH regulated, there is chemiotaxis of the immune cells as DCs and macrophages, and a reduction in the secretion of cytokine IL-10 with inhibition of tumor progression. At the same time, with PI3K–AKT inhibition, there is no stimulus of hypoxia-inducible factor 1α (HIF-1α) and, consequently, a reduction in mitogenic/angiogenic inductors

Final considerations

This review work explored the potential antitumor effects of RA in different cancer contexts and brought up the hypothesis of its pharmacological application in the purinergic system as a treatment for CM. Nevertheless, for being an unprecedented work, the scarcity of articles evidencing the action of this compound in this signaling pathway requires further studies to be corroborated and proved. In addition, although there is consistent data on this anticancer potential in in vitro models, there are few articles portraying this experience in *in vivo* models and, in particular, in skin cancer.

Given this scenario, it is extremely important that the pharmacological potential of this phenolic acid as an option is further explored, especially when considering its aggressiveness, lack of effective therapeutic options, and ease of finding this compound in different sources. In spite of this, the results explored so far are positive, which allows us to believe in a new path to be followed when it comes to CM treatment.

That said, the literature indicates that the purinergic system is involved in the physiopathology of cancer, such as in CM. Moreover, RA seems to have an anticancer effect in different cancer contexts and to act on purinergic signaling. Considering this evidence, we hypothesize that RA can be an effective candidate for the adjuvant treatment of CM acting as a modulating molecule of purinergic cell pathways. On the one hand, there is a possibility, through P2X7 blocking by RA, of mitigating the Warburg effect, which negatively interferes on the microenvironment due to releasing exacerbated lactate from the glycolysis pathway, which leads

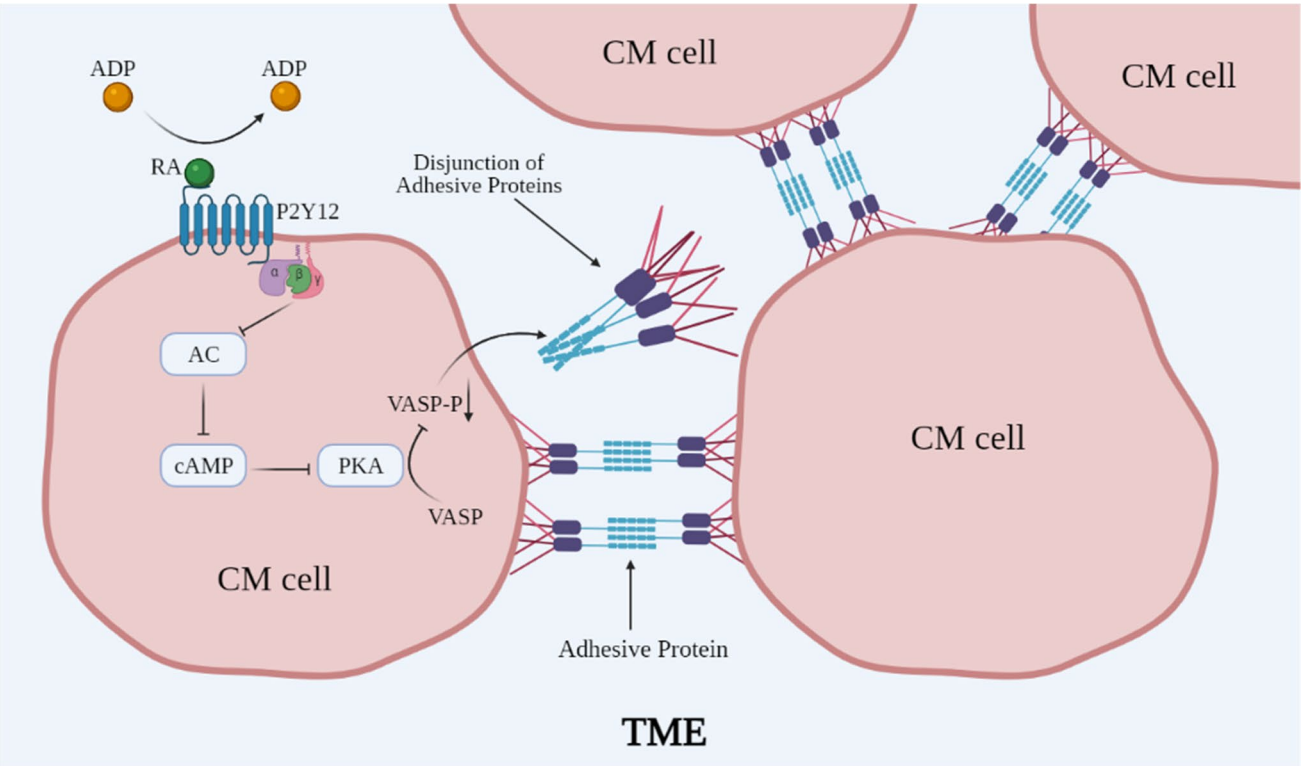


Fig. 5 Hypothesis of RA antagonism in P2Y12 receptor acting against progression of tumoral events. The ADP-like antagonism effect of RA on metabotropic purine receptor P2Y type 12 (P2Y12) prevents ADP-ligand, thus avoiding the intracellular cascade linked to activation of adenylate cyclase (AC) and its second messenger, cyclic adenosine monophosphate (cAMP), which does not promote activation of protein kinase A (PKA) to vasodilator-stimulated phosphoprotein (VASP) phosphorylation. All that intracell signaling leads to downregulation of the adhesive proteins' expression in the plasma membrane, reducing tumor mass formation in TME

to pH alteration and suppression of tumor-killing mechanisms, such as the immune response. On the other hand, an interesting alternative is to modulate the P2Y12 receptor by the RA antagonic molecule, reducing the formation of adhesive molecules to prevent adherence in tumor cells, and tumor mass inhibition, in turn constraining tumor progression and metastasis. In this way, our proposals for CM treatment based on targeting purinergic signaling permeate the integral practice, going from intracell to TME, which can be more effective than the techniques hitherto employed.

Undoubtedly, much is still to be discovered and elucidated about this promising compound, mainly concerning the pharmacological perspectives for CM, this paper being an interesting work baseline to support more research and, perhaps, promote new evidence for the development of clinical trials in human beings, which is very important in the improvement of CM treatment.

Funding The authors would like to thank Universidade Federal da Fronteira Sul – UFFS for the partnership.

Data availability Not applicable.

Declarations

Conflicts of interest Gilnei Bruno da Silva declares that she has no conflict of interest.
Milena Ayumi Yamauchi declares that she has no conflict of interest.
Daniela Zanini declares that she has no conflict of interest.
Margarete Dulce Bagatini declares that she has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent All authors are in agreement with the content of the manuscript and with the submission.

References

1. IARC - International Agency of Research in Cancer (2020). CANCER TODA Available in: http://gco.iarc.fr/today/home. Accessed in: may. 2021.
2. Paddock LE et al (2016) Skin self-examination and long-term melanoma survival. Melanoma Res 26(4):401–408. https://doi.org/10.1097/CMR.0000000000000255
3. INCA - Instituto Nacional do Câncer (Brazil). Estimative 2020: cancer incidence in Brazil. [Brasília, DF]: Instituto Nacional do

Câncer, 2018. Available in: https://www.inca.gov.br/publicacoes/livros/estimativa-2020-incidencia-de-cancer-no-brasil. Accessed May 2021
4. INCA - Instituto Nacional do Câncer (Brazil). Cancer of skin melanoma. [Brasília, DF]: Instituto Nacional do Câncer, 2021. Available in: https://www.inca.gov.br/tipos-de-cancer/cancer-de-pele-melanoma. Acessed May 2021
5. Carr S, Smith C, Wernberg J (2020) Epidemiology and risk factors of melanoma. Surg Clin North Am 100(1):1–12. https://doi.org/10.1016/j.suc.2019.09.005
6. Siegel R, Naishadham D, Jemal A (2013) Cancer statistics, 2013. CA: A Cancer Journal for Clinicians, 63(1)11–30. https://doi.org/10.3322/caac.21166.
7. Wilson, M. A.; Schuchter, L. M. Chemotherapy for melanoma. In: KAUFMAN, H. L.; MEHNERT, J. M. (Eds.). Melanoma. Cancer Treatment and Research. Cham: Springer International Publishing, 2016;167:209–229. https://doi.org/10.1007/978-3-319-22539-5_8.
8. Schadendorf, D. et al. Melanoma. The Lancet, 392(10151)971–984, sept. 2018. https://doi.org/10.1016/S0140-6736(18)31559-9.
9. Gao Z, Dong K, Zhang H (2014) The roles of CD73 in cancer. Biomed Res Int 1–9:2014. https://doi.org/10.1155/2014/460654
10. Ferrari D, Malavasi F, Antonioli L (2017) A purinergic trail for metastases. Trends Pharmacol Sci 38(3):277–290. https://doi.org/10.1016/j.tips.2016.11.010
11. Di Virgilio F et al (2018) Extracellular ATP and P2 purinergic signalling in the tumour microenvironment. Nat Rev Cancer 18(10):601–618. https://doi.org/10.1038/s41568-018-0037-0
12. Zhang, Y. et al. Anticancer effects of rosmarinic acid in OVCAR-3 ovarian cancer cells are mediated via induction of apoptosis, suppression of cell migration and modulation of lncRNA MALAT-1 expression. Journal of B.U.ON.: official journal of the Balkan Union of Oncology, 23(3)763–768, 2018.
13. Pelinson LP et al (2019) Antiproliferative and apoptotic effects of caffeic acid on Sk-Mel-28 human melanoma cancer cells. Mol Biol Rep 46(2):2085–2092. https://doi.org/10.1007/s11033-019-04658-1
14. Wang L et al (2019) Rosmarinic acid inhibits proliferation and invasion of hepatocellular carcinoma cells SMMC 7721 via PI3K/AKT/mTOR signal pathway. Biomed Pharmacother 120:109443. https://doi.org/10.1016/j.biopha.2019.109443
15. Anwar, S. et al (2020) Rosmarinic acid exhibits anticancer effects via MARK4 inhibition. Sci Rep 10(1)10300. https://doi.org/10.1038/s41598-020-65648-z
16. JIN, B. et al. Detailed studies on the anticancer action of rosmarinic acid in human Hep-G2 liver carcinoma cells: evaluating its effects on cellular apoptosis, caspase activation and suppression of cell migration and invasion. Journal of B.U.ON.: official journal of the Balkan Union of Oncology, 25(3)1383–1389, 2020.
17. Messeha SS et al (2020) Rosmarinic acid-induced apoptosis and cell cycle arrest in triple-negative breast cancer cells. Eur J Pharmacol 885:173419. https://doi.org/10.1016/j.ejphar.2020.173419
18. Li Y et al (2020) Screening for the antiplatelet aggregation quality markers of Salvia yunnanensis based on an integrated approach. J Pharm Biomed Anal 188:113383. https://doi.org/10.1016/j.jpba.2020.113383
19. Nguyen AV, Soulika AM (2019) The dynamics of the skin's immune system. International Journal of Molecular Sciences, 20(8). https://doi.org/10.3390/ijms20081811
20. Enninga EAL et al (2017) Survival of cutaneous melanoma based on sex, age, and stage in the United States, 1992–2011. Cancer Med 6(10):2203–2212. https://doi.org/10.1002/cam4.1152
21. INCA - Instituto Nacional do Câncer (Brazil). Online mortality atlas. (Brasília, DF): Instituto Nacional do Câncer, 2019. Available in: https://mortalidade.inca.gov.br/MortalidadeWeb/pages/Modelo08/consultar.xhtml#panelResultado. Accessed May 2021
22. Rebecca, V. W.; Somasundaram, R.; Herlyn, M. Pre-clinical modeling of cutaneous melanoma. Nature Communications, 11(1)2858, 2020. https://doi.org/10.1038/s41467-020-15546-9.
23. Michielin O et al (2019) Cutaneous melanoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 30(12):1884–1901. https://doi.org/10.1093/annonc/mdz411
24. Davis LE, Shalin SC, Tackett AJ (2019) Current state of melanoma diagnosis and treatment. Cancer Biol Ther 20(11):1366–1379. https://doi.org/10.1080/15384047.2019.1640032
25. Liu, V. et al. Pathologic characteristics of melanoma (2020). UpToDate. Available in: https://www.uptodate.com/contents/pathologic-characteristics-of-melanoma?search=diagnos is%20of%20cutaneous%20melanoma&source=search_result&selectedTitle=2~150&usage_ty pe=default&display_rank=2. Accessed May 2021
26. Cabrera R, Recule F (2018) Unusual clinical presentations of malignant melanoma: a review of clinical and histologic features with special emphasis on dermatoscopic Findings. Am J Clin Dermatol 19(Suppl 1):15–23. https://doi.org/10.1007/s40257-018-0373-6
27. Swetter, S; Geller, A. C. Melanoma: clinical features and diagnosis. UpToDate. 2020. Available in: https://www.uptodate.com/contents/melanoma-clinical-features-and-diagnosis. Accessed May 2021
28. Elder DE et al (2020) The 2018 World Health Organization classification of cutaneous, mucosal, and uveal melanoma: detailed analysis of 9 distinct subtypes defined by their evolutionary pathway. Arch Pathol Lab Med 144(4):500–522. https://doi.org/10.5858/arpa.2019-0561-RA
29. Volkovova, K. et al. Associations between environmental factors and incidence of cutaneous melanoma. Review. Environmental Health, 11(Suppl 1)S12, 2012. https://doi.org/10.1186/1476-069X-11-S1-S12.
30. Sample A, He YY (2018) Mechanisms and prevention of UV-induced melanoma. Photodermatol Photoimmunol Photomed 34(1):13–24. https://doi.org/10.1111/phpp.12329
31. Paulson KG et al (2020) Age-specific incidence of melanoma in the United States. JAMA Dermatol 156(1):57–64. https://doi.org/10.1001/jamadermatol.2019.3353
32. Rastrelli M et al (2014) Melanoma: epidemiology, risk factors, pathogenesis, diagnosis and classification. In Vivo (Athens, Greece) 28(6):1005–1011
33. Barbosa KBF et al (2010) Estresse oxidativo: conceito, implicações e fatores modulatórios. Rev Nutr 23(4):629–643. https://doi.org/10.1590/S1415-52732010000400013
34. Schmatz R (2011) Efeitos do resveratrol, do suco de uva e do vinho tinto nos biomarcadores de estresse oxidativo e na atividade de ectoenzimas em ratos diabéticos. 2011. Thesis (PhE in Byological Science: Toxicological Biochemistry) - Universidade Federal de Santa Maria, Santa Maria - RS
35. Kudryavtseva, A. V. et al. Mitochondrial dysfunction and oxidative stress in aging and cancer. Oncotarget, 7(29)44879–44905, 2016. https://doi.org/10.18632/oncotarget.9821.
36. Pizzino G et al (2017) Oxidative stress: harms and benefits for human health. Oxid Med Cell Longev 2017. https://doi.org/10.1155/2017/8416763
37. CANNAVÒ, S. P. et al. The role of oxidative stress in the biology of melanoma: A systematic review. Pathology - Research and Practice 215(1)21–28, 2019. https://doi.org/10.1016/j.prp.2018.11.020.

38. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144(5):646–674. https://doi.org/10.1016/j.cell.2011.02.013
39. Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100(1):57–70. https://doi.org/10.1016/S0092-8674(00)81683-9
40. Liberti MV, Locasale JW (2016) The Warburg effect: how does it benefit cancer cells? Trends Biochem Sci 41(3):211–218. https://doi.org/10.1016/j.tibs.2015.12.001
41. Hsu PP, Sabatini DM (2008) Cancer cell metabolism: Warburg and beyond. Cell 134(5):703–707. https://doi.org/10.1016/j.cell.2008.08.021
42. Scott DA et al (2011) Comparative metabolic flux profiling of melanoma cell lines. J Biol Chem 286(49):42626–42634. https://doi.org/10.1074/jbc.M111.282046
43. Icard, P.; Lincet, H. A global view of the biochemical pathways involved in the regulation of the metabolism of cancer cells. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 1826(2)423–433, 2012. https://doi.org/10.1016/j.bbcan.2012.07.001.
44. Vander Heiden MG, Cantley LC, Thompson CB (2009) Understanding the Warburg Effect: the metabolic requirements of cell proliferation. Science 324(5930):1029–1033. https://doi.org/10.1126/science.1160809
45. Hall, A. et al. Dysfunctional oxidative phosphorylation makes malignant melanoma cells addicted to glycolysis driven by the V600EBRAF oncogene. Oncotarget, 4(4)584–599, 30 apr. 2013. https://doi.org/10.18632/oncotarget.965.
46. Lyssiotis CA, Kimmelman AC (2017) Metabolic interactions in the tumor microenvironment. Trends Cell Biol 27(11):863–875. https://doi.org/10.1016/j.tcb.2017.06.003
47. Jones W, Bianchi K (2015) Aerobic glycolysis: beyond proliferation. Front Immunol 6. https://doi.org/10.3389/fimmu.2015.00227
48. Fischer GM et al (2018) Metabolic strategies of melanoma cells: mechanisms, interactions with the tumor microenvironment, and therapeutic implications. Pigment Cell Melanoma Res 31(1):11–30. https://doi.org/10.1111/pcmr.12661
49. Hinshaw DC, Shevde LA (2019) The tumor microenvironment innately modulates cancer progression. Can Res 79(18):4557–4566. https://doi.org/10.1158/0008-5472.CAN-18-3962
50. Whitton B et al (2018) Vacuolar ATPase as a potential therapeutic target and mediator of treatment resistance in cancer. Cancer Med 7(8):3800–3811. https://doi.org/10.1002/cam4.1594
51. Chen J et al (2021) Warburg effect is a cancer immune evasion mechanism against macrophage immunosurveillance. Front Immunol 11:621757. https://doi.org/10.3389/fimmu.2020.621757
52. Tan Z et al (2015) The Monocarboxylate transporter 4 is required for glycolytic reprogramming and inflammatory response in macrophages. J Biol Chem 290(1):46–55. https://doi.org/10.1074/jbc.M114.603589
53. Gottfried E et al (2006) Tumor-derived lactic acid modulates dendritic cell activation and antigen expression. Blood 107(5):2013–2021. https://doi.org/10.1182/blood-2005-05-1795
54. Frauwirth KA et al (2002) The CD28 Signaling pathway regulates glucose metabolism. Immunity 16(6):769–777. https://doi.org/10.1016/S1074-7613(02)00323-0
55. Payen VL et al (2020) Monocarboxylate transporters in cancer. Molecular. Metabolism 33:48–66. https://doi.org/10.1016/j.molmet.2019.07.006
56. Fischer K et al (2007) Inhibitory effect of tumor cell–derived lactic acid on human T cells. Blood 109(9):3812–3819. https://doi.org/10.1182/blood-2006-07-035972
57. Harmon C et al (2019) Lactate-mediated acidification of tumor microenvironment Induces apoptosis of liver-resident NK cells in colorectal liver metastasis. Cancer Immunol Res 7(2):335–346. https://doi.org/10.1158/2326-6066.CIR-18-0481
58. Morrot A et al (2018) Metabolic symbiosis and immunomodulation: how tumor cell-derived lactate may disturb innate and adaptive immune responses. Front Oncol 8:81. https://doi.org/10.3389/fonc.2018.00081
59. Levine AJ, Puzio-Kuter AM (2010) The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes. Science 330(6009):1340–1344. https://doi.org/10.1126/science.1193494
60. Czarnecka, A. M. et al. Targeted therapy in melanoma and mechanisms of resistance. International Journal of Molecular Sciences, 21(13)4576, 2020. https://doi.org/10.3390/ijms21134576.
61. Pasquali, S. et al. Systemic treatments for metastatic cutaneous melanoma. The Cochrane Database of Systematic Reviews, 2018(2)CD011123, 2018. https://doi.org/10.1002/14651858.CD011123.pub2.
62. Moreira, A. et al. Current melanoma treatments: where do we stand? Cancers, 13(2)221, 2021. https://doi.org/10.3390/cancers13020221.
63. Namikawa, K.; Yamazaki, N. Targeted therapy and immunotherapy for melanoma in Japan. Current Treatment Options in Oncology, 20(1)7, 2019. 10.1007 / s11864–019–0607–8.
64. Ramos-Casals, M. et al. Immune-related adverse events of checkpoint inhibitors. Nature Reviews. Disease Primers, 6(1)38, 2020. https://doi.org/10.1038/s41572-020-0160-6.
65. Eddy, K.; Chen, S. Overcoming immune evasion in melanoma. International Journal of Molecular Sciences, 21(23)8984, 2020. https://doi.org/10.3390/ijms21238984.
66. Luke JJ et al (2017) Targeted agents and immunotherapies: optimizing outcomes in melanoma. Nat Rev Clin Oncol 14(8):463–482. https://doi.org/10.1038/nrclinonc.2017.43
67. Hartman, R. I.; LIN, J. Y. Cutaneous melanoma-a review in detection, staging, and management. Hematology/Oncology Clinics of North America, 33(1)25–38, 2019. https://doi.org/10.1016/j.hoc.2018.09.005.
68. Curti BD, Faries MB (2021) Recent advances in the treatment of melanoma. N Engl J Med 384(23):2229–2240. https://doi.org/10.1056/NEJMra2034861
69. Kasakovski, D. et al. Advances in targeting cutaneous melanoma. Cancers, 13(9)2090, 2021. 10.3390 / cancers13092090.
70. Baust JM et al (2017) Best practices in cell culture: an overview. In Vitro Cellular & Developmental Biology - Animal 53(8):669–672. https://doi.org/10.1007/s11626-017-0177-7
71. Mirabelli P, Coppola L, Salvatore M (2019) Cancer cell lines are useful model systems for medical research. Cancers 11(8). https://doi.org/10.3390/cancers11081098
72. Burnstock G et al (2004) Cellular distribution and function of P2 receptor subtypes in different systems. Int Rev Cytol 240:31–304. https://doi.org/10.1016/S0074-7696(04)40002-3
73. Atkinson B et al (2006) Ecto-nucleotidases of the CD39/NTPDase family modulate platelet activation and thrombus formation: potential as therapeutic targets. Blood Cells Mol Dis 36:217–222. https://doi.org/10.1016/j.bcmd.2005.12.025
74. Burnstock G, Verkhratsky A (2012). Purinergic signalling and the nervous system. https://doi.org/10.1007/978-3-642-28863-0
75. Visovatti SH et al (2012) Increased CD39 nucleotidase activity on microparticles from patients with idiopathic pulmonary arterial hypertension. PLoS ONE 7:408–429. https://doi.org/10.1371/journal.pone.0040829
76. Dos Anjos F et al (2020) Potential therapeutic role of purinergic receptors in cardiovascular disease mediated by SARS-CoV-2. J Immunol Res 1–14:2020. https://doi.org/10.1155/2020/8632048
77. Burnstock G (2006) Purinergic signalling - an overview. Novartis Found Symp 276:26–48

78. Burnstock G (2007) Physiology and pathophysiology of purinergic neurotransmission. Physiol Rev 87(2):659–797. https://doi.org/10.1152/physrev.00043.2006
79. Burnstock G, Knight GE, Greig AVH (2012) Purinergic signaling in healthy and diseased skin. J Investig Dermatol 132:526–546. https://doi.org/10.1038/jid.2011.344
80. Leal, D. B. R. et al. Characterization of NTPDase (NTPDase1; ectoapyrase; ecto- diphosphohydrolase; CD39; EC 3.6.1.5) activity in human lymphocytes. Biochimica et Biophysica Acta - General Subjects, 1721(1–3)9–15, 2005. https://doi.org/10.1016/j.bbagen.2004.09.006.
81. Burnstock, G. Purine and purinergic receptors. Brain and Neuroscience Advances, 2: 239821281881749, jan. 2018. https://doi.org/10.1177/2398212818817494.
82. Di Virgilio F (2012) Purines, purinergic receptors, and cancer. Can Res 72(21):5441–5447. https://doi.org/10.1158/0008-5472.CAN-12-1600
83. Pfaffenzeller MS, Franciosi MLM, Cardoso AM (2020) Purinergic signaling and tumor microenvironment in cervical cancer. Purinergic Signalling 16(1):123–135. https://doi.org/10.1007/s11302-020-09693-3
84. Yegutkin, G. G. Nucleotide-and nucleoside-converting ectoenzymes: important modulators of purinergic signalling cascade. Biochimica et Biophysica Acta (BBA) – Molecular Cell Research, 1783:673–694, 2008. https://doi.org/10.1016/j.bbamcr.2008.01.024.
85. Zimmerman H et al (2007) Ectonucleotidases, molecular properties and functional impact. An R Acad Nac Farm 73:537–566
86. Bagatini MD et al (2018) The impact of purinergic system enzymes on noncommunicable, neurological, and degenerative diseases. J Immunol Res 1–21:2018. https://doi.org/10.1155/2018/4892473
87. Burnstock G, Di Virgilio F (2013) Purinergic signalling and cancer. Purinergic Signalling 9(4):491–540. https://doi.org/10.1007/s11302-013-9372-5
88. Xie R et al (2014) The P2Y2 nucleotide receptor mediates the proliferation and migration of human hepatocellular carcinoma cells induced by ATP. J Biol Chem 289:19137–19149. https://doi.org/10.1074/jbc.M113.540047
89. Joo, Y. N.; et al. P2Y2R activation by nucleotides released from the highly metastatic breast cancer cell MDA-MB-231 contributes to pre-metastatic niche formation by mediating lysyl oxidase secretion, collagen crosslinking, and monocyte recruitment. Oncotarget 2014; 5: 9322–9334. https://doi.org/10.18632/oncotarget.2427.
90. Aliagas E, Vidal A, Texido L, Ponce J, Condom E, Martin-Satue M (2014) High expression of ecto-nucleotidases CD39 and CD73 in human endometrial tumors. Mediators Inflamm 2014:509027. https://doi.org/10.1155/2014/509027
91. Cappellari AR et al (2012) Characterization of ectonucleotidases in human medulloblastoma cell lines: ecto-5'NT/CD73 in metastasis as potential prognostic factor. PLoS ONE 7:e47468. https://doi.org/10.1371/journal.pone.0047468
92. Di Virgilio F, Adinolfi E (2017) Extracellular purines, purinergic receptors and tumor growth. Oncogene 36(3):293–303. https://doi.org/10.1038/onc.2016.206
93. Longhi MS et al (2013) Biological functions of ecto-enzymes in regulating extracellular adenosine levels in neoplastic and inflammatory disease states. J Mol Med (Berl) 91:165–172. https://doi.org/10.1007/s00109-012-0991-z
94. Zanini, D. et al. Ectoenzymes and cholinesterase activity and biomarkers of oxidative stress in patients with lung cancer. Molecular and Cellular Biochemistry, 374(1–2)137–2013. https://doi.org/10.1007/s11010-012-1513-6.
95. Zanini, D. et al. ADA activity is decreased in lymphocytes from patients with advanced stage of lung cancer. Medical Oncology, 36(9)78, 2019. https://doi.org/10.1007/s12032-019-1301-1.
96. Ledderose C et al (2016) Cutting off the power: inhibition of leukemia cell growth by pausing basal ATP release and P2X receptor signaling? Purinergic Signaling 12(3):439–451. https://doi.org/10.1007/s11302-016-9510-y
97. Mânica A et al (2019) The signaling effects of ATP on melanoma-like skin cancer. Cell Signal 59:122–130. https://doi.org/10.1016/j.cellsig.2019.03.021
98. Hu L-P et al (2019) Targeting purinergic receptor P2Y2 prevents the growth of pancreatic ductal adenocarcinoma by inhibiting cancer cell glycolysis. Clin Cancer Res 25(4):1318–1330. https://doi.org/10.1158/1078-0432.CCR-18-2297
99. Zhang Y et al (2019) Highly-expressed P2X7 receptor promotes growth and metastasis of human HOS/MNNG osteosarcoma cells via PI3K/Akt/GSK3β/β-catenin and mTOR/HIF1α/VEGF signaling. Int J Cancer 1068–1082. https://doi.org/10.1002/ijc.32207
100. Hevia MJ et al (2019) Differential effects of purinergic signaling in gastric cancer-derived cells through P2Y and P2X receptors. Front Pharmacol 10:612. https://doi.org/10.3389/fphar.2019.00612
101. Burnstock G (2011) Introductory overview of purinergic signalling. Front Biosci E3(3):896–900. https://doi.org/10.2741/e298
102. Burnstock, G. Short- and long-term (trophic) purinergic signalling. Philosophical Transactions of the Royal Society B: Biological Sciences, 371(1700)20150422, 2016. https://doi.org/10.1098/rstb.2015.0422.
103. Stagg J, Smyth MJ (2010) Extracellular adenosine triphosphate and adenosine in cancer. Oncogene 29(39):5346–5358. https://doi.org/10.1038/onc.2010.292
104. Burnstock G (2015) Blood cells: an historical account of the roles of purinergic signalling. Purinergic Signalling 11(4):411–434. https://doi.org/10.1007/s11302-015-9462-7
105. Cekic C, Linden J (2016) Purinergic regulation of the immune system. Nat Rev Immunol 16(3):177–192. https://doi.org/10.1038/nri.2016.4
106. Ghiringhelli F et al (2009) Activation of the NLRP3 inflammasome in dendritic cells induces IL-1β–dependent adaptive immunity against tumors. Nat Med 15(10):1170–1178. https://doi.org/10.1038/nm.2028
107. Bian S et al (2013) P2X7 integrates PI3K/AKT and AMPK-PRAS40-mTOR signaling pathways to mediate tumor cell death. PLoS ONE 8:60184. https://doi.org/10.1371/journal.pone.0060184
108. Antonioli L et al (2014) Adenosine pathway and cancer: where do we go from here? Expert Opin Ther Targets 18:973–977. https://doi.org/10.1517/14728222.2014.925883
109. Manica A et al (2018) High levels of extracellular ATP lead to chronic inflammatory response in melanoma patients. J Cell Biochem 119(5):3980–3988. https://doi.org/10.1002/jcb.26551
110. White N et al (2005) P2Y purinergic receptors regulate the growth of human melanomas. Cancer Lett 224(1):81–91. https://doi.org/10.1016/j.canlet.2004.11.027
111. Gebremeskel S et al (2015) The reversible P2Y12 inhibitor ticagrelor inhibits metastasis and improves survival in mouse models of cancer: P2Y12 Inhibitor Ticagrelor Inhibits Metastasis. Int J Cancer 136(1):234–240. https://doi.org/10.1002/ijc.28947
112. Ruzsnavszky O et al (2011) UV-B induced alteration in purinergic receptors and signaling on HaCaT keratinocytes. J Photochem Photobiol, B 105(1):113–118. https://doi.org/10.1016/j.jphotobiol.2011.07.009
113. Merighi S et al (2002) Adenosine receptors as mediators of both cell proliferation and cell death of cultured human melanoma

cells. J Investig Dermatol 119(4):923–933. https://doi.org/10.1046/j.1523-1747.2002.00111.x
114. Cekic C et al (2014) Myeloid expression of adenosine A_{2A} receptor suppresses T and NK cell responses in the solid tumor microenvironment. Can Res 74(24):7250–7259. https://doi.org/10.1158/0008-5472.CAN-13-3583
115. Fishman, P. et al. A3 adenosine receptor as a target for cancer therapy: Anti-Cancer Drugs, 13(5)437–443, 2002. https://doi.org/10.1097/00001813-200206000-00001.
116. Koszałka, P. et al. Specific activation of A3, A2A and A1 adenosine receptors in CD73-knockout mice affects B16F10 melanoma growth, neovascularization, angiogenesis and macrophage infiltration. PLOS ONE, 11(3)e0151420, 2016. https://doi.org/10.1371/journal.pone.0151420.
117. Jiang, T. et al. Comprehensive evaluation of NT5E/CD73 expression and its prognostic significance in distinct types of cancers. BMC Cancer, 18(1)267, 2018. https://doi.org/10.1186/s12885-018-4073-7.
118. Sadej R, Spychala J, Skladanowski AC (2006) Expression of ecto-5′-nucleotidase (eN, CD73) in cell lines from various stages of human melanoma. Melanoma Res 16(3):213–222. https://doi.org/10.1097/01.cmr.0000215030.69823.11
119. Monteiro, I. et al. CD73 expression and clinical significance in human metastatic melanoma. Oncotarget, 9(42)26659–26669, 2018. https://doi.org/10.18632/oncotarget.25426.
120. Forte G et al (2012) Inhibition of CD73 improves B cell-mediated anti-tumor immunity in a mouse model of melanoma. J Immunol 189(5):2226–2233. https://doi.org/10.4049/jimmunol.1200744
121. Sadej R, Skladanowski AC (2012) Dual, enzymatic and non-enzymatic, function of ecto-5'-nucleotidase (eN, CD73) in migration and invasion of A375 melanoma cells. Acta Biochim Pol 59(4):647–652
122. Aung TN et al (2017) Understanding the effectiveness of natural compound mixtures in cancer through their molecular mode of action. Int J Mol Sci 18(3). https://doi.org/10.3390/ijms18030656
123. Noel B et al (2020) Role of natural compounds in preventing and treating breast cancer. Front Biosci (Schol Ed) 12:137–160
124. Ouyang, L. et al. Plant natural products: from traditional compounds to new emerging drugs in cancer therapy. Cell Proliferation, 47(6)506, 2014. https://doi.org/10.1111/cpr.12143.
125. Muller AG et al (2019) Delivery of natural phenolic compounds for the potential treatment of lung cancer. DARU Journal of Pharmaceutical Sciences 27(1):433–449. https://doi.org/10.1007/s40199-019-00267-2
126. Luo YA et al (2020) Anticancer effects of rosmarinic acid in human oral cancer cells is mediated via endoplasmic reticulum stress, apoptosis, G2/M cell cycle arrest and inhibition of cell migration. JBUON 25(2):1245–1250
127. Radziejewska I, Supruniuk K, Bielawska A (2021) Anti-cancer effect of combined action of anti-MUC1 and rosmarinic acid in AGS gastric cancer cells. Eur J Pharmacol 902. https://doi.org/10.1016/j.ejphar.2021.174119
128. Nunes S et al (2017) Therapeutic and nutraceutical potential of rosmarinic acid-cytoprotective properties and pharmacokinetic profile. Crit Rev Food Sci Nutr 57(9):1799–1806. https://doi.org/10.1080/10408398.2015.1006768
129. Pramanik KC et al (2013) Caffeic acid phenethyl ester suppresses melanoma tumor growth by inhibiting PI3K/AKT/XIAP pathway. Carcinogenesis 34(9):2061–2070. https://doi.org/10.1093/carcin/bgt154
130. Karthikkumar V et al (2015) Rosmarinic acid inhibits DMH-induced cell proliferation in experimental rats. J Basic Clin Physiol Pharmacol 26(2). https://doi.org/10.1515/jbcpp-2014-0044.
131. Yang K et al (2020) Rosmarinic acid inhibits migration, invasion, and p38/AP-1 signaling via miR-1225-5p in colorectal cancer cells. J Recept Signal Transduct 1–10. https://doi.org/10.1080/10799893.2020.1808674
132. Brewer MS (2011) Natural antioxidants: sources, compounds, mechanisms of action, and potential applications. Compr Rev Food Sci Food Saf 10(4):221–247. https://doi.org/10.1111/j.1541-4337.2011.00156.x
133. Elufioye, T. O.; Habtemariam, S. Hepatoprotective effects of rosmarinic acid: Insight into its mechanisms of action. Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie, 112,:108600,. https://doi.org/10.1016/j.biopha.2019.108600.
134. Pubchem. National Center for Biotechnology Information. Chemical information about rosmarinic acid (RA). Available in: https://pubchem.ncbi.nlm.nih.gov. Accessed May. 2021
135. Fialová S. et al (2019) Derivatization of rosmarinic acid enhances its in vitro antitumor, antimicrobial and antiprotozoal properties. Molecules, 24(6). https://doi.org/10.3390/molecules24061078
136. Petersen M (2013) Rosmarinic acid: new aspects. Phytochem Rev 12(1):207–227. https://doi.org/10.1007/s11101-013-9282-8
137. Hitle M. et al (2020) Rosmarinic acid–human pharmacokinetics and health benefits. Planta Med a-1301–8648,. https://doi.org/10.1055/a-1301-8648
138. Luo C et al (2020) A review of the anti-inflammatory effects of rosmarinic acid on inflammatory diseases. Front Pharmacol 11. https://doi.org/10.3389/fphar.2020.00153
139. Yahfoufi N. et al (2018) The immunomodulatory and anti-inflammatory role of polyphenols. Nutrients, 10(11). https://doi.org/10.3390/nu10111618
140. Jang, Y. G.; Hwang, K. A.; Choi, K. C. Rosmarinic acid, a component of rosemary tea, induced the cell cycle arrest and apoptosis through modulation of HDAC2 expression in prostate cancer cell lines. Nutrients, 10(11)1784, 2018. https://doi.org/10.3390/nu10111784.
141. Han YH, Kee JY, Hong SH (2018) Rosmarinic acid activates AMPK to inhibit metastasis of colorectal cancer. Front Pharmacol 9:68. https://doi.org/10.3389/fphar.2018.00068
142. Ozturk H et al (2014) Protective effects of rosmarinic acid against renal ischaemia/reperfusion injury in rats. J Pak Med Assoc 64(3):260–265
143. Sevgi K, Tepe B, Sarikurkcu C (2015) Antioxidant and DNA damage protection potentials of selected phenolic acids. Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association 77:12–21. https://doi.org/10.1016/j.fct.2014.12.006
144. Swarup V et al (2007) Antiviral and anti-inflammatory effects of rosmarinic acid in an experimental murine model of Japanese encephalitis. Antimicrob Agents Chemother 51(9):3367–3370. https://doi.org/10.1128/AAC.00041-07
145. Wei Y et al (2018) Rosmarinic acid mitigates lipopolysaccharide-induced neuroinflammatory responses through the inhibition of TLR4 and CD14 expression and NF-κB and NLRP3 inflammasome activation. Inflammation 41(2):732–740. https://doi.org/10.1007/s10753-017-0728-9
146. Lembo S et al (2014) The modulatory effect of ellagic acid and rosmarinic acid on ultraviolet-B-induced cytokine/chemokine gene expression in skin keratinocyte (HaCaT) cells. Biomed Res Int 1–8:2014. https://doi.org/10.1155/2014/346793
147. Rocha J et al (2015) Anti-inflammatory effect of rosmarinic acid and an extract of *Rosmarinus officinalis* in rat models of local and systemic inflammation. Basic Clin Pharmacol Toxicol 116(5):398–413. https://doi.org/10.1111/bcpt.12335
148. Friedman, T. The Effect of rosmarinic acid on immunological and neurological systems: a basic science and clinical review. Journal of Restorative Medicine, 4(1)50–59, 2015. https://doi.org/10.14200/jrm.2015.4.0105.
149. Marinho, S. et al. Anti-inflammatory effects of rosmarinic acid-loaded nanovesicles in acute colitis through modulation of

NLRP3 inflammasome. Biomolecules, 11(2)162, 26 Jan. 2021. https://doi.org/10.3390/biom11020162.
150. Han Y et al (2019) Rosmarinic inhibits cell proliferation, invasion and migration via up-regulating miR-506 and suppressing MMP2/16 expression in pancreatic cancer. Biomed Pharmacother 115:108878. https://doi.org/10.1016/j.biopha.2019.108878
151. Cao W et al (2016) Rosmarinic acid inhibits inflammation and angiogenesis of hepatocellular carcinoma by suppression of NF-κB signaling in H22 tumor-bearing mice. J Pharmacol Sci 132(2):131–137. https://doi.org/10.1016/j.jphs.2016.09.003
152. Oğuz A et al (2020) Investigation of antioxidant effects of rosmarinic acid on liver, lung and kidney in rats: a biochemical and histopathological study. Folia Morphol 79(2):288–295. https://doi.org/10.5603/FM.a2019.0087
153. Olivares, A. et al. Effect of rosmarinic acid and ionizing radiation on glutathione in melanoma B16F10 cells: a translational opportunity. Antioxidants, 9(12)1291, 2020. https://doi.org/10.3390/antiox9121291.
154. Xu Y et al (2010) Anti-invasion effect of rosmarinic acid via the extracellular signal-regulated kinase and oxidation–reduction pathway in Ls174-T cells. J Cell Biochem 111(2):370–379. https://doi.org/10.1002/jcb.22708
155. Zou J et al (2018) Discovery of a novel ERp57 inhibitor as antiplatelet agent from Danshen *(Salvia miltiorrhiza)*. Evidence-Based Complementary and Alternative Medicine 1–9:2018. https://doi.org/10.1155/2018/9387568
156. Heidary Arash, E. et al. MARK4 inhibits Hippo signaling to promote proliferation and migration of breast cancer cells. EMBO reports, 18(3)420–436, 2017. https://doi.org/10.15252/embr.201642455.
157. Liu, Z. et al. Mark4 promotes oxidative stress and inflammation via binding to PPARγ and activating NF-κB pathway in mice adipocytes. Scientific Reports, 6(1)21382, 2016. https://doi.org/10.1038/srep21382.
158. Helenius, M.; Jalkanen, S.; Yegutkin, G. G. Enzyme-coupled assays for simultaneous detection of nanomolar ATP, ADP, AMP, adenosine, inosine and pyrophosphate concentrations in extracellular fluids. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1823 (10)1967–1975, 2012. https://doi.org/10.1016/j.bbamcr.2012.08.001.
159. Mcallister SS, Weinberg RA (2010) Tumor-host interactions: a far-reaching relationship. J Clin Oncol 28(26):4022–4028. https://doi.org/10.1200/JCO.2010.28.4257
160. Munn, D. H.; BRONTE, V. Immune suppressive mechanisms in the tumor microenvironment. Current Opinion in Immunology, 39:1–6, 2016. https://doi.org/10.1016/j.coi.2015.10.009.
161. Allard B et al (2017) The ectonucleotidases CD39 and CD73: novel checkpoint inhibitor targets. Immunol Rev 276(1):121–144. https://doi.org/10.1111/imr.12528
162. Sawayama H et al (2014) Clinical impact of the Warburg effect in gastrointestinal cancer (Review). Int J Oncol 45(4):1345–1354. https://doi.org/10.3892/ijo.2014.2563
163. Campos-Contreras, A. Del R.; Díaz-Muñoz, M.; Vázquez-Cuevas, F. G (2020) Purinergic signaling in the hallmarks of cancer. Cells, 9(7). https://doi.org/10.3390/cells9071612
164. FENG, L. et al. Vascular CD39/ENTPD1 directly promotes tumor cell growth by scavenging extracellular adenosine triphosphate. Neoplasia, 13(3)206-IN2, 2011. https://doi.org/10.1593/neo.101332.
165. OHSHIMA, Y. et al. γ-Irradiation induces P2X7 receptor-dependent ATP release from B16 melanoma cells. Biochimica et Biophysica Acta (BBA) - General Subjects, 1800(1)40–46, 2010. https://doi.org/10.1016/j.bbagen.2009.10.008.
166. Adinolfi E et al (2018) The P2X7 receptor: a main player in inflammation. Biochem Pharmacol 151:234–244. https://doi.org/10.1016/j.bcp.2017.12.021
167. De Marchi, E. et al. P2X7 Receptor as a therapeutic target. In: Advances in Protein Chemistry and Structural Biology. Elsevier, 2016. 104:39–79. https://doi.org/10.1016/bs.apcsb.2015.11.004.
168. Han S, Yang S et al (2015) Anti-Warburg effect of rosmarinic acid via miR-155 in gastric cancer cells. Drug Design, Dev Ther 2695. https://doi.org/10.2147/DDDT.S82342
169. Korybalska, K. et al. The role of purinergic P2Y12 receptor blockers on the angiogenic properties of endothelial cells: an in vitro study. Journal of Physiology and Pharmacology, 2018. https://doi.org/10.26402/jpp.2018.4.06.
170. Cho MS et al (2017) Role of ADP receptors on platelets in the growth of ovarian cancer. Blood 130(10):1235–1242. https://doi.org/10.1182/blood-2017-02-769893
171. Bellefeuille SD, Molle CM, Gendron F-P (2019) Reviewing the role of P2Y receptors in specific gastrointestinal cancers. Purinergic Signalling 15(4):451–463. https://doi.org/10.1007/s11302-019-09678-x
172. Hemminki O, Dos Santos JM, Hemminki A (2020) Oncolytic viruses for cancer immunotherapy. J Hematol Oncol 13. https://doi.org/10.1186/s13045-020-00922-1
173. Ballerini P et al (2018) P2Y12 receptors in tumorigenesis and metastasis. Front Pharmacol 9:66. https://doi.org/10.3389/fphar.2018.00066
174. Gershenwald, J. E.; Scolyer, R. A. Melanoma staging: American Joint Committee on Cancer (AJCC) 8th Edition and Beyond. Annals of Surgical Oncology, 25(8)2105–2110, 2018. https://doi.org/10.1245/s10434-018-6513-7.
175. Waer, C. N. et al. Rosmarinic acid/ blue light combination treatment inhibits head and neck squamous cell carcinoma *in vitro*. Anticancer Research, 40(2)751–758, 2020. https://doi.org/10.21873/anticanres.14006.
176. Ozgun G, Ozgun E (2019) The cytotoxic concentration of rosmarinic acid increases MG132-induced cytotoxicity, proteasome inhibition, autophagy, cellular stresses, and apoptosis in HepG2 cells. Hum Exp Toxicol 39(4):514–523. https://doi.org/10.1177/0960327119896614

3.2 MANUSCRITO 1

Rosmarinic acid decreases viability, inhibits migration and modulates expression of apoptosis-related CASP8/CASP3/NLRP3 genes in human metastatic melanoma cells

Gilnei Bruno da Silva, Daiane Manica, Alana Patrícia da Silva, Filomena Marafon, Marcelo Moreno, Margarete Dulce Bagatini

Submetido à Revista *Chemico-Biological Interactions*.

Rosmarinic acid decreases viability, inhibits migration and modulates expression of apoptosis-related CASP8/CASP3/NLRP3 genes in human metastatic melanoma cells

Gilnei Bruno da Silva[1], Daiane Manica[1], Alana Patrícia da Silva[2], Filomena Marafon[1], Marcelo Moreno[1], Margarete Dulce Bagatini[1*]

[1] Postgraduate Program in Biomedical Sciences, Federal University of Fronteira Sul, Chapecó, SC, Brazil.

[2] Postgraduate Program in Food Science and Technology, Federal University of Fronteira Sul, Laranjeiras do Sul, PR, Brazil.

[*]Corresponding authors. E-mail: margaretebagatini@yahoo.com.br.

Abstract: cutaneous melanoma is the most aggressive type of skin cancer; it is difficult to treat, and has been highlighted in recent years due to increasing numbers of cases worldwide. The use of antitumoral therapeutics for this neoplasm has been associated with severe side effects, low quality of life, and resistance. We aimed in this study to explore the effect of the phenolic compound RA on human metastatic melanoma cells. SK-MEL-28 melanoma cells were treated for 24 hours with different concentrations of RA. In parallel, PBMCs also were treated with RA under the same experimental conditions to verify the cytotoxic effect on non-tumoral cells. Then, we assessed cell viability and migration, levels of intracellular and extracellular ROS, as well as NOx, NPSH, and PSH. Gene expression of the caspase 8, caspase 3 and NLRP3 inflammassome was evaluated by RT-qPCR. The enzymatic activity of the caspase 3 protein was assessed by a sensitive fluorescent assay. Fluorescence microscopy was employed to corroborate the effects of RA on melanoma cell viability, mitochondria transmembrane potential and apoptotic bodies formation. We found that RA potently reduces melanoma cell viability and migration after 24 hours of treatment. On the other hand, it has no cytotoxic effect on non-tumoral cells. The fluorescence micrographics indicated that RA reduces transmembrane potential of mitochondria and induces apoptotic bodies formation. Moreover, RA significantly decreases intracellular and extracellular ROS levels, and increases the antioxidant defenders NPSH and PSH. A remarkable feature found in our study was that RA strongly upregulates the gene expression of the caspase 8 and caspase 3, and downregulates NLRP3 inflammassome expression. Similar to gene expression, RA greatly increases the enzymatic activity of caspase 3 protein. Taken together, we have shown for the first time that RA reduces cell viability and migration of human metastatic melanoma cells, in addition to modulates apoptosis-related gene expression. We suggest that RA may have the potential to be used in a therapeutic perspective, particularly for CM cell treatment.

Keywords: Skin cancer. Polyphenols. Anti-cancer effect. Caspases. Apoptosis. Inflammassome.

Highlights:

- RA decreases viability and inhibits migration of human metastatic melanoma cells;
- RA reduces transmembrane potential of mitochondria and induces apoptotic bodies formation;
- RA decreases extracellular and intracellular ROS, and improvements in NPSH and PSH levels;
- RA modulates expression of apoptosis-related genes and increases the enzymatic activity of caspase 3 protein;
- We suggest that RA may have the potential to be used in a therapeutic CM perspective.

1 Introduction

Cutaneous melanoma (CM) is an aggressive and difficult to treat subtype of skin cancer, which originates from malignant melanocyte mutations. It may spread to other organs in a process called metastasis [1]. The incidence of melanoma has continued to increase worldwide and deserves attention [2]. This pathology is linked to excessive or unprotected sunbathing, which leads to DNA mutations by ultraviolet (UV) radiation [3].

Nowadays, cancer immunotherapies, such as checkpoint inhibitors, are routinely used as adjuvant and neoadjuvant treatments in melanoma patients, and have improved previously poor survival [4]. However, the use of this therapeutic strategy is associated with severe side effects and poor quality of life. Moreover, there is a risk of resistance immediately after treatment for several months [5]. Thus, there is an urgent need for new drugs or adjuvant therapies to improve patient survival and health quality [6].

In this context, the potential of phenolic compounds to be used in cancer as therapeutic adjuvants have been highlighted in recent years, and rosmarinic acid (RA), a phenolic acid, has been indicated to treat CM [7]. Several studies have shown that RA exhibits anticancer effects, such as in breast cancer [8], prostate cancer [9], and hepatocellular carcinoma [10]. In addition to these anticancer effects, it seems to be an antioxidant compound [11]. With this, more research should be carried out to clearly elucidate the possibilities regarding cell signalling pathways in which the RA may exert a pharmacological action against cancer.

Therefore, in this study, we aimed to explore the effects of the phenolic compound RA on the human metastatic melanoma cell line SK-MEL-28. We assessed the potential of RA to affect the viability and migration of cells, oxidative stress and antioxidant markers, and searched for a plausible cell signalling pathway to explain the observed cell death. We hypothesised that the mechanisms of apoptosis and the inhibition of cell migration might be due to the ability of RA to alter the expression of some apoptotic genes and enzymatic apoptotic effectors.

2 Material and methods

2.1 Chemicals and reagents

All chemicals and reagents used for this study were of analytical grade, purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA) and Merck (Darmstadt, Germany). RA (99% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture plates and flasks used for culture procedures were obtained from Gibco™ Thermo Fisher Scientific (Grand Island, NY, USA) and Invitrogen Life Technologies (Carlsbad, CA, USA). Molecular biology reagents were purchased from Invitrogen and Applied Biosystems (Waltham, Massachusetts, USA). Fluorescent images were captured using a fluorescent microscope (Nikon® Eclipse TS2-FL).

2.2 Cell culture and RA treatment

The human metastatic melanoma cell line SK-MEL-28 was purchased from the Cell Bank of Rio de Janeiro (BCRJ), Brazil. Cells were grown in flasks with Dulbecco's modified Eagle's medium (DMEM - high glucose with L-glutamine) containing antibiotics and an antifungal (penicillin/streptomycin) and supplemented with 10% fetal bovine serum. The cells were grown under adequate conditions in a humidified and controlled atmosphere of 5% carbon dioxide (CO_2) at 37°C. Peripheral blood mononuclear cells (PBMCs) were cultivated in Roswell Park Memorial Institute (RPMI) culture medium under the same conditions as the cancer cells. RA was dissolved in the appropriate culture medium to obtain different concentrations and the cells were treated for 24 hours at concentrations of 50 µM, 100 µM, 200 µM, 400 µM and 800 µM, based on a previous study performed by Anwar et al. [12], with adaptations. The negative control group cells received only culture medium.

2.3 Cell viability by MTT assay

The MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay was used according to a study carried out by Mosmann [13]. Briefly, melanoma cells were seeded in 96-well plates, in 4 replicates, at a density of 1×10^5 cells/well and treated with RA. Then, the supernatant was removed, and the cells were washed once with phosphate-buffered saline (PBS) (0.1 M, pH 7.4) to avoid any interference from the compound used in the treatment. The MTT (Sigma-Aldrich) reagent (5 mg/ml) dissolved in PBS was added and the plates were incubated for 2 hours at 37°C. Subsequently, the supernatant was discarded and 200 µL of DMSO was added in order to dissolve the formazan crystals generated by the reduction of the MTT salt by the viable cells. The absorbance was measured at 570 nm using a SpectraMax® i3 Multimode Plate Reader 96 microplates (Molecular Devices, Sunnyvale, CA, USA). The results were expressed as percentage (%) of cell viability relative to control.

2.4 Cell viability by fluorescence microscopy assay

Acridine orange (AO) was used for this assay, a fluorophore which is taken up by viable cells and stains double-stranded (ds) and single-stranded (ss) nucleic acids. When AO diffuses into DNA, it emits green fluorescence with intensity proportional to viable cells [14]. Thus, after treatment, cells were seeded at a density of approximately $1x10^5$ cells/well, in 96-well plates, washed twice with PBS and stained with AO. The readings were taken under a fluorescence microscope (480-490 nm), in triplicates, magnification of 10x, and adjusted for brightness and contrast linearly by the software Imagej®. The results were expressed as percentage (%) of fluorescence intensity relative to control.

2.5 Measurement of mitochondrial transmembrane potential (ΔΨm)

This assay was executed using tetramethylrhodamine ethyl ester (TMRE), which is a cationic dye substance that accumulates in active mitochondria and emits red fluorescence when excited at 550 nm, which is proportional to mitochondria transmembrane potential [15]. Briefly, cells were seeded at a density of approximately $1x10^5$ cells/well, in 96-well plates, washed twice with PBS, and then, incubated with 100 µL of a solution of Hank's balanced salts (HBSS) contain TMRE (20 nM) for 30 minutes. The readings were taken under a fluorescence microscope, in triplicates, magnification of 10x, and adjusted for brightness and contrast linearly by the software

Imagej®. The results were expressed as units of mitochondrial transmembrane potential (ΔΨm) in relation to control.

2.5 Detection of apoptotic bodies

Apoptosis is a process clearly elucidated for promote cell morphological changes including nuclear condensation, membrane bleeding, DNA fragmentation and formation of apoptotic bodies [16,17]. To evaluate apoptotic bodies formation, we stained the RA-treated melanoma cells with AO and taken fluorescence micrographs under a fluorescence microscope (480-490 nm; magnification of 10X). The photomicrographs were converted into grayscale to make more clear observation of apoptotic bodies morphotype and DNA fragmentations. The qualitatively analysis were made after auto-adjusts for brightness and contrast by the software Imagej®.

2.6 Wound-healing assay

The wound healing assay was performed as described by Justus et al. [18] to evaluate tumour cell migration. A monolayer of cells was seeded at a density of $1x10^6$ cells/well in 6-well plates. After reaching 100% confluence, a scratch was made with a sterile 200 µL tip and an initial photo was taken by optical microscopy at a magnification of 4x. After the treatment period, the supernatant was removed, the cells were washed with saline, and a new image was captured. The final images were adjusted for brightness and contrast linearly by the software Imagej®. The results were expressed as percentage (%) of wound-healing closure in relation to control.

2.7 Oxidative stress assays

For oxidative stress assays melanoma cells were seeded in 6-well plates, at densities of $1×10^6$ cells/well and treated with different concentrations of RA. After the exposure period, the supernatant was collected to perform the following analysis in triplicate.

2.7.1 Detection of extracellular and intracellular ROS

To detect reactive oxygen species (ROS), a commercial Fluorometric ROS kit detection (Sigma® Life Science, Darmstadt, Germany) was used, following the manufacturer's protocol. The product of the fluorometric reaction proportional to the amount of ROS present after 24 hours of treatment was measured at excitation = 490

and emission = 520 nm (Thermo Scientific™ Varioskan™ LUX). The results are expressed as ROS percentage (%) relative to control.

2.7.2 Detection of NOx levels

Nitric oxide (NOx) is a reactive nitrogen species (RNS) that can react with oxygen species (ROS) and biological molecules [19]. Due to its very short half-life, it is difficult to determine it and the end degradation products, nitrate/nitrite, are more suitable for detection [20]. Thus, we employed a modified Griess method to assess the reduction of nitrate to nitrite, according to a previous study [21] with adaptations. Firstly, the Griess reagent was made by the addition of 2% sulphanilamide and 0.2% N-1-naftiletilenodiamino-bicloridrato (NED) to a 5% orthophosphoric acid (H_2PO_4) medium. Then, 50 µL samples were mixed to 50 µL of vanadium chloride (VCl_3) at 0.08% and 50 µL of Griess reagent and incubated for 37ºC at 20 min. In principle, sulphanilamide reacts with the nitrite in the sample to form a diazonium salt which reacts with the NED to produce the purple-azo-dye product measured by absorbance at 540 nm. A calibration curve was prepared, and the results are expressed in µM.

2.7.3 Determination of PSH and NPSH levels

Both levels of thiols were determined according to Ellman [22] with adaptations. This method consists of the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) measured at 412 nm. For the total thiol (PSH) assay 40 µL of supernatant was added in 96-well plate to 200 µL of potassium phosphate buffer (PPB) (1M, pH 6,8) and 20 µL of DTNB and read immediately. The same experimental procedure was carried out for non-protein thiol (NPSH), except the samples were deproteinized by the addition of 10% trichloroacetic acid (TCA) before analysis. The colorimetric results were determined using a cysteine standard curve and expressed in µM.

2.8 Assessment of caspase 8, caspase 3 and NLRP3 inflammassome gene expression

Gene expression was assessed by real-time quantitative polymerase chain reaction (RT-qPCR) analysis. RNA was obtained from cell culture samples with TRIzol™ reagent (Invitrogen™) and quantified spectrophotometrically (NanoDrop™ Thermo Fisher Scientific). Reverse transcription into cDNA was performed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™), by the addition to

each 10 µL sample 10 µL of a mix containing 1 µL of MultiScribe™ Reverse Transcriptase. The steps of the reaction were: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, and a final hold step of 4°C for 30 minutes, performed using a thermal cycler. The RT-qPCR reaction was performed using 17 µL of a mix containing the SYBR Green PCR Master Mix (Applied Biosystems™) and 3 µL of the cDNA sample. The parameters used were a pre-activation step of 10 minutes at 95°C, followed by a cycling step of 40 cycles at 95°C for 15 seconds, 60°C for 60 seconds, and finally a melting step with a melting curve of 60°C to 95°C with an increase of 1°C every 5 seconds. The relative expression of each gene was represented as the fold expression in relation to the control and calculated using the comparative $^{\Delta\Delta}$CT value. GAPDH was used as the housekeeping gene to normalise gene expression. The forward and reverse sequences of oligos (5′-3′) used for each gene were as follows: GAPDH (F): CTCCTCACAGTTGCCATGTA; GAPDH (R): GTTGAGCACAGGGTACTTTATTG; CASP8 (F): AGGAGCTGCTCTTCCGAATT; CASP8 (R): CCCTGCCTGGTGTCTGAAGT; CASP3 (F): TTTGAGCCTGAGCAGAGACATG; CASP3 (R): TACCAGTGCGTATGGAGAAATGG; NLRP3 (F): CCATCGGCAAGACCAAGA; NLRP3 (R): ACAGGCTCAGAATGCTCATC.

2.9 Enzymatic activity of caspase 3 protein

A sensitive commercial fluorometric caspase 3 kit assay (Sigma® Life Science, Darmstadt, Germany) was used to assess enzyme activity, following the manufacturer's protocol. This assay is based on the hydrolysis of the specific peptide substrate by the caspase 3 protein, which results in a fluorescent product proportional to the activity of caspase 3 after 24 hours of RA treatment. Readings were measured at excitation = 360 and emission = 460 nm (Thermo Scientific™ Varioskan™ LUX) and expressed as relative fluorescence units (ΔRFU) in relation to control.

2.10 Statistical analysis

All measurements were statistically performed by analysis of variance followed by the appropriate *post hoc* test using GraphPad Prism 9 software. All data are expressed as mean ± standard deviation. The differences between the groups in relation to the studied variables were evaluated through the analysis of unpaired t-tests and one-way ANOVA. The differences in the probability of rejection of the null hypothesis at <5% ($p<0.05$) were considered statistically significant. Statistical

significance was defined for p-values of *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

3 Results

3.1 RA reduces the viability of cutaneous melanoma cells and has the opposite effect on non-tumoral cells

Figure 1 (A-B) shows the viability of tumoral SK-MEL-28 cells and non-tumoral PBMCs treated for 24 hours with RA. The RA concentrations 200 μM, 400 μM, and 800 μM significantly reduced the viability of CM cells compared to control (p<0.0001) (Fig. 1A). In PBMCs, RA showed no potential to reduce viability; on the contrary, it progressively increased cell viability as the concentration was increased (Fig. 1B).

Complementary to the MTT assay, we also assessed cell viability by fluorescence microscopy of melanoma cells (Fig. 2A). After treatment, melanoma cells presented significant reduction of fluorescence intensity at 200 μM (p<0.05), 400 μM and 800 μM (p<0.01) in comparison to control. This corroborates the previous results found with the MTT assay regarding the effect of RA on cell viability.

3.2 RA decreases the transmembrane potential of mitochondria

Our study also investigated whether that RA could exhibit effects on transmembrane potential of mitochondria from melanoma cells. As shows in Figure 2B, melanoma cells treated with RA had greatly reduction of fluorescence intensity at 100 μM, 200 μM, 400 μM, 800 μM (p<0.0001) when compared to control. This results confirms that RA exerts effects on mitochondria's functioning and converges to cell viability assays employed.

3.3 RA induces the formation of apoptotic bodies

We qualitatively detected the ability of RA to form apoptotic bodies, as shown in Figure 3. Compared to control, treatment with RA, in addition to decreasing the number of cells, induced the formation of apoptotic bodies with DNA fragmentation expressively at 400 μM and 800 μM.

3.4 RA inhibits cell migration

Figure 4 shows melanoma cell migration with and without RA treatment, in ascending concentrations, and the respective statistical analysis. There was a

significant decrease in wound closure at 100 μM ($p<0.001$) and more pronounced inhibition of closure at 200 μM, 400 μM, and 800 μM of RA treatment in relation to control ($p<0.0001$).

3.5 RA decreases ROS and improves PSH and NPSH levels

Figure 5 (A-E) shows the oxidative stress markers extracellular and intracellular ROS and NOx, and the levels of the antioxidants NPSH and PSH. After 24 hours of treatment, RA highly decreased the levels of intracellular and intracellular ROS at all concentrations tested relative to control ($p<0.0001$) (Fig. 5A-B). On the other hand, RA improved NPSH levels at 50 μM and 200 μM ($p<0.05$), and significantly increased NPSH at 400 μM ($p<0.01$) (Fig. 5C). Likewise, PSH also increased compared to control in treated melanoma cells; however, these levels were highest at 400 μM ($p<0.01$) and 800 μM ($p<0.001$) (Fig. 5D). There was no statistical difference significance for the NOx marker compared to control (Fig. 5E).

3.6 RA increases gene expression of caspase 8 and caspase 3, and decreases NLRP3 inflammassome

Figure 6 (A-C) shows the results of assays used to assess the caspase 8, caspase 3 and NLRP3 inflammassome gene expression in melanoma cells. After 24 hours, RA treatment greatly increased caspase 8 expression in all tested concentrations ($p<0.0001$) in comparison to control (Fig. 6A). Likewise, RA treatment significantly increased caspase 3 expression at 800 μM ($p<0.0001$) and increased at 200 μM and 400 μM ($p<0.001$) (Fig. 6B). On the other hand, RA provoked high decreasing in NLRP3 expression in all tested concentrations ($p<0.0001$) (Fig. 6C).

3.7 RA increases the enzymatic activity of the caspase 3 protein

Similar to gene expression, the enzymatic activity of the caspase 3 protein was highly increased in 800 μM ($p<0.0001$) and significantly increased at 400 μM ($p<0.001$) compared to control (Fig. 6D). This results also converges to cell viability and migration assays employed along our study.

4 Discussion

Findings from literature have highlighted the RA exhibits antitumor potential in a number of cancer contexts, such as hepatocellular carcinoma [10], ovarian [23],

prostate [9], breast cancer [8], colorectal [24], and skin cancer [25]. In addition, this phenolic acid has also been shown to participate in redox balance reactions [26]. However, the real effectiveness of RA in cutaneous melanoma and the cell signalling pathways involved still is poorly researched. Based on this, in the current study, we showed that RA decreased the viability and migration of human metastatic melanoma cells, modulates caspase 8, caspase 3 and NLRP3 inflammassome gene expression, as well as increases the enzymatic activity of caspase 3 protein.

To demonstrate this, we treated SK-MEL-28 cells with differing concentrations of RA for 24 hours to assess the antitumor effect. In parallel, we also treated PBMCs to verify the cytotoxic effects on non-tumoral cells under the same experimental conditions. Complementarily, we performed cell viability assay by fluorescence microscopy using AO as fluorochromes. We found that RA, in the range of 100 μM to 800 μM, significantly decreased melanoma cell viability, while showing no cytotoxic effects on PBMCs. Fluorescence microscopy corroborated the reduction in cell viability by the progressively lower fluorescence intensity. Anwar et al. [12] previously showed that RA has a cytotoxic effect by reducing MDA-MB-231 and A549 cell viability, and moreover showed a lack of toxic activity in non-cancer cells. At similar concentrations of RA used in our study, an *in vitro* experiment performed by Jang et al. [9] also found decreasing cell viability in two prostate cancer lineages, i.e. PC-3 and DU145. Another study that treated CT26 colorectal cancer cells showed that RA reduces cell viability at 200 μM in 24 hours [24].

Into cells, adenosine triphosphate (ATP) is produced by mitochondria in a process known as oxidative phosphorylation. This process requires the active transfer of positively charged protons across the inner mitochondrial membrane, resulting in an inner negative charge, known as the mitochondrial transmembrane potential [27]. The mitochondrial transmembrane potential dysfunction has been shown involved in several diseases, such as in autism [28], Alzheimer [29], and cancer [30]. Thus, for being involvement both in normal conditions and in cancer contexts, we assessed the transmembrane potential of mitochondria from RA-treated melanoma cells and found high reduction of the potential of mitochondria.

Cell death plays important role in many cases of physiological and pathological scenario, as in regulation of homeostatic state of multicellular organisms and tissues. Among the possibilities of cell death described, the apoptosis, pyroptosis and necroptosis are the well-known forms [31]. The apoptosis process has been shown to

promote cell morphological changings, which including nuclear condensation, membrane bleeding, DNA fragmentation and formation of apoptotic bodies [16,17]. In this context, we employed a qualitatively scan for possible apoptotic bodies formation in melanoma cells from effect of RA. Interestly, besides reduced cell number, we verified presence of apoptotic bodies in RA-treated melanoma cells with DNA fragmentation and visible cytoplasm modifications, more evident at 400 μM and 800 μM.

As well-know, CM is an aggressive type of tumour that affects the skin; mainly due to its highly metastatic nature, CM has a low survival rate [32]. In this context, metastasis, a complex biological process that involves invasion, migration, and intravasation into the blood, allows tumorigenic cells to spread out to organs distant from the original lesion [33]. It is known that this process involves interactions among ligand proteins presenting into extracellular matrices (ECM) of tumoral and non-tumoral cells [34]. For this reason, we employed a wound-healing assay to verify the capacity of RA to inhibit melanoma cell migration. Starting 100 μM, we observed significant inhibition of cell migration in our study. In research performed by Han et al. [24], the migration of murine colon carcinoma and human colon carcinoma cells was also inhibited, in agreement with our results. Studies that used RA to treat tumorigenic cells also confirmed its effects on migration cell parameters [8,35].

Furthermore, oxidative stress has been pointed out as an initiator of cancer via damage to DNA, proteins, and lipids, leading to mutagenesis, alterations in cell signalling, proliferation, survival, and stress resistance. However, it also may limit and kill a number of cancers [36,37]. In this context, considering that the mechanism of action some chemotherapy drugs is based on the overproduction of ROS [38,39], and that oxidative stress can act in two different manners on cancer, we assessed the oxidative stress markers ROS and NOx as a way to explain our results. However, we found significantly decreased levels of both extracellular and intracellular ROS. A previous study showed that RA has cytoprotective effects by scavenging intracellular ROS in human HaCaT keratinocyte cells [40]. Also, it has been shown that RA is not capable of inducing ROS generation and might be considered an antioxidant compound [41]. Besides ROS analysis, NOx levels did not show statistical significance, a similar result to the study performed by Waer et al. who used RA in head and neck squamous cell carcinoma [42]. This results, matches to the reduced transmembrane potential of mitochondria found in our present study.

Under homeostatic conditions, simultaneous with oxidative species formation, the tissues offset oxidative damage with the production of antioxidants [43]. The thiol system comprises important antioxidant compounds that contain a sulfhydryl group (-SH), such as glutathione (GSH) and cysteine/cystine [44,45]. Taking into consideration that our oxidative stress marker levels were reduced after the treatment of melanoma cells with RA and importance of thiols in redox balance, was assessed NPSH and PSH levels. We found increasing NPSH levels at 200 µM and 400 µM, while at 400 µM and 800 µM PSH was greatly increased. Although we have not found data in the literature about the influence of RA on thiol levels, one study showed that RA increases levels of GSH in melanoma B16F10 cells under ionising radiation [46]. Thus, these results on oxidative and antioxidant markers confirm that RA is a potent antioxidant phenolic compound.

Since our results showed that RA significantly decreased the viability and migration of cutaneous melanoma cells, but improved antioxidants and decreased levels of pro-oxidants, we did not have a clear cell signalling pathway to explain the exact mechanism by which RA exhibited antitumor effects on cells. In this sense, a cell death signalling pathway involving a group of proteins that act as apoptosis mediators named aspartate-specific cysteine proteases (caspases) has been evidenced [47]. Of the apoptosis-related caspases, the major effector protein is caspase 3, which is cleaved into an enzymatically active form, after activation by pro-apoptotic caspase 8 signal in canonical form [48]. Thus, we assessed gene expression of these caspases by RT-qPCR and found that RA strongly increased caspase 8 and caspase 3 gene expression in human metastatic melanoma cells. In a convergent way, we also detected increased enzymatic activity of caspase 3 at the same concentrations as those that RA increased the gene expression. A previous study also showed the potential of RA in increasing caspase 3 activity in SK-MEL-2 cells [49]. Similarly, RA has been shown to promote upregulating caspase 3 protein and gene expression in PC-3 and DU145 cells [9]. A study performed by Jin et al. [35] showed that RA inhibits proliferation, migration, and invasion of Hep-G2 liver cancer cells through the overexpression of caspase 3. All these set of molecular results are consistent to our micrographs findings about apoptotic bodies formation.

In contrast to studies and our findings, some data from the literature bring to light that the mechanisms by which RA leads to apoptosis are not restricted to the modulation of caspases. Messeha et al. [8], for example, indicated that RA-induced

apoptosis in MDA-MB-468 triple-negative breast cancer cell lines were caspase-independent. Another study also showed that RA induced apoptosis in colorectal cancer via the AMPK pathway [24]. Furthermore, apoptosis and the suppression of cell migration were associated with the effect of RA on the expression of lncRNA MALAT-1 in OVCAR-3 cells [23].

Among the possible molecular pathways that acts as pro-apoptotic signals further to the caspases, are the multiprotein complexes that play role in cellular homeostasis and cell death, known as inflammasomes [50]. The NLR pyrin domain containing protein 3 (NLRP3) is the most widely inflammasome type elucidated in literature [51]. The NLRP3 activation commonly occurs by the ROS pathway [52], but evidences also have been shown the possible interconnection between caspase 8 and inflammassomes activation [53]. Recently results showed aberrant overactivation of NLRP3 inflammasome in the cancer of lung and bladder [54,55]. Considering the its importance in cancer context, we searched for NLRP3 gene expression after melanoma cells being treated with RA. With this, we found, by RT-qPCR, that RA highly reduced NLRP3 gene expression in treated melanoma cells in all tested concentrations. In a research performed by Feng et al. [56] was demonstrated a possible effect of NLRP3 inflammasome acting as resistance promoter against drug-chemotherapy 5-fluorouracil in oral squamous cell carcinoma, and authors suggested it that may be targeting by adjuvants substances. Thus, our results confirm that RA is an interesting polyphenol candidate to be used in a therapeutic-target to ameliorates the overexpression of NLRP3 inflammasome.

Taken together, our results provided is evidence that RA exhibits effects on melanoma cells reducing cell viability, inhibiting migration, modulating apoptosis-related genes and increasing enzymatic activity of caspase 3 protein.

5 Conclusion

We have shown for the first time that RA reduces cell viability and human metastatic melanoma cell migration. RA upregulates caspase 8 and caspase 3 gene expression, as well as the enzymatic activity of caspase 3 protein. In addition, RA downregulates NLRP3 inflammasome in cutaneous melanoma cells. All these elements are crucial in the regulation of apoptotic events in several tumoral cells and we have proved that RA modulates their gene expression. Finally, RA has effects by decreasing intracellular and extracellular levels of ROS and increasing the antioxidant

defenders NPSH and PSH. This finding supports the notion that RA is a natural phenolic compound with antioxidant properties and anticancer effects. We further suggest that RA may have potential to be used in a therapeutic perspective, particularly for the treatment of CM cells.

Funding: This work is funded by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (MDB; Grant: 310606/2021-7), Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina (FAPESC) (MDB; Grant: 2021TR1543), Universidade Federal da Fronteira Sul (MDB). GBS and DM also thank Fundo de Apoio à Manutenção e ao Desenvolvimento da Educação Superior (FUMDES/UNIEDU) by the postgraduate scholarships.

Author contributions:

Conceptualisation: GBS.

Methodology: GBS, DM, APS, FM.

Investigation: GBS, DM.

Visualisation: MDB and MM.

Funding acquisition: MDB.

Project administration: GBS, DM and MDB.

Supervision: MDB.

Writing - original draft: GBS.

Writing - review, and editing: GBS and MDB.

All authors reviewed and approved the final version.

Code availability: Not applicable.

Data and materials availability: All data are available in the main text or supplementary materials.

Conflicts of interest: The authors have no relevant financial or non-financial interests to disclose.

Ethics approval: Not applicable.

Consent to publish: Not applicable.

References

[1] Cummins, D.L., Cummins, J.M., Pantle, H., Silverman, M.A., Leonard, A.L., Chanmugam, A., 2006. Cutaneous malignant melanoma. Mayo Clinic Proceedings 81, 500-507. https://doi.org/10.4065/81.4.500

[2] Markovic, S.N., Erickson, L.A., Rao, R.D., McWilliams, R.R., Kottschade, L.A., Creagan, E.T., Weenig, R.H., Hand, J.L., Pittelkow, M.R., Pockaj, B.A., Bardia, A., Vachon, C.M., Schild, S.E., Laman, S.D., Maples, W.J., Pulido, J.S., Cameron, J.D., 2007. Malignant melanoma in the 21st century, Part 1: Epidemiology, risk factors, screening, prevention, and diagnosis. Mayo Clinic Proceedings 82, 364-380. https://doi.org/10.4065/82.3.364

[3] Carr, S., Smith, C., Wernberg, J., 2020. Epidemiology and risk factors of melanoma. Surgical Clinics of North America 100, 1-12. https://doi.org/10.1016/j.suc.2019.09.005

[4] O'Day, S.J., Hamid, O., Urba, W.J., 2007. Targeting cytotoxic T-lymphocyte antigen-4 (CTLA-4): A novel strategy for the treatment of melanoma and other malignancies. Cancer 110, 2614-2627. https://doi.org/10.1002/cncr.23086

[5] Hwang, S.-Y., Chae, J.-I., Kwak, A.-W., Lee, M.-H., Shim, J.-H., 2020. Alternative options for skin cancer therapy via regulation of AKT and related signaling pathways. IJMS 21, 6869. https://doi.org/10.3390/ijms21186869

[6] Schadendorf, D., van Akkooi, A.C.J., Berking, C., Griewank, K.G., Gutzmer, R., Hauschild, A., Stang, A., Roesch, A., Ugurel, S., 2018. Melanoma. The Lancet 392, 971-984. https://doi.org/10.1016/S0140-6736(18)31559-9

[7] da Silva, G.B., Yamauchi, M.A., Zanini, D., Bagatini, M.D., 2022. Novel possibility for cutaneous melanoma treatment by means of rosmarinic acid action on purinergic signaling. Purinergic Signalling 18, 61-81. https://doi.org/10.1007/s11302-021-09821-7

[8] Messeha, S.S., Zarmouh, N.O., Asiri, A., Soliman, K.F.A., 2020. Rosmarinic acid-induced apoptosis and cell cycle arrest in triple-negative breast cancer cells. European Journal of Pharmacology 885, 173419. https://doi.org/10.1016/j.ejphar.2020.173419

[9] Jang, Y.-G., Hwang, K.-A., Choi, K.-C., 2018. Rosmarinic acid, a component of rosemary tea, induced the cell cycle arrest and apoptosis through modulation of

HDAC2 expression in prostate cancer cell lines. Nutrients 10, 1784. https://doi.org/10.3390/nu10111784

[10] Wang, L., Yang, H., Wang, C., Shi, X., Li, K., 2019. Rosmarinic acid inhibits proliferation and invasion of hepatocellular carcinoma cells SMMC 7721 via PI3K/AKT/mTOR signal pathway. Biomedicine & Pharmacotherapy 120, 109443. https://doi.org/10.1016/j.biopha.2019.109443

[11] Sevgi, K., Tepe, B., Sarikurkcu, C., 2015. Antioxidant and DNA damage protection potentials of selected phenolic acids. Food and Chemical Toxicology 77, 12-21. https://doi.org/10.1016/j.fct.2014.12.006

[12] Anwar, S., Shamsi, A., Shahbaaz, M., Queen, A., Khan, P., Hasan, G.M., Islam, A., Alajmi, M.F., Hussain, A., Ahmad, F., Hassan, Md.I., 2020. Rosmarinic acid exhibits anticancer effects via MARK4 inhibition. Sci Rep 10, 10300. https://doi.org/10.1038/s41598-020-65648-z

[13] Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55-63. https://doi.org/10.1016/0022-1759(83)90303-4

[14] McGahon, A.J., Martin, S.J., Bissonnette, R.P., Mahboubi, A., Shi, Y., Mogil, R.J., Nishioka, W.K., Green, D.R., 1995. Chapter 9 The End of the (Cell) Line: Methods for the Study of Apoptosis in Vitro, in: Methods in Cell Biology. Elsevier, pp. 153-185. https://doi.org/10.1016/S0091-679X(08)61929-9

[15] Joshi, D.C., Bakowska, J.C., 2011. Determination of mitochondrial membrane potential and reactive oxygen species in live rat cortical neurons. JoVE 2704. https://doi.org/10.3791/2704

[16] Tang, D., Kang, R., Berghe, T.V., Vandenabeele, P., Kroemer, G., 2019. The molecular machinery of regulated cell death. Cell Res 29, 347–364. https://doi.org/10.1038/s41422-019-0164-5

[17] Kerr, J.F.R., 1971. Shrinkage necrosis: A distinct mode of cellular death. J. Pathol. 105, 13–20. https://doi.org/10.1002/path.1711050103

[18] Justus, C.R., Leffler, N., Ruiz-Echevarria, M., Yang, L.V., 2014. In vitro cell migration and invasion assays. JoVE 51046. https://doi.org/10.3791/51046

[19] Romitelli, F., Santini, S.A., Chierici, E., Pitocco, D., Tavazzi, B., Amorini, A.M., Lazzarino, G., Di Stasio, E., 2007. Comparison of nitrite/nitrate concentration in human plasma and serum samples measured by the enzymatic batch Griess assay, ion-pairing HPLC and ion-trap GC-MS: The importance of a correct removal of proteins in the Griess assay. Journal of Chromatography B 851, 257-267. https://doi.org/10.1016/j.jchromb.2007.02.003

[20] Zahedi Asl, S., Ghasemi, A., Azizi, F., 2008. Serum nitric oxide metabolites in subjects with metabolic syndrome. Clinical Biochemistry 41, 1342-1347. https://doi.org/10.1016/j.clinbiochem.2008.08.076

[21] Tatsch, E., Bochi, G.V., Pereira, R. da S., Kober, H., Agertt, V.A., Anraku de Campos, M.M., Gomes, P., Duarte, M.M.M.F., Moresco, R.N., 2011. A simple and inexpensive automated technique for measurement of serum nitrite/nitrate. Clinical Biochemistry 44, 348-350. https://doi.org/10.1016/j.clinbiochem.2010.12.011

[22] Ellman, G.L., 1959. Tissue sulfhydryl groups. Archives of Biochemistry and Biophysics 82, 70-77. https://doi.org/10.1016/0003-9861(59)90090-6

[23] Zhang, Y., Hu, M., Liu, L., Cheng, X.-L., Cai, J., Zhou, J., Wang, T., 2018. Anticancer effects of Rosmarinic acid in OVCAR-3 ovarian cancer cells are mediated via induction of apoptosis, suppression of cell migration and modulation of lncRNA MALAT-1 expression. J BUON 23, 763-768.

[24] Han, Y.-H., Kee, J.-Y., Hong, S.-H., 2018. Rosmarinic acid activates AMPK to inhibit metastasis of colorectal cancer. Front. Pharmacol. 9, 68. https://doi.org/10.3389/fphar.2018.00068

[25] Alcaraz, M., Alcaraz-Saura, M., Achel, D.G., Olivares, A., López-Morata, J.A., Castillo, J., 2014. Radiosensitizing effect of rosmarinic acid in metastatic melanoma B16F10 cells. Anticancer Res 34, 1913-1921.

[26] Amoah, S., Sandjo, L., Kratz, J., Biavatti, M., 2016. Rosmarinic acid - Pharmaceutical and clinical aspects. Planta Med 82, 388-406. https://doi.org/10.1055/s-0035-1568274

[27] Crowley, L.C., Christensen, M.E., Waterhouse, N.J., 2016. Measuring Mitochondrial Transmembrane Potential by TMRE Staining. Cold Spring Harb Protoc 2016, pdb.prot087361. https://doi.org/10.1101/pdb.prot087361

[28] Rose, S., Niyazov, D.M., Rossignol, D.A., Goldenthal, M., Kahler, S.G., Frye, R.E., 2018. Clinical and Molecular Characteristics of Mitochondrial Dysfunction in Autism Spectrum Disorder. Mol Diagn Ther 22, 571–593. https://doi.org/10.1007/s40291-018-0352-x

[29] Gowda, P., Reddy, P.H., Kumar, S., 2022. Deregulated mitochondrial microRNAs in Alzheimer's disease: Focus on synapse and mitochondria. Ageing Research Reviews 73, 101529. https://doi.org/10.1016/j.arr.2021.101529

[30] Gong, Y., Luo, S., Fan, P., Zhu, H., Li, Y., Huang, W., 2020. Growth hormone activates PI3K/Akt signaling and inhibits ROS accumulation and apoptosis in granulosa cells of patients with polycystic ovary syndrome. Reprod Biol Endocrinol 18, 121. https://doi.org/10.1186/s12958-020-00677-x

[31] Kist, M., Vucic, D., 2021. Cell death pathways: intricate connections and disease implications. The EMBO Journal 40. https://doi.org/10.15252/embj.2020106700

[32] Paddock, L.E., Lu, S.E., Bandera, E.V., Rhoads, G.G., Fine, J., Paine, S., Barnhill, R., Berwick, M., 2016. Skin self-examination and long-term melanoma survival. Melanoma Research 26, 401-408. https://doi.org/10.1097/CMR.0000000000000255

[33] Vanharanta, S., Massagué, J., 2013. Origins of metastatic traits. Cancer Cell 24, 410-421. https://doi.org/10.1016/j.ccr.2013.09.007

[34] Trepat, X., Chen, Z., Jacobson, K., 2012. Cell Migration, in: Terjung, R. (Ed.), Comprehensive Physiology. Wiley, pp. 2369-2392. https://doi.org/10.1002/cphy.c110012

[35] Jin, B., Liu, J., Gao, D., Xu, Y., He, L., Zang, Y., Li, N., Lin, D., 2020. Detailed studies on the anticancer action of rosmarinic acid in human Hep-G2 liver carcinoma cells: evaluating its effects on cellular apoptosis, caspase activation and suppression of cell migration and invasion. J BUON 25, 1383-1389.

[36] Gill, J.G., Piskounova, E., Morrison, S.J., 2016. Cancer, oxidative stress, and metastasis. Cold Spring Harb Symp Quant Biol 81, 163-175. https://doi.org/10.1101/sqb.2016.81.030791

[37] Toyokuni, S., Okamoto, K., Yodoi, J., Hiai, H., 1995. Persistent oxidative stress in cancer. FEBS Letters 358, 1-3. https://doi.org/10.1016/0014-5793(94)01368-B

[38] Pourahmad, J., Amirmostofian, M., Kobarfard, F., Shahraki, J., 2009. Biological reactive intermediates that mediate dacarbazine cytotoxicity. Cancer Chemother Pharmacol 65, 89-96. https://doi.org/10.1007/s00280-009-1007-8

[39] Amirmostofian, M., Pourahmad Jaktaji, J., Soleimani, Z., Tabib, K., Tanbakosazan, F., Omrani, M., Kobarfard, F., 2013. Synthesis and molecular-cellular mechanistic study of pyridine derivative of dacarbazine. Iran J Pharm Res 12, 255-265.

[40] Fernando, P.M.D.J., Piao, M.J., Kang, K.A., Ryu, Y.S., Hewage, S.R.K.M., Chae, S.W., Hyun, J.W., 2016. Rosmarinic acid attenuates cell damage against UVB radiation-induced oxidative stress via enhancing antioxidant effects in human HaCaT cells. Biomolecules & Therapeutics 24, 75-84. https://doi.org/10.4062/biomolther.2015.069

[41] Wu, C.-F., Hong, C., Klauck, S.M., Lin, Y.-L., Efferth, T., 2015. Molecular mechanisms of rosmarinic acid from Salvia miltiorrhiza in acute lymphoblastic leukemia cells. Journal of Ethnopharmacology 176, 55-68. https://doi.org/10.1016/j.jep.2015.10.020

[42] Waer, C.N., Kaur, P., Tumur, Z., Hui, D.D., Le, B., Guerra, C., Henson, B., Seleem, D., Lewis, J., 2020. Rosmarinic acid/ blue light combination treatment inhibits head and neck squamous cell carcinoma *in vitro*. Anticancer Res 40, 751-758. https://doi.org/10.21873/anticanres.14006

[43] Sies, H., 2015. Oxidative stress: a concept in redox biology and medicine. Redox Biology 4, 180-183. https://doi.org/10.1016/j.redox.2015.01.002

[44] Rebrin, I., Sohal, R.S., 2008. Pro-oxidant shift in glutathione redox state during aging☆. Advanced Drug Delivery Reviews 60, 1545-1552. https://doi.org/10.1016/j.addr.2008.06.001

[45] Sen, C.K., Packer, L., 2000. Thiol homeostasis and supplements in physical exercise. The American Journal of Clinical Nutrition 72, 653S-669S. https://doi.org/10.1093/ajcn/72.2.653S

[46] Olivares, A., Alcaraz-Saura, M., Achel, D.G., Alcaraz, M., 2020. Effect of rosmarinic acid and ionizing radiation on glutathione in melanoma B16F10 cells: A translational opportunity. Antioxidants 9, 1291. https://doi.org/10.3390/antiox9121291

[47] Julien, O., Wells, J.A., 2017. Caspases and their substrates. Cell Death Differ 24, 1380-1389. https://doi.org/10.1038/cdd.2017.44

[48] Stennicke, H.R., Salvesen, G.S., 1998. Properties of the caspases. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology 1387, 17-31. https://doi.org/10.1016/S0167-4838(98)00133-2

[49] Jin, S., Kim, K.C., Kim, J.-S., Jang, K.-I., Hyun, T.K., 2020. Anti-melanoma activities and phytochemical compositions of *Sorbus commixta* fruit extracts. Plants 9, 1076. https://doi.org/10.3390/plants9091076

[50] Zhang, J., Wirtz, S., 2022. Does Pyroptosis Play a Role in Inflammasome-Related Disorders? IJMS 23, 10453. https://doi.org/10.3390/ijms231810453

[51] Mangan, M.S.J., Olhava, E.J., Roush, W.R., Seidel, H.M., Glick, G.D., Latz, E., 2018. Targeting the NLRP3 inflammasome in inflammatory diseases. Nat Rev Drug Discov 17, 588–606. https://doi.org/10.1038/nrd.2018.97

[52] Dostert, C., Pétrilli, V., Van Bruggen, R., Steele, C., Mossman, B.T., Tschopp, J., 2008. Innate Immune Activation Through Nalp3 Inflammasome Sensing of Asbestos and Silica. Science 320, 674–677. https://doi.org/10.1126/science.1156995

[53] Schwarzer, R., Jiao, H., Wachsmuth, L., Tresch, A., Pasparakis, M., 2020. FADD and Caspase-8 Regulate Gut Homeostasis and Inflammation by Controlling MLKL- and GSDMD-Mediated Death of Intestinal Epithelial Cells. Immunity 52, 978-993.e6. https://doi.org/10.1016/j.immuni.2020.04.002

[54] Kong, H., Wang, Y., Zeng, X., Wang, Z., Wang, H., Xie, W., 2015. Differential expression of inflammasomes in lung cancer cell lines and tissues. Tumor Biol. 36, 7501–7513. https://doi.org/10.1007/s13277-015-3473-4

[55] Poli, G., Brancorsini, S., Cochetti, G., Barillaro, F., Egidi, M.G., Mearini, E., 2015. Expression of inflammasome-related genes in bladder cancer and their association with cytokeratin 20 messenger RNA. Urologic Oncology: Seminars and Original Investigations 33, 505.e1-505.e7. https://doi.org/10.1016/j.urolonc.2015.07.012

[56] Feng, X., Luo, Q., Zhang, H., Wang, H., Chen, W., Meng, G., Chen, F., 2017. The role of NLRP3 inflammasome in 5-fluorouracil resistance of oral squamous cell carcinoma. J Exp Clin Cancer Res 36, 81. https://doi.org/10.1186/s13046-017-0553-x

Figures and Captions

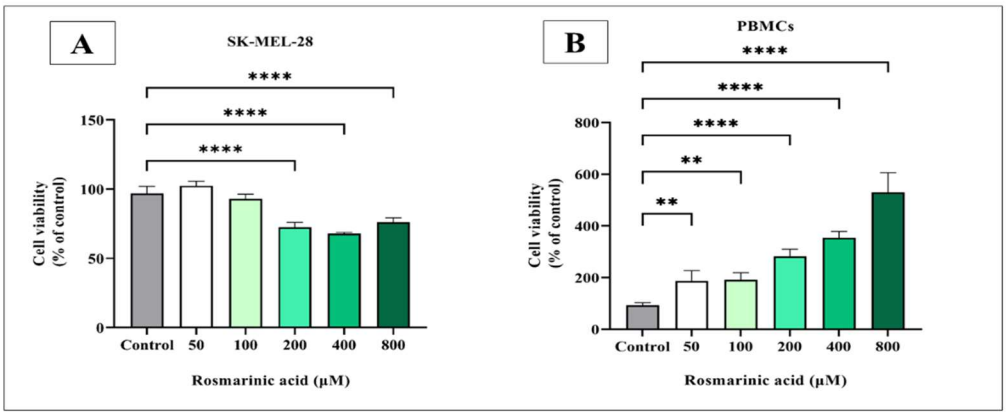


Fig. 1 – Cell viability of SK-MEL-28 and PBMCs. The tumoral and non-tumoral cell viability was evaluated by the MTT assay. RA significantly decreased SK-MEL-28 cells viability (A) at 200 µM, 400 µM and 800 µM, while it had no cytotoxic effects on PBMCs (B). *(p<0.05); ** (p<0.01); *** (p<0.001); **** (p<0.0001). All data indicate differences from the control group.

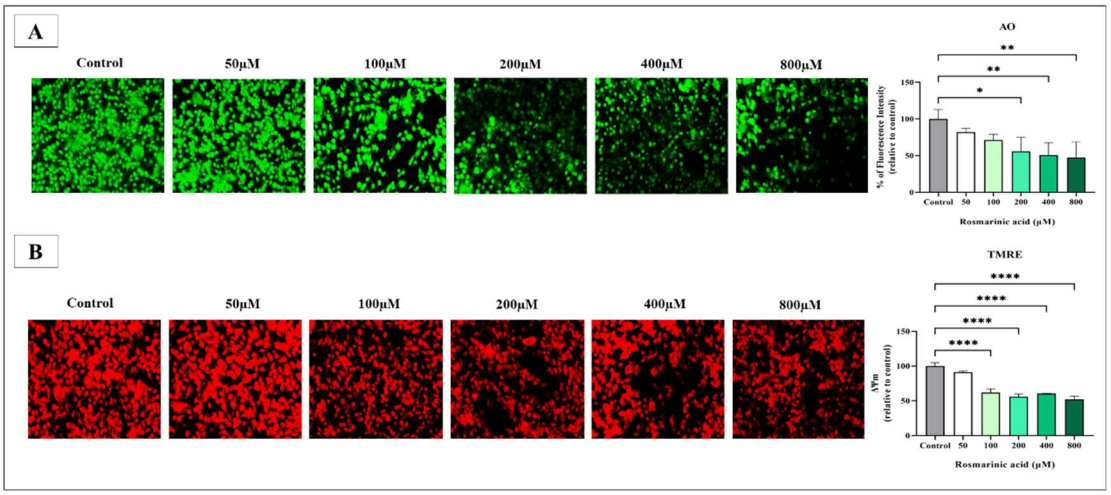


Fig. 2 – Fluorescence microscopy assay for cell viability and transmembrane potential of mitochondria. Melanoma cells was stained with AO, for cell viability assay, and TMRE, for mitochondria transmembrane potential assay, and analysed by fluorescence microscopy. After 24 hours, melanoma cells stained with AO showed decreasing in fluorescence intensity at 200 µM, 400 µM and 800 µM concentrations when compared to untreated control cells (A). Similarly, cells stained with TMRE showed significant reduction in transmembrane potential of mitochondria as RA treatment concentration increased (B). *(p<0.05); ** (p<0.01); ***(p<0.001); ****

(p<0.0001). All data indicate differences from the control group. Microscope magnification = 10x.

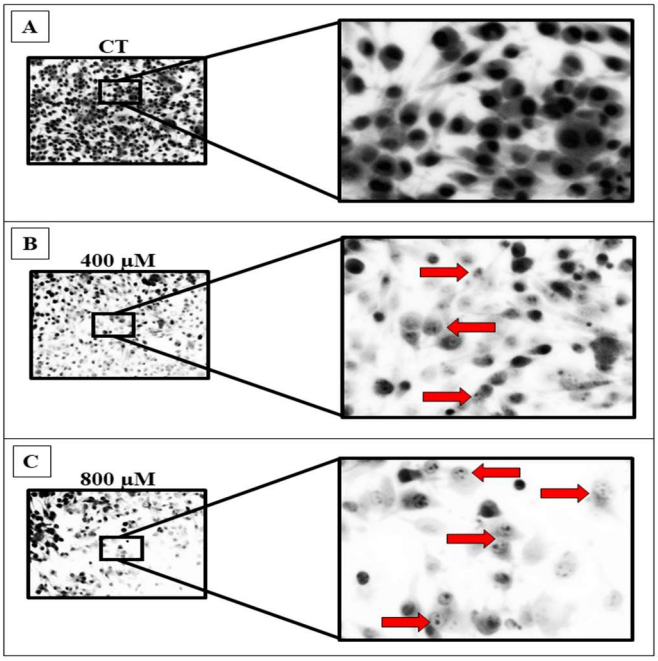


Fig. 3 – Formation of apoptotic bodies assay. After 24 hours of RA treatment, melanoma cells were stained with AO and photomicrographs was taken. The images were adjusted by Imagej software to grayscale and qualitatively analysed. RA induced formation of apoptotic bodies, with visible cytoplasm modifications and DNA fragmentation, more evident at 400 µM (B) and 800 µM (C) in relation to control. All data indicate differences from the control group. Microscope magnification = 10x.

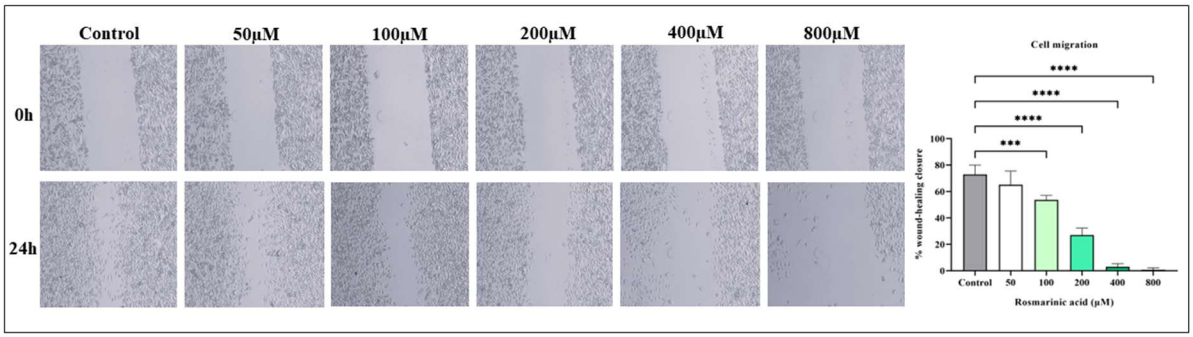


Fig. 4 – Migration of melanoma cells. Melanoma cell migration was evaluated by the wound-healing assay. After a cell monolayers formation, a scratch was made, cells were treated with RA for 24 hours, and photomicrography was taken. RA greatly prevented wound closure in the range from 100 µM to 800 µM. * (p<0.05); ** (p<0.01);

*** (p<0.001); **** (p<0.0001). All data indicate differences from the control group. Microscope magnification = 4x.

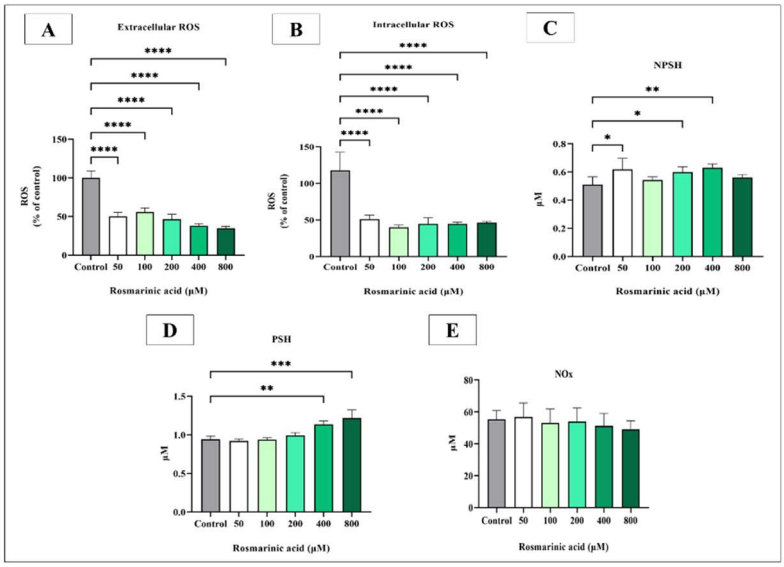


Fig. 5 – Pro-oxidant and antioxidant markers levels from melanoma cells. Treatment of melanoma cells with RA significantly decreased intracellular (A) and extracellular (B) ROS levels. On the other hand, NPSH (C) levels were increased at 200 µM and 400 µM, and PSH (D) levels were increased at 400 µM and 800 µM. There was no statistical significance in NOx (E) levels. * (p<0.05); ** (p<0.01); *** (p<0.001); **** (p<0.0001). All data indicate differences from the control group.

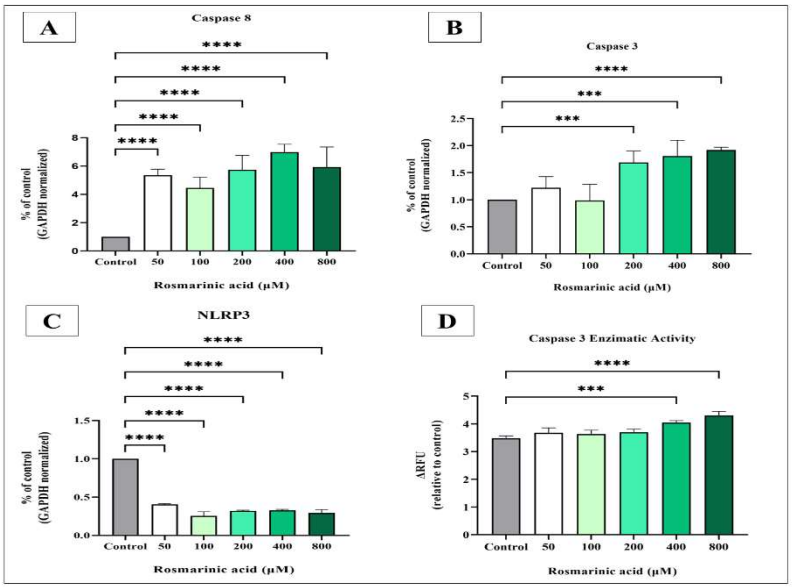


Fig. 6 – Gene expression and enzymatic activity of caspase 3 protein. RT-qPCR was used to assess gene expression of caspase 8, caspase 3 and NLRP3 inflammasome. Enzyme activity was evaluated using a fluorescence assay. RA significantly increased gene expression of caspase 8 (A) and caspase 3 (B). In contrast, RA significantly reduced NLRP3 inflammasome expression gene expression (C) at all concentrations tested. Caspase 3 protein enzymatic activity was highly increased at 400 μM and 800 μM. * ($p<0.05$); ** ($p<0.01$); *** ($p<0.001$); **** ($p<0.0001$). All data indicate differences from the control group.

3.3 MANUSCRITO 2

The effect of rosmarinic acid on cell viability and purinergic system components of human metastatic melanoma cells

Gilnei Bruno da Silva, Daiane Manica, Filomena Marafon, Marcelo Moreno, Margarete Dulce Bagatini

Manuscrito em Progresso (Possível submissão à Revista *Human Cell*).

The effect of rosmarinic acid on cell viability and purinergic system components of human metastatic melanoma cells

Gilnei Bruno da Silva[1], Daiane Manica[1], Filomena Marafon[1], Marcelo Moreno[1], Margarete Dulce Bagatini[1*]

[1] Post-graduate Program in Biomedical Sciences, Federal University of Fronteira Sul, Chapecó, SC, Brazil.

[*]Corresponding authors. E-mail: margaretebagatini@yahoo.com.br.

Abstract: cancer cases have increasing and CM has been highlighted due to massively contributes with statistics cancer data of world and its high ability to metastasize. Researches have been shown that RA is a promisor phenolic compound in antineoplastic context. Thus, in this study we aimed to evaluate the antineoplastic effect of RA on viability, and possible modulatory effect on the components of the purinergic system of CM cells. For this, CM cells SK-MEL-28 lineage was treated for 24 hours with different concentrations of RA. Then, we assessed cell viability, gene and protein expression of ectonucleotidases CD39 and CD73, hydrolytic enzymatic activity of CD39, CD73 and ADA, and extracellular ATP levels. RA strongly reduced CM cells viability after 24 hours of RA treatment in a dose-dependent manner. Relative to the purinergic system, RA downregulated gene and protein expression of CD73, while had no effect on CD39 expression. Similarly, RA decreased enzymatic activity of CD39 on ATP hydrolysis, CD73 on AMP hydrolysis, and ADA on Ado breakdown. There was no statistical significance for extracellular ATP levels. Taken together all this set out findings, we found that RA reduces viability of CM cells and, for the first time, that RA have significant effect on purinergic signaling in melanoma cells.

Keywords: Melanoma. Phenolic compound. Anticancer. Purinergic signaling. Ectonucleotidase.

Highlights:

- We found that RA reduces CM cell viability;
- We found for the first time that RA exhibits effect on purinergic signaling in CM cells;
- RA modulates gene and protein expression of the ectonucleotidase CD73;
- Furthermore, RA modulates enzymatic activity of the ectonucleotidases CD39, CD73 and ADA;
- We suggest that RA is a possible phenolic acid that may be used as an adjuvant therapy to target the purinergic system in association with anticancer drugs on CM context.

1 Introduction

Cases and incidence of cancer, a dread disease, have spread out worldwide in the 21st century [1]. Among them, cutaneous melanoma (CM) has become a public health problem due to massively contributes with statistics cancer data of world and its high ability to metastasize [2,3]. This neoplasm pathology has its origin from malignant transformation of epidermal melanocytes main to excessive or unprotected sunbathing, which leads to DNA mutations by ultraviolet (UV) radiation [4,5].

Even new therapeutic possibilities currently found, several side effects and possible pharmacological resistance still limit the effectiveness in the treatment of cancer [6], and when it comes to stages III and IV of CM, these problems are more pronounced [7]. In this context, phenolic compounds, such as rosmarinic acid (RA), have been indicated as promising in an adjuvant therapeutic perspective associated to pharmacological used [8]. Curiously, this phenolic acid is easily and naturally found in plants from *Boraginaceae* and *Lamiaceae* families, such as rosemary (*Rosmarinus officinalis L.)* [9]. Some researches already evidenced the anticancer effect of RA in colon carcinoma [10], prostate cancer [11], and breast cancer [12]. Despite this, in the field of CM, the anticancer effects of RA are still poorly studied.

Recently, the purinergic system, a ubiquitous and sophisticated cell–cell communication extracellular signaling pathway [13], has been shown to play important role in cancers pathophysiology, such as in lung cancer [14], glioblastoma [15] and melanoma [16,17]. Are involved in this cellular signaling molecules that include manly the nucleotides adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), and the nucleoside adenosine (Ado), whose levels are regulated by ectonucleotidases NTPDase (CD39), 5′-nucleotidase (CD73) and Adenosine deaminase (ADA). The signaling molecules action on P1 and P2 receptors implicates in several cellular outcomes [13,18].

Although other signaling pathways were showed solid association with the anticancer action of RA, the potential for purinergic signaling modulation had no yet been explored. Thus, we aimed to evaluate the effect of RA on viability and modulatory effect on the components of the purinergic system of CM cells. Our hypothesis is that RA modulates purinergic signaling ectonucleotidases, from their expression to enzymatic activity, leading to a feasible antineoplastic conditions with reduction in cell viability.

2 Material and methods

2.1 Chemicals, reagents and equipment

All chemicals and reagents used were of analytical grade, purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA) and Merck (Darmstadt, Germany). RA (99% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture plates and flasks used for culture procedures were obtained from Gibco™ Thermo Fisher Scientific (Grand Island, NY, USA) and Invitrogen Life Technologies (Carlsbad, CA, USA). Molecular biology reagents were purchased from Invitrogen and Applied Biosystems (Waltham, Massachusetts, USA). Flow cytometry analysis was carried out in an Accuri™ C6 Plus cytometer (BD Biosciences) and analyzed by the FlowJo V10 software.

2.2 Cell culture and RA treatment

The human metastatic melanoma cell line SK-MEL-28 was purchased from the Cell Bank of Rio de Janeiro (BCRJ), Brazil. Cells were grown in flasks with Dulbecco's modified Eagle's medium (DMEM - high glucose with L-glutamine) containing antibiotics and an antifungal (penicillin/streptomycin) and supplemented with 10% fetal bovine serum. The cells were grown under adequate conditions in a humidified and controlled atmosphere of 5% carbon dioxide (CO_2) at 37°C. RA was dissolved in the appropriate culture medium to obtain different concentrations and the cells were treated for 24 hours at concentrations of 50 µM, 100 µM, 200 µM, 400 µM and 800 µM, based on a previous study performed by Anwar et al. [19], with adaptations. The negative control group cells received only culture medium.

2.3 Cell viability by MTT assay

The MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay was used according to a study carried out by Mosmann [20]. SK-MEL-28 cells were seeded in 96-well plates, in 4 replicates, at a density of 1×10^5 cells/well and treated with RA. Then, the supernatant was removed, and the cells were washed once with phosphate-buffered saline (PBS) (0.1 M, pH 7.4) to avoid any interference from the compound used in the treatment. The MTT (Sigma-Aldrich) reagent (5 mg/ml) dissolved in PBS was added and the plates were incubated for 2 hours at 37°C. Then, the supernatant was discarded and 200 µL of DMSO was added in order to dissolve the formazan crystals generated by the reduction of the MTT salt by the viable cells.

The absorbance was measured at 570 nm using a SpectraMax® i3 Multimode Plate Reader 96 microplates (Molecular Devices, Sunnyvale, CA, USA). The results were expressed as percentage (%) of cell viability relative to control.

2.4 Assessment of CD39 and CD73 gene expression

Gene expression was assessed by real-time quantitative polymerase chain reaction (RT-qPCR) analysis. RNA was obtained from cell culture samples with TRIzol™ reagent (Invitrogen™) and quantified spectrophotometrically (NanoDrop™ Thermo Fisher Scientific). Reverse transcription into cDNA was performed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™), by the addition to each 10 µL sample 10 µL of a mix containing 1 µL of MultiScribe™ Reverse Transcriptase. The steps of the reaction were: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, and a final hold step of 4°C for 30 minutes, performed using a thermal cycler. The RT-qPCR reaction was performed using 17 µL of a mix containing the SYBR Green PCR Master Mix (Applied Biosystems™) and 3 µL of the cDNA sample. The parameters used were a pre-activation step of 10 minutes at 95°C, followed by a cycling step of 40 cycles at 95°C for 15 seconds, 60°C for 60 seconds, and finally a melting step with a melting curve of 60°C to 95°C with an increase of 1°C every 5 seconds. The relative expression of each gene was represented as the fold expression in relation to the control and calculated using the comparative $^{\Delta\Delta}$CT value. GAPDH was used as the housekeeping gene to normalise gene expression. The forward and reverse sequences of oligos (5′-3′) used for each gene were as follows: GAPDH (F): CTCCTCACAGTTGCCATGTA; GAPDH (R): GTTGAGCACAGGGTACTTTATTG; CD39 (F): GCCCTGGTCTTCAGTGTATTAG; CD39 (R): CTGGCATAACCTACCTACTCTTTC; CD73 (F): GTGCCTTTGATGAGTCAGGTAG; CD73 (R): TTCCTTTCTCTCGTGTCCTTTG.

2.5 Assessment of CD39 and CD73 protein expression

Cells were maintained in culture flasks until the confluence phase, followed by dissociation using trypsin and were counted immediately in a hemocytometer. Then 1x10^6 cells were centrifuged for 5 minutes at 400g and washed twice with PBS with 10% FBS. The sediments were suspended and incubated for 30 minutes with purified mouse anti-human CD39 and anti-human CD73 antibodies (BD Pharmingen TM) (1:10). The same number of cells was incubated without antibodies (negative control).

All samples were washed with PBS, and 10.000 events were immediately acquired by flow cytometry (BD ACCURI C6) and analyzed by FlowJo V10 software. The results were expressed as percentage (%) of CD39 or CD73 positive cells relative to control.

2.6 Enzymatic activity of CD39, CD73 and ADA

The hydrolysis of nucleotides such as ATP/ADP (CD39), AMP (CD73), and the nucleoside Ado (ADA) was employed to evaluate the alterations in purinergic system enzymes activities. Briefly, after protein adjustments of CM cells, 20 µL of samples was added to the reaction mixture of each enzyme and pre-incubated at 37 °C for 10 min. The enzymatic reaction was initiated by adding the specific substrates for each enzyme: ATP and ADP for CD39 and AMP for CD73. After incubation at 37 °C for 70 min, the reactions were stopped by the addition of trichloroacetic acid (TCA, 10%), and the released inorganic phosphate due to ATP, ADP, and AMP hydrolysis was determined by using malachite green as the colorimetric reagent. A standard curve was prepared with KH_2PO_4. Controls were performed to correct for non-enzymatic hydrolysis. The absorbance was measured at 630 nm, and enzyme-specific activities were reported as nmol/Pi/min/mg of protein [21,22]. For the ADA, activity was performed based on the measurement of ammonia produced when this enzyme acts in the excess of adenosine, following a previously published method [23]. In brief, 50 µL of cell suspension reacted with 21 mmol/L of adenosine (pH 6.5) at 37 °C for 60 min. After the incubation period, the reaction was stopped by the addition of 167.8 mM sodium nitroprusside, 106.2 mM phenol, and a sodium hypochlorite solution. Lastly, absorbance was read at 620 nm, and values were expressed as units/liter (U/L).

2.7 Extracellular ATP determination

Molecular Probes® ATP Determination Kit (Invitrogen™) an extremely sensitive bioluminescence assay was used with recombinant firefly luciferase and its substrate D-luciferin. The assay is based on luciferase's requirement for ATP in producing light – emission maximum ~560 nm at pH 7.8 [24]. We combined the components of the reaction as follows in order to make a standard reaction solution and adjust the volumes according to particular requirements. Each reaction contained 1,25 µg/mL of firefly luciferase, 50 µM D-luciferin and 1 mM DTT in 1× Reaction Buffer. After 15 minutes of incubation, luminescence was measured. An ATP standard curve was prepared in concentrations from 1 nM to 1 µM.

2.8 Protein determination

The method of Bradford [25] was employed for protein determination using bovine serum albumin as standard and the protein samples were adjusted according to each assay as mg/mL.

2.9 Statistical analysis

All measurements were statistically performed by analysis of variance followed by the appropriate *post hoc* test using GraphPad Prism 9 software. All data are expressed as mean ± standard deviation. The differences between the groups in relation to the studied variables were evaluated through the analysis one-way ANOVA. The differences in the probability of rejection of the null hypothesis at <5% (*P<0.05*) were considered statistically significant. Statistical significance was defined for p-values of **P<0.05*, ***P<0.01*, ****P<0.001*, and *****P<0.0001*.

3 Results

3.1 Effect of RA on CM cells viability

The viability results of the CM cell line SK-MEL-28 after 24 hours of RA treatment evaluated by the MTT assay are shown in Figure 1A. RA significantly reduced CM cell viability in a progressively dose-dependent manner at 100 μM (*P<0.01*), 200 μM (*P<0.001*), 400 μM (*P<0.0001*) and 800 μM (*P<0.0001*) compared to untreated CT cells. The 400 μM and 800 μM concentrations had a similar dose response, while the lowest compound dose tested (50 μM) was not statistically significant. This results showed that RA exhibits strong effect on viability of CM cells.

3.2 Effect of RA on the ectonucleotidases expression in CM cells

We searched for a possible RA modulatory effect on expression of the CD39 and CD73 in melanoma cells, which are shown in Figures 2 and 3. For this, we firstly assessed gene expression by RT-qPCR. After 24 hours of RA treatment the gene expression of CD73 was downregulated at 50 μM (*P<0.001*), 100 μM (*P<0.0001*), 200 μM (*P<0.01*), 400 μM (*P<0.001*), 800 μM (*P<0.0001*) in comparison to CT (Fig. 3A). On the other hand, no statistical significance was detected for changes in CD39 gene expression (Fig. 2A). To make our findings robustness, we complementary assessed protein expression of ectonucleotidases by flow cytometry. Similar to gene expression results, CD73 protein expression was reduced at 100 μM (*P<0.05*) and 800 μM

(*P<0.01*) when compared to CT (Fig. 3B-C), while CD39 protein expression was not statistically significant (Fig. 2B-C). Thus, our findings confirm that RA have significant modulatory effect on purinergic system ectoenzymes, specifically on CD73 expression in CM cells.

3.4 Effect of RA on enzymatic activity of ectonucleotidases and extracellular ATP levels in CM cells

Thinking about elucidating effect of RA on the full purinergic signaling cascade, we further used an assay for enzymatic activity of CD39, CD73 and ADA, and measured the extracellular levels of ATP (Fig. 1B-F). All tested concentrations of RA greatly reduced CD39 enzymatic activity on ATP hydrolysis in CM cells (*P<0.0001*) (Fig. 1B). On AMP hydrolysis, CD73 activity was highly reduced at 800 μM (*P<0.0001*) compared to untreated cells (Fig. 1C). In relation to ADA activity, RA treatment also was capable to reduce of nucleoside Ado breakdown at 800 μM (*P<0.001*). There was no statistical significance for changes in activity of CD39 on ADP hydrolysis (Fig. 1C) and extracellular ATP levels (Fig. 1F).

4 Discussion

The involvement of purinergic signaling on pathophysiology of cancer was previous showed and include the CM [14,16]. Furthermore, especially in advanced stages of CM, there is poor prognosis due to insufficient effectiveness of available treatments [26,27]. Even if the highlighted evidences about RA antineoplastic potential, researches looking into the effects of RA on purinergic signaling in CM cells has yet to be explored. Thus, in this study we proved that RA decreases SK-MEL-28 cells viability, and modulates CD73 expression, as well as CD39, CD73 and ADA enzymatic activity.

For cell viability evaluation we employed the MTT assay and found that RA decreased SK-MEL-28 cells in a dose-dependent manner. A study also found that antiproliferative effect of RA occurs in a dose- and time-dependent manner in breast cancer cell lineages [12]. Jin et al. [28] related dose-dependent manner to action of plant extract fraction whose contained RA. Another study involving CT26 colorectal cancer cells showed that RA reduces cell viability specifically at 200 μM after 24 hours of treatment [29]. Thus, our results reinforce the antineoplastic effect of RA and add new findings in CM context.

Subsequently, we searching for a possible modulatory effect of RA on purinergic system components. In this research we found that RA significantly reduced gene expression of the CD73 ectoenzyme in a range of 100 μM to 800 μM. On the other hand, it did not have a significant effect on the gene expression of the CD39 enzyme. Human melanomas have an overexpression of CD39 compared to normal melanocytes [30]. High CD73 expression rates have been reported in patients with advanced melanoma [31]. Thus, the results related to the expression of the CD39 and CD73 ectonucleotidases found in this study bring to light the possibility of the therapeutic application of RA in the purinergic modulation in the CM.

Since the gene expression of ectonucleotidases was altered by treatment with RA, we also searched for protein expression to make our findings robustness. Curiously, at the treatment concentration of 800 μM there was a significant reduction in the expression of CD73, while for CD39 there was no statistically significant difference. It is known that the CD73 enzyme is responsible for converting AMP into Ado, and thus exerts important control over the immune response against tumors [32]. In an animal model, the effect on antitumor immunity and tumor size reduction was related to CD73 inhibition [33] Given the importance of this ectonucleotidase, the potential of RA on the reduction in gene and protein expression of CD73 in CM is evident and may be explored by clinical trials studies.

Alterations in the enzymatic activity of ectonucleotidases were highlighted in previous studies involving patients with lung cancer [34], uterine cervical neoplasia [35], and melanoma [16]. Considering the citations above and the results presented in this work, which indicated that RA is a phenolic compound that modulates the gene and protein expression of components of the purinergic system, we advanced towards to understand in detail the action on the purinergic enzymatic cascade. For this, assays of the enzymatic activity of ectonuceotidases were carried out. After 24 hours of treatment, there was interference of RA in the enzymatic activity of CD39, with a significant reduction in ATP hydrolysis in all concentrations. With regard to ADP hydrolysis, RA did not exhibit significant effects. Furthermore, at a concentration of 800 μM of RA, a reduction in AMP hydrolysis was detected, which occurs through the action of CD73.

At the end of the purinergic cascade, Ado nucleoside formation is tightly regulated by the CD73/ADA enzyme axis. This is because, as previously seen, CD73 promotes the conversion of AMP into Ado, while ADA ectonucleotidase converts Ado

into inosine by an irreversible deamination reaction. Thus, the ADA ectoenzyme plays a crucial role in controlling Ado levels [36]. The presence of increased levels of Ado in TME is related to tumor immunosuppression [37]. Reduced ADA activity and an increase in P1 receptor expression (activated by Ado) in patients with advanced-stage lung cancer has been attributed to pro-tumor effects by increasing IL-6 and TNF-α levels, and decreasing IL-17 and INF-γ [14]. In this research, a significant reduction of the enzymatic activity of ADA was verified in 800 μM of treatment. However, in opposition to the studies mentioned above, the findings linked to the expression of CD73, as well as the enzymatic activities of the ectonucleotidases, explain that the purinergic cascade is more prone to a decrease in Ado levels than to an increase.

Interestingly, it is known that ATP release by melanoma cells increases CD39 expression [38]. On the other hand, ATP can interact with P2X7 receptors and promote cell death [39]. In this context, studies have indicated that ATP can lead to the death of cancer cells in different ways [40]. Considering the results of this work for the purinergic parameters and the pleiotropic role of ATP, possible alterations in the extracellular levels of this nucleotide after treatment of SK-MEL-28 cells with RA were sought. However, there was no statistical significance for this parameter.

5 Conclusion

We found that RA reduces viability of CM cells and, unprecedented-like manner, we have shown that RA have significant effect on purinergic signaling in melanoma cells. RA downregulates gene and protein expression of ectonucleotidase CD73, as well as decreases hydrolytic enzymatic activity of CD39 (ATP), CD73 (AMP) and ADA (Ado). Considering all this set out findings, RA is a possible phenolic acid that may be used as an adjuvant therapy to target the purinergic system in association with anticancer drugs on CM context.

Funding: This work is funded by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (MDB; Grant: 310606/2021-7), Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina (FAPESC) (MDB; Grant: 2021TR1543), Universidade Federal da Fronteira Sul (MDB). GBS and DM also thank Fundo de Apoio à Manutenção e ao Desenvolvimento da Educação Superior (FUMDES/UNIEDU) by the postgraduate scholarships.

Author contributions:

Conceptualisation: GBS.
Methodology: GBS, DM, and FM.
Investigation: GBS and DM.
Visualisation: MDB and MM.
Funding acquisition: MDB.
Project administration: GBS and MDB.
Supervision: MDB.
Writing - original draft: GBS.
Writing - review, and editing: GBS.
All authors reviewed and approved the final version.
Code availability: Not applicable.
Data and materials availability: All data are available in the main text or supplementary materials.
Conflicts of interest: The authors have no relevant financial or non-financial interests to disclose.
Ethics approval: Not applicable.
Consent to publish: Not applicable.

References

[1] Roy, P. S., Saikia, B. J. (2016). Cancer and cure: A critical analysis. Indian J. Cancer 53(3), 441-442. https://doi.org/10.4103/0019-509x.200658.

[2] Markovic, S.N., et al. (2007). Malignant melanoma in the 21st century, Part 1: Epidemiology, risk factors, screening, prevention, and diagnosis. Mayo Clinic Proceedings 82, 364-380. https://doi.org/10.4065/82.3.364.

[3] Paddock, L.E., et al. (2016). Skin self-examination and long-term melanoma survival. Melanoma Research 26, 401-408. https://doi.org/10.1097/CMR.0000000000000255.

[4] Carr, S., Smith, C., Wernberg, J. (2020). Epidemiology and risk factors of melanoma. Surgical Clinics of North America 100, 1-12. https://doi.org/10.1016/j.suc.2019.09.005.

[5] A.L. Milac, G. Negroiu, The multiple roles of tyrosinase-related protein-2/L-dopachrome tautomerase in melanoma: biomarker, therapeutic target, and molecular driver in tumor progression, Hum. Ski. Cancers – Pathways, Mech. Targets Treat. InTech, 2018, https://doi.org/10.5772/intechopen.70513.

[6] Hwang, S.-Y., Chae, J.-I., Kwak, A.-W., Lee, M.-H., Shim, J.-H. (2020). Alternative options for skin cancer therapy via regulation of AKT and related signaling pathways. IJMS 21, 6869. https://doi.org/10.3390/ijms21186869.

[7] Michielin, O., et al. (2019). Cutaneous melanoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 30, 884–1901. https://doi.org/10.1093/annonc/ mdz411

[8] Silva, G.B. da, Yamauchi, M.A., Zanini, D., Bagatini, M.D., 2022. Novel possibility for cutaneous melanoma treatment by means of rosmarinic acid action on purinergic signaling. Purinergic Signalling 18, 61-81. https://doi.org/10.1007/s11302-021-09821-7.

[9] Nunes, S. et al. (2017). Therapeutic and nutraceutical potential of rosmarinic acid-Cytoprotective properties and pharmacokinetic profile. Critical Reviews in Food Science and Nutrition 57(9), 1799–1806. https://doi.org/10.1080/10408398.2015.1006768.

[10] Karthikkumar, V. et al. (2015). Rosmarinic acid inhibits DMH-induced cell proliferation in experimental rats. Journal of Basic and Clinical Physiology and Pharmacology 26(2). https://doi.org/10.1515/jbcpp-2014-0044.

[11] Jang, Y.-G., Hwang, K.-A., Choi, K.-C. (2018). Rosmarinic acid, a component of rosemary tea, induced the cell cycle arrest and apoptosis through modulation of HDAC2 expression in prostate cancer cell lines. Nutrients 10, 1784. https://doi.org/10.3390/nu10111784.

[12] Messeha, S.S., Zarmouh, N.O., Asiri, A., Soliman, K.F.A. (2020). Rosmarinic acid-induced apoptosis and cell cycle arrest in triple-negative breast cancer cells. European Journal of Pharmacology 885, 173419. https://doi.org/10.1016/j.ejphar.2020.173419.

[13] Burnstock G., Verkhratsky A (2012). Purinergic signaling, and the nervous system, Springer. ISBN:3642288626. https://doi.org/10. 1007/978-3-642-28863-0.

[14] Zanini, D. et al. (2019). ADA activity is decreased in lymphocytes from patients with advanced stage of lung cancer. Medical Oncology 36(9), 78. https://doi.org/10.1007/s12032-019-1301-1.

[15] Azambuja J.H., et al. (2018). CD73 downregulation decreases in vitro and in vivo glioblastoma growth. Mol Neurobiol 56:3260–3279. https://doi.org/10. 1007/s12035-018-1240-4.

[16] Mânica, A. et al. (2019). The signaling effects of ATP on melanoma-like skin cancer. Cellular Signalling 59, 122–130. https://doi.org/10.1016/j.cellsig.2019.03.021.

[17] Manica, A. et al. (2018). High levels of extracellular ATP lead to chronic inflammatory response in melanoma patients. Journal of Cellular Biochemistry 119(5), 3980–3988. https://doi.org/10.1002/jcb.26551.

[18] Burnstock G, Knight GE (2004). Cellular distribution and function of P2 receptor subtypes in diferent systems. Int Rev Cytol 240:31–304. https://doi.org/10.1016/S0074-7696(04)40002-3.

[19] Anwar, S., et al. (2020). Rosmarinic acid exhibits anticancer effects via MARK4 inhibition. Sci Rep 10, 10300. https://doi.org/10.1038/s41598-020-65648-z.

[20] Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55-63. https://doi.org/10.1016/0022-1759(83)90303-4.

[21] Pilla C, et al. (1996). ATP diphosphohydrolase activity (apyrase, EC 3.6.1.5) in human blood platelets. Platelets 7:225–230. https:// doi.org/10.3109/09537109609023582.

[22] Lunkes G.I., et al. (2003). Enzymes that hydrolyze adenine nucleotides in diabetes and associated pathologies. Thromb Res 109:189–194. https://doi.org/10.1016/s0049-3848(03)00178-6.

[23] Giusti G, Galanti B (1984) Colorimetric method. HU Bergmeyer, Methods of Enzymatic Analysis, 3rd edn. Verlag Chemie, Weinheim, pp 315–323.

[24] Karamohamed S, Guidotti G (2001) Bioluminometric method for real-time detection of ATPase activity. Biotechniques 31:420–425.

[25] Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248 (1976).

[26] Weber, J.S., et al. (2015). Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. Lancet Oncol. 16(4), 375-384. https://doi.org/10.1016/S1470-2045(15)70076-8.

[27] Soengas, M.S.; Lowe, S.W. (2003). Apoptosis and melanoma chemoresistance. Oncogene 22, 3138-3151. https://doi.org/10.1038/sj.onc.1206454

[28] Jin, S., et al. (2020). Anti-melanoma activities and phytochemical compositions of *Sorbus commixta* fruit extracts. Plants 9, 1076. https://doi.org/10.3390/plants9091076

[29] Han, Y.-H., Kee, J.-Y., Hong, S.-H., 2018. Rosmarinic acid activates AMPK to inhibit metastasis of colorectal cancer. Front. Pharmacol. 9, 68. https://doi.org/10.3389/fphar.2018.00068.

[30] Dzhandzhugazyan, K.N., Kirkin, A.F., P.T.S., Zeuthen, J. (1998). Ectoatp diphosphohydrolase/cd39 is overexpressed in differentiated human melanomas. febs let 430, 227–230.

[31] Monteiro, I. et al. (2018). CD73 expression and clinical significance in human metastatic melanoma. Oncotarget 9(42), 26659–26669. https://doi.org/10.18632/oncotarget.25426.

[32] Beavis P.A., et al. (2012). CD73: a potent suppressor of antitumor immune responses. Trends Immunol 33(5), 231-23.

[33] Forte, G. et al. (2012). Inhibition of CD73 Improves B Cell-Mediated Anti-Tumor Immunity in a Mouse Model of Melanoma. The Journal of Immunology, 189(5), 2226–2233. https://doi.org/10.4049/jimmunol.1200744.

[34] Zanini, D. et al. (2012). Lung cancer alters the hydrolysis of nucleotides and nucleosides in platelets. Biomedicine & Pharmacotherapy 66(1), 40-45. http://dx.doi.org/10.1016/j.biopha.2011.09.003

[35] Maldonado P.A., et al. (2010). Nucleotide degrading enzymes in platelets from uterine cervical neoplasia patients treated with conization or radiotherapy. Biomed Pharmacother 64(7), 499– 504. https://doi.org/10.1016/j.biopha.2010.02.007.

[36] Blackburn M.R., et al. (2005). Adenosine deaminase deficiency: metabolic basis of immune deficiency and pulmonary inflammation. Adv Immunol 86, 1–41.

[37] Di Virgilio, F. (2013). Purines, Purinergic Receptors, and Cancer. Cancer Research, 72(21), 5441–5447. https://doi.org/10.1158/0008-5472.CAN-12-1600.

[38] Kretz M., et al. (2009). ATP release by B16 melanomas upregulate the expression of the ectonucleotidase CD39 on Treg. J Invest Dermatol, 129.

[39] Raffaghello, L., et al. (2006). The P2X7 receptor sustains the growth of human neuroblastoma cells through a substance P-dependent mechanism. Cancer Res. 66, 907–914.

[40] Burnstock, G., Di Virgilio, F. (2013). Purinergic signalling and cancer. Purinergic Signalling 9(4), 491–540. https://doi.org/10.1007/s11302-013-9372-5.

Figure captions

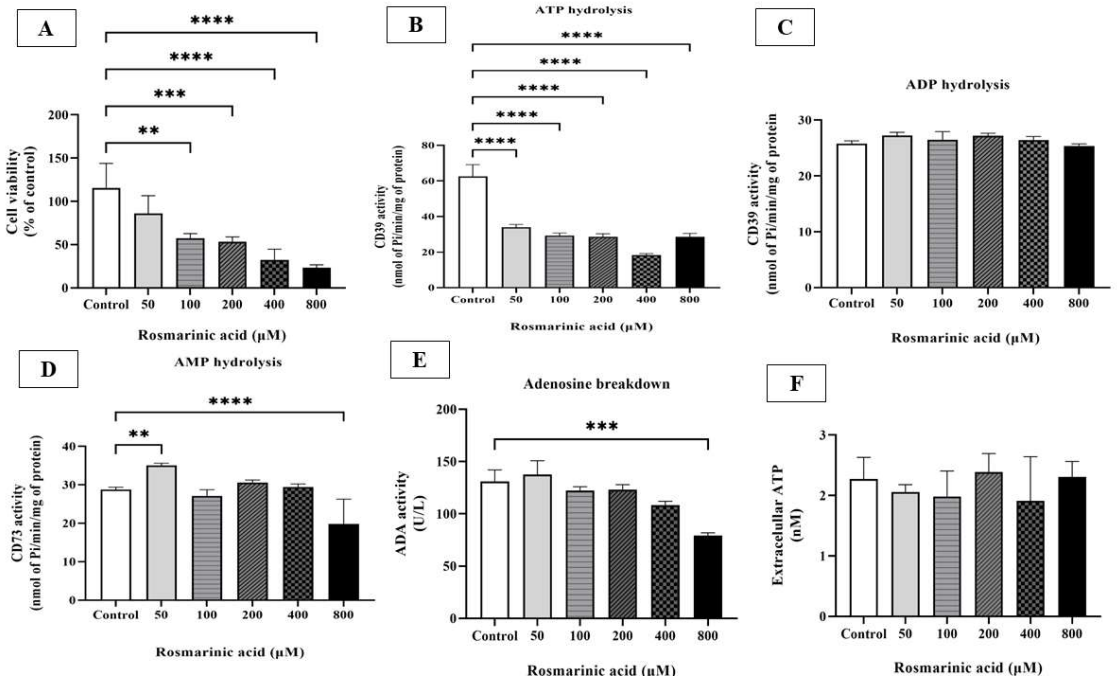


Fig. 1 – Cell viability, ectonucleotidase enzymatic activity, and extracellular ATP levels of CM cells. RA decreased SK-MEL-28 cell viability in a dose-dependent manner in range of 100 to 800 µM of treatment (A). All tested concentrations of RA significantly reduced the CD39 enzymatic activity on ATP hydrolysis (B). CD73 activity on AMP hydrolysis was reduced at 800 µM (D). The enzymatic activity of ADA was reduced at 800 µM (E). There was no statistical significance for CD39 activity on ADP hydrolysis (C) and for extracellular ATP levels (F). * ($p<0.05$); ** ($p<0.01$); *** ($p<0.001$); **** ($p<0.0001$). All data indicate differences from the control group.

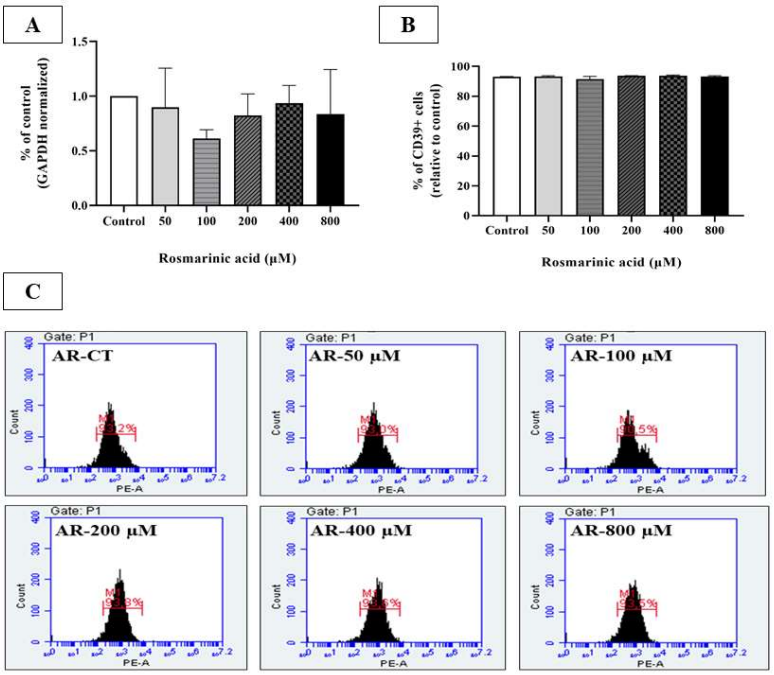


Fig. 2 – Expression of ectonucleotidase CD39. There was no statistical significance for both gene expression (A) and protein expression (B-C) of ectonucleotidase CD39 in SK-MEL-28 cells after 24 hours of RA treatment * ($p<0.05$); ** ($p<0.01$); *** ($p<0.001$); **** ($p<0.0001$). All data indicate differences from the control group.

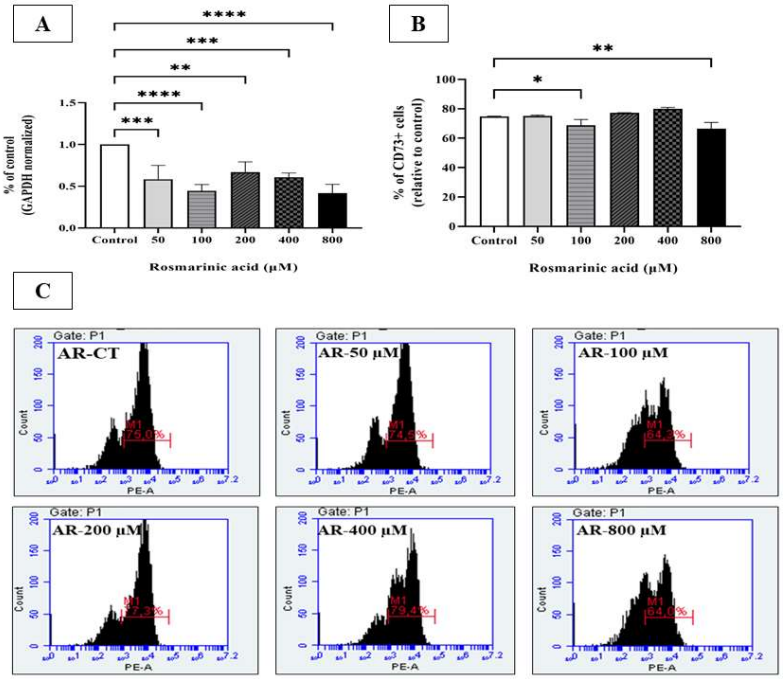


Fig. 3 – Expression of ectonucleotidase CD73. All tested concentrations of RA highly reduced CD73 gene expression (A), while protein expression was reduced at 100 µM and 800 µM of treatment (B-C). * ($p<0.05$); ** ($p<0.01$); *** ($p<0.001$); **** ($p<0.0001$). All data indicate differences from the control group.

4 DISCUSSÃO

O MC é um problema de saúde pública que tem ganhado notoriedade nos últimos anos devido ao aumento dos casos e a sua alta capacidade de metastização (MARKOVIC et al., 2007; PADDOCK et al., 2016). Mesmo com o surgimento de novas possibilidades terapêuticas, diversos efeitos colaterais e possíveis resistências farmacológicas ainda limitam a efetividade no tratamento do câncer (HWANG et al., 2020), e com isso os pesquisadores têm despendido esforços na busca de novas estratégias terapêuticas antitumorais (MULDER; LIMA; MIRANDA, 2013).

Nesse sentido, como explanado previamente, evidências provenientes da literatura científica têm demonstrado que o AR possui potencial antitumoral em diversos contextos patológicos. Convergentemente, o montante de achados obtidos por meio desta dissertação endossa os trabalhos precedentes e explicita que o AR também possui efeitos significativos no contexto do MC, reduzindo a viabilidade celular, modulando a expressão gênica e proteica de mediadores apoptóticos, participando do equilíbrio redox, bem como modulando a sinalização purinérgica.

No que tange as pesquisas que se dedicaram a compreender o potencial do AR numa perspectiva terapêutica antitumoral, Zhang et al. (2018) elucidaram que esse composto possui efeitos anticâncer em células tumorais de ovário. Também foi demonstrado que o AR induz a morte de células de câncer de próstata (JANG et al., 2018). Da mesma forma, esse ácido fenólico foi indicado como indutor da morte de células de câncer mamário (MESSEHA et al., 2020). Corroborando os achados, no presente estudo, após o tratamento das células de melanoma cutâneo SK-MEL-28 por 24 horas com AR, observou-se um efeito citotóxico com redução na viabilidade das células tumorais. Em células mononucleares de sangue periférico, as quais foram utilizadas como controle não tumoral, esse efeito não foi observado. Com isso, esses resultados agregam-se a literatura e mostram de forma inédita que o AR exerce efeito antitumoral sobre células de MC.

Na dinâmica tumoral, as células podem sofrer um importante processo chamado de metástase, que compreender um complexo processo biológico em que as células tumorais migram e invadem regiões adjacentes às lesões iniciais, podendo ganhar acesso a todos os sistemas corporais por meio da circulação (VANHARANTA; MASSAGUÉ, 2013). Como esclarecido em outras seções deste trabalho, o MC é um tipo de câncer com alta capacidade metastática, o que diminui a sobrevida dos

acometidos. Em uma pesquisa com células de carcinoma de colón, evidenciou-se que o AR inibe a migração celular (HAN et al., 2018). O mesmo efeito foi comprovado no contexto do câncer de mama (MESSEHA et al., 2020) e carcinoma hepático (JIN et al., 2020). Neste trabalho, notou-se que o efeito na inibição da migração das células SK-MEL-28 foi progressivamente mais significativo conforme o aumento das concentrações de AR. Os resultados apresentados elucidam pela primeira vez que o AR inibe a migração de células de melanoma.

Um dos possíveis mecanismos de ação das drogas anticâncer é através da produção de EROs, que levam a degeneração de estruturas biológicas importantes, como o DNA (AMIRMOSTOFIAN et al., 2013). Nesse estudo lançou-se mão da verificação dos níveis de moléculas pró-oxidantes e antioxidantes após o tratamento das células tumorais com AR. Assim, verificou-se uma expressiva redução nos níveis intracelulares e extracelulares de EROs, acompanhado de um aumento nos níveis de PSH e NPSH, o que indica que o AR neutraliza moléculas pró-oxidantes e melhora os níveis de moléculas antioxidantes em concentrações mais elevadas. Esses achados confirmam que o AR é um potente agente antioxidante, como já ilustrado por Sevgi, Tepe, Sarikurkcu (2015), e Oğuz et al. (2020).

Diversas formas de indução à morte celular têm sido atribuídas ao AR, como através da via PI3K/AKT/mTOR (WANG et al., 2019). Outra via de atuação do AR já conhecida é por meio da expressão de lncRNA MALAT-1 (ZHANG et al., 2018). Existem estudos que mostram que o AR induz a apoptose independente de caspases (HAN et al., 2018). Em contraste, neste trabalho de dissertação, todas as concentrações testadas de AR foram capazes de aumentar significativamente a expressão gênica da caspase 8. Para a caspase 3, esse efeito só foi observado nas concentrações de 200 µM, 400 µM e 800 µM de tratamento. Além disso, o AR aumentou a atividade enzimática da caspase 3 nas concentrações de 400 µM e 800 µM. Nas concentrações de 400 µM e 800 µM o AR induziu à formação de corpos apoptóticos. Dessa forma, fica evidenciado que esse composto fenólico é multifacetado quanto aos seus mecanismos de ação no campo de estudo tumoral.

Nos últimos anos, componentes envolvidos na sinalização celular de diversos processos biológicos, conhecido como inflamassomas, têm ganhado destaque, principalmente o NLRP3. Inflamassomas são complexos multiproteicos que desempenham papel na homeostase e na morte celular (ZHANG et al., 2022). Expressão aberrante de NLRP3 foi detectado no contexto tumoral, como no câncer

de pulmão, e podem indicar um possível efeito pró-tumoral (KONG et al., 2015). Neste estudo, o tratamento com AR reduziu significativamente a expressão gênica do NLRP3 nas células de MC em todas as concentrações testadas. Curiosamente, Akhter et al. (2022) encontraram que o AR é capaz de inibir a ativação da via NLRP3 em ratos no qual foi induzido lesão renal aguda. Dada a importância desse complexo proteico na progressão do câncer e a capacidade do AR em modular a expressão do NLRP3, os achados aqui apresentados enaltecem esta pesquisa.

Além das vias de sinalização celular elucidadas até aqui, a sinalização purinérgica tem sido mostrada por desempenhar um papel relevante no câncer e no MC por diversos pesquisadores. Isso porque é ubiquamente expressa em quase todas as células do corpo humano (BURSNTOCK et al., 2006), e assim, as células tumorais e do sistema imune interagem por essa via no TME. Zanini et al. (2012) mostraram alterações na sinalização purinérgica em pacientes com câncer de pulmão. Essas alterações, que envolvem a atividade enzimática das ectonucleotidases, também foram destacadas por Mânica et al. (2019) em um estudo experimental com células sanguíneas provenientes de pacientes com melanoma.

Apesar do exposto acima, a busca pelos efeitos do AR na sinalização purinérgica de células de MC ainda não havia sido explorada. Nesta pesquisa descobriu-se que o AR diminuiu significativamente a expressão gênica da ectoenzima CD73 em uma faixa de 100 µM a 800 µM. Por outro lado, não apresentou efeito significativo na expressão gênica da enzima CD39. Melanomas humanos apresentam uma super-expressão da CD39 em comparação a melanócitos normais (DZHANDZHUGAZYAN; KIRKIN; ZEUTHEN, 1998). Altas taxas de expressão da CD73 foram relatadas em pacientes em grau avançado de melanoma (MONTEIRO et al., 2018). Dessa forma, os resultados relacionados à expressão das ectonucleotidases CD39 e CD73 encontrados com este estudo trazem à tona a possibilidade da aplicação terapêutica do AR na modulação purinérgica no MC.

Também foi verificada a expressão proteica dessas ectoenzimas nas células tratadas com AR. Da mesma forma, na concentração de 800 µM de tratamento houve redução significativa na expressão de CD73, enquanto que para CD39 não houve diferença estatisticamente significante. É sabido que a enzima CD73 é responsável pela conversão do AMP em Ado, e assim exerce importante controle na resposta imune contra os tumores (BEAVIS et al., 2012). Em modelo animal, o efeito na imunidade antitumoral e redução do tamanho do tumor foi relacionada com a inibição

da CD73 (FORTE et al. 2012). Dada a importância desta ectonucleotidase, fica evidente o potencial do AR sobre a redução na expressão gênica e proteica da CD73 no MC.

Alterações na atividade enzimática das ectonucleotidases foram destacadas em estudo prévios envolvendo pacientes com câncer de pulmão (ZANINI et al., 2012), neoplasia cervical uterina (MALDONADO et al., 2010), e no melanoma (MÂNICA et al., 2019). Considerando o exposto e os resultados acima apresentados, os quais indicaram que o AR é um composto fenólico modulador da expressão gênica e proteica de componentes do sistema purinérgico, objetivou-se compreender detalhadamente a ação sobre a cascata enzimática purinérgica. Para isso, realizaram-se ensaios da atividade enzimática das ectonuceotidases. Após 24 horas de tratamento, observou-se interferência do AR na atividade enzimática da CD39, com redução significativa na hidrólise de ATP em todas as concentrações. No que diz respeito a hidrólise do ADP, o AR não exibiu efeitos significativos. Ainda, na concentração de 800 µM de AR, detectou-se uma redução na hidrólise do AMP, a qual ocorre pela ação da CD73. É importante reforçar que alterações na atividade enzimática das ectonucleotidases não está restrita apenas às neoplasias, como destacado por Baldissarelli et al. (2017), que mostraram um aumento na atividade enzimática purinérgica de pacientes com hipotireoidismo.

No final da cascata purinérgica, a formação do nucleosídeo Ado é estreitamente regulada pelo eixo enzimático CD73/ADA. Isso porque, como visto previamente, a CD73 promove a conversão do AMP em Ado, enquanto que a ectonucleotidase ADA converte o Ado em inosina por uma reação irreversível de desaminação. Assim, a ectoenzima ADA tem papel crucial no controle dos níveis de Ado (BLACKBURN et al., 2005). A presença de níveis aumentados de Ado no TME está relacionada com imunossupressão tumoral (DI VIRGILIO et al., 2012). A atividade reduzida da ADA e um aumento na expressão do receptor de P1 (ativado pelo Ado) em pacientes com câncer de pulmão em estágio avançado foi atribuída a efeitos pró-tumorais por aumentar os níveis de IL-6 e TNF-α, e diminuição IL-17 and INF-γ (ZANINI et al., 2019). Nesta pesquisa constatou-se uma significativa redução da atividade enzimática da ADA em 800 µM de tratamento. Porém, em oposição aos estudos citados acima, os achados vinculados a expressão da CD73, bem como as atividades enzimáticas das ectonucleotidases, explicam que a cascata purinérgica está mais propensa à diminuição dos níveis de Ado que ao aumento.

Interessantemente, é sabido que a liberação de ATP por células de melanoma aumenta a expressão da CD39 (KRETZ et al., 2009). Por outro lado, o ATP pode interagir com os receptores P2X7 e promover a morte celular (RAFFAGHELLO et al., 2006). Nesse âmbito, estudos têm indicado que o ATP pode levar a morte das células cancerígenas por vias diferentes (BURNSTOCK; DI VIRGILIO, 2013). Considerando-se os resultados deste trabalho para os parâmetros purinérgicos e o papel pleiotrópico do ATP, buscou-se por possíveis alterações nos níveis extracelulares desse nucleotídeo após o tratamento das células SK-MEL-28 com AR. Contudo, não se verificou significância estatística para esse parâmetro.

5 CONCLUSÃO E CONSIDERAÇÕES FINAIS

Considerando o conjunto de resultados aqui apresentados, conclui-se que o ácido rosmarínico:

a) Possui efeito citotóxico nas células tumorais, reduzindo a viabilidade celular, e não é citotóxico para células não tumorais (CMSPs);

b) Inibe a migração das células de MC, parâmetro envolvido no processo de metástase;

c) É um forte composto antioxidante que age reduzindo os níveis intracelulares e extracelulares de EROs, e melhora os níveis das moléculas antioxidantes PSH e NPSH;

d) Modula a expressão gênica dos mediadores de apoptose caspases 8 e 3, e do inflamassoma NRLP3, bem como induz a formação de corpos apoptóticos;

e) Modula a atividade enzimática da caspase 3;

f) Participa da sinalização purinérgica, por modular a expressão gênica e proteica da ectonucleotidase CD73, e a atividade enzimática da CD39, CD73 e ADA;

Assim, o AR mostrou-se um forte candidato a ser utilizado numa perspectiva terapêutica adjuvante no tratamento do MC. Para tanto, sugere-se que sejam desenvolvidos mais estudos clínicos para a compreensão detalhada dos seus efeitos, mecanismos de ação sistêmica e possíveis reações adversas.

6 PERSPECTIVAS FUTURAS

Além dos resultados já obtidos ainda se pretende:

a) Realizar um ensaio para verificação de apoptose;

b) Detectar os níveis das citocinas IL-2, IL-4, IL-6, IL-1β, TNF-α e INF-ɣ;

c) Verificar a expressão gênica e proteica dos receptores envolvidos na sinalização purinérgica e sinalizadores secundários de morte celular.

REFERÊNCIAS

AKHTE, J., et al. NLRP3 infammasome in rosmarinic acid-aforded attenuation of acute kidney injury in mice. **Nature***,* v. 12, n. 1313, 2022. https://doi.org/10.1038/s41598-022-04785-z

AMIRMOSTOFIAN, M., et al. Synthesis and molecular-cellular mechanistic study of pyridine derivative of dacarbazine. **Iran J Pharm Res**, 12, 255-265, 2013.

ANTONIOLI, L.; et al. Adenosine pathway and cancer: where do we go from here? **Expert Opinion on Therapeutic Targets**, v. 18, p. 973-977, 2014. https://doi.org/10.1517/14728222.2014.925883.

ANWAR, S. et al. Rosmarinic Acid Exhibits Anticancer Effects via MARK4 Inhibition. **Scientific Reports**, v. 10, n. 1, p. 10300, dez. 2020. https://doi.org/10.1038/s41598-020-65648-z.

ALIAGAS E., et al. High expression of ecto-nucleotidases CD39 and CD73 in human endometrial tumors. **Mediators Inflamm** 2014; 2014: 509027. https://doi.org/10.1155/2014/509027.

AUNG, T. N. et al. Understanding the Effectiveness of Natural Compound Mixtures in Cancer through Their Molecular Mode of Action. **International Journal of Molecular Sciences,** v. 18, n. 3, mar. 2017.

ATKINSON, B.; et al. Ecto-nucleotidases of the CD39/NTPDase family modulate platelet activation and thrombus formation: potential as therapeutic targets. **Blood Cells, Molecules and Diseases**, v. 36, p. 217-222, 2006. https://doi.org/10.1016/j.bcmd.2005.12.025.

AZZAM, E.I.; JAY-GERIN, J. P.; PAIN, D. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. **Cancer Lett**, v**.** 327, p.48–60, 2012.

BAGATINI, M. D., et al. The Impact of Purinergic System Enzymes on Noncommunicable, Neurological, and Degenerative Diseases. **Journal of Immunology Research**, v. 2018, p.1-21, 2018. https://doi.org/10.1155/2018/4892473.

BAGATINI, M.D., et al. **Oxidative Stress: Noxious but Also Vital**. In: Atukeren P, editor. Novel Prospects in Oxidative and Nitrosative Stress [Internet]. InTech; 2018. http://10.5772/intechopen.73394

BALCH, C.M., et al. Final version of 2009 AJCC melanoma staging and classification. **Journal of Clinical Oncology**, v. 27, n. 36, p. 6199- 6206, 2009.

BALDISSARELLI J., et al. Post-thyroidectomy hypothyroidism increases the expression and activity of ectonucleotidases in platelets: Possible involvement of reactive oxygen species. **Platelets,** http://dx.doi.org/10.1080/09537104.2017.1361017

BARBOSA, K. B. F.; et al. Estresse oxidativo: conceito, implicações e fatores modulatórios. **Revista de Nutrição,** v. 23, n. 4, p. 629–643, ago. 2010. https://doi.org/10.1590/S1415-52732010000400013.

BARBER, D.L., et al. Restoring function in exhausted CD8 T cells during chronic viral infection. **Nature**, v. 439, p. 682–687, 2006.

BAUMEISTER, S.H, et al. Coinhibitory Pathways in Immunotherapy for Cancer. **Annu. Rev. Immunol**, v. 34, p. 539–573, 2016.

BEAVIS P.A., et al. CD73: a potent suppressor of antitumor immune responses. **Trends Immunol**, v. 33, n. 5, p. 231-23, 2012.

BELTRÃO-BRAGA, P. C. B., et al. **Aspectos moleculares da transformação celular: conceitos e implicações**. In: Waitzberg, D. L., Dieta, nutrição e câncer. São Paulo: Atheneu, p. 1-187, 2004.

BIAN, S.; et al. P2X7 integrates PI3K/AKT and AMPK-PRAS40-mTOR signaling pathways to mediate tumor cell death. **PLoS ONE**, v. 8, p. 60184, 2013. https://doi.org/10.1371/journal.pone.0060184.

BIRBEN, E. et al. Oxidative stress and antioxidant defense. **The World Allergy Organization Journal**, v. 5, n. 1, p. 9–19, jan. 2012.

BLACKBURN M.R., et al. Adenosine deaminase deficiency: metabolic basis of immune deficiency and pulmonary inflammation. **Adv Immunol**, v. 86, p.1–41, 2005.

BOS, R., et al. Functional differences between low- and highaffinity CD8+ T cells in the tumor environment. **OncoImmunology**, v. 1, p. 1239–1247, 2012.

BREWER, M.S. Natural antioxidants: sources, compounds, mechanisms of action, and potential applications. **Compr Rev Food Sci Food Saf,** v. 10, n. 4, p. 221–247, 2011. https://doi.org/10.1111/j.1541-4337.2011.00156.x.

BURNSTOCK, G. Short- and long-term (trophic) purinergic signalling. **Philosophical Transactions of the Royal Society B: Biological Sciences**, v. 371, n. 1700, p. 20150422, ago. 2016. https://doi.org/10.1098/rstb.2015.0422.

BURNSTOCK, G. Blood cells: an historical account of the roles of purinergic signalling. **Purinergic Signalling**, v. 11, n. 4, p. 411–434, dez. 2015. https://doi.org/10.1007/s11302-015-9462-7.

BURNSTOCK, G.; et al. Cellular distribution and function of P2 receptor subtypes in different systems. **International Review of Cytology**, v. 240, p.31-304, 2004. https://doi.org/10.1016/S0074-7696(04)40002-3.

BURNSTOCK, G.; VERKHRATSKY, A. **Purinergic signalling and the nervous system,** 2012. https://doi.org/10.1007/978-3-642-28863-0.

BURNSTOCK, G. Purinergic signalling - an overview. **Novartis Foundation Symposium**, v. 276, p. 26-48, 2006.

BURNSTOCK, G. Physiology and pathophysiology of purinergic neurotransmission. **Physiological Reviews**, v. 87, n. 2, p. 659-797, 2007. https://doi.org/10.1152/physrev.00043.2006.

BURNSTOCK, G.; KNIGHT, G. E.; GREIG, A. V. H. Purinergic signaling in healthy and diseased skin. **Journal of Investigative Dermatology**, v. 132, p. 526-546, 2012. https://doi.org/10.1038/jid.2011.344.

BURNSTOCK, G. Purine and purinergic receptors. **Brain and Neuroscience Advances**, v. 2, p. 239821281881749, jan. 2018. https://doi.org/10.1177/2398212818817494.

BURNSTOCK, G.; DI VIRGILIO, F. Purinergic signalling and cancer. **Purinergic Signalling**, v. 9, n. 4, p. 491–540, dez. 2013. https://doi.org/10.1007/s11302-013-9372-5.

BURNSTOCK, G. Introductory overview of purinergic signalling. **Frontiers in Bioscience**, v. E3, n. 3, p. 896–900, 2011. https://doi.org/10.2741/e298.

CABRERA, R.; RECULE, F. Unusual Clinical Presentations of Malignant Melanoma: A Review of Clinical and Histologic Features with Special Emphasis on Dermatoscopic Findings. **American Journal of Clinical Dermatology**, v. 19, n. Suppl 1, p. 15–23, 2018. https://doi.org/10.1007/s40257-018-0373-6.

CAO, W. et al. Rosmarinic acid inhibits inflammation and angiogenesis of hepatocellular carcinoma by suppression of NF-κB signaling in H22 tumor-bearing mice. **Journal of Pharmacological Sciences**, v. 132, n. 2, p. 131–137, out. 2016. https://doi.org/10.1016/j.jphs.2016.09.003.

CANNAVÒ, S. P. et al. The role of oxidative stress in the biology of melanoma: A systematic review. **Pathology - Research and Practice**, v. 215, n. 1, p. 21–28, jan. 2019.

CAPPELLARI A. R.; et al. Characterization of ectonucleotidases in human medulloblastoma cell lines: ecto-5'NT/CD73 in metastasis as potential prognostic factor. **PLoS One** 2012; 7: e47468. https://doi.org/10.1371/journal.pone.0047468.

CARR, S.; SMITH, C.; WERNBERG, J. Epidemiology and Risk Factors of Melanoma. **Surgical Clinics of North America**, v. 100, n. 1, p. 1–12, fev. 2020. https://doi.org/10.1016/j.suc.2019.09.005.

CEKIC, C. et al. Myeloid Expression of Adenosine A_{2A} Receptor Suppresses T and NK Cell Responses in the Solid Tumor Microenvironment. **Cancer Research**, v. 74, n. 24, p. 7250–7259, dez. 2014. https://doi.org/10.1158/0008-5472.CAN-13-3583.

CEKIC, C.; LINDEN, J. Purinergic regulation of the immune system. **Nature Reviews Immunology**, v. 16, n. 3, p. 177–192, mar. 2016. https://doi.org/10.1038/nri.2016.4.

CILDIR, G.; et al. Chronic adipose tissue inflammation: All immune cells on the stage. **Trends Mol. Med**., v. 19, p. 487–500, 2013.

COSTA, C. M. da, et al. A simple automated procedure for thiol measurement in human serum samples. **Jornal Brasileiro de Patologia e Medicina Laboratorial**, v. 42, n. 5, 2006.

COUSSENS, L. M.; WERB, Z. Inflammation and cancer. **Nature**, v. 420, n. 6917, p. 860–867, dez. 2002.

CURTI, B. D.; FARIES, M. B. Recent Advances in the Treatment of Melanoma. **New England Journal of Medicine**, v. 384, n. 23, p. 2229–2240, jun. 2021. https://doi.org/10.1056/NEJMra2034861.

CZARNECKA, A. M. et al. Targeted Therapy in Melanoma and Mechanisms of Resistance. **International Journal of Molecular Sciences**, v. 21, n. 13, p. 4576, jun. 2020. https://doi.org/10.3390/ijms21134576.

DAVIS, L. E.; SHALIN, S. C.; TACKETT, A. J. Current state of melanoma diagnosis and treatment. **Cancer Biology & Therapy**, v. 20, n. 11, p. 1366–1379, ago. 2019. https://doi.org/10.1080/15384047.2019.1640032.

DI VIRGILIO, F. et al. Extracellular ATP and P2 purinergic signalling in the tumour microenvironment. **Nature Reviews. Cancer**, v. 18, n. 10, p. 601–618, out. 2018. https://doi.org/10.1038/s41568-018-0037-0.

DI VIRGILIO, F.; ADINOLFI, E. Extracellular purines, purinergic receptors and tumor growth. **Oncogene**, v. 36, n. 3, p. 293–303, jan. 2017. https://doi.org/10.1038/onc.2016.206.

DI VIRGILIO, F. et al. Extracellular ATP and P2 purinergic signalling in the tumour microenvironment. **Nature Reviews. Cancer**, v. 18, n. 10, p. 601–618, out. 2018. https://doi.org/10.1038/s41568-018-0037-0.

DI VIRGILIO, F. Purines, Purinergic Receptors, and Cancer. **Cancer Research**, v. 72, n. 21, p. 5441–5447, nov. 2012. https://doi.org/10.1158/0008-5472.CAN-12-1600.

DI VIRGILIO, F.; ADINOLFI, E. Extracellular purines, purinergic receptors and tumor growth. **Oncogene**, v. 36, n. 3, p. 293–303, jan. 2017. https://doi.org/10.1038/onc.2016.206.

DZHANDZHUGAZYAN, K.N., KIRKIN, A.F., P.T.S., ZEUTHEN, J. EctoATP diphosphohydrolase/CD39 is overexpressed in differentiated human melanomas. **FEBS Lett**, v. 430, p. 227–230, 1998.

EDDY, K.; CHEN, S. Overcoming Immune Evasion in Melanoma. **International Journal of Molecular Sciences**, v. 21, n. 23, p. 8984, nov. 2020. https://doi.org/10.3390/ijms21238984

ENNINGA, E. A. L. et al. Survival of cutaneous melanoma based on sex, age, and stage in the United States, 1992–2011. **Cancer Medicine**, v. 6, n. 10, p. 2203–2212, set. 2017. https://doi.org/10.1002/cam4.1152.

ELDER, D. E., et al. The 2018 World Health Organization Classification of Cutaneous, Mucosal, and Uveal Melanoma: Detailed Analysis of 9 Distinct Subtypes Defined by Their Evolutionary Pathway. **Archives of Pathology & Laboratory Medicine**, v. 144, n. 4, p. 500–522, fev. 2020. https://doi.org/10.5858/arpa.2019-0561-RA.

ELUFIOYE, T. O.; HABTEMARIAM, S. Hepatoprotective effects of rosmarinic acid: Insight into its mechanisms of action. **Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie**, v. 112, p. 108600, abr. 2019. https://doi.org/10.1016/j.biopha.2019.108600.

FERRARI, D.; MALAVASI, F.; ANTONIOLI, L. A Purinergic Trail for Metastases. **Trends in Pharmacological Sciences**, v. 38, n. 3, p. 277–290, mar. 2017. https://doi.org/10.1016/j.tips.2016.11.010.

FISHMAN, P. et al. A3 adenosine receptor as a target for cancer therapy: **Anti-Cancer Drugs**, v. 13, n. 5, p. 437–443, jun. 2002. https://doi.org/10.1097/00001813-200206000-00001.

FORTE, G. et al. Inhibition of CD73 Improves B Cell-Mediated Anti-Tumor Immunity in a Mouse Model of Melanoma. **The Journal of Immunology**, v. 189, n. 5, p. 2226–2233, set. 2012. https://doi.org/10.4049/jimmunol.1200744.

FIALOVÁ, S. et al. Derivatization of Rosmarinic Acid Enhances its in vitro Antitumor, Antimicrobial and Antiprotozoal Properties. **Molecules**, v. 24, n. 6, mar. 2019. https://doi.org/10.3390/molecules24061078.

FRIEDMAN, T. The Effect of Rosmarinic Acid on Immunological and Neurological Systems: A Basic Science and Clinical Review. **Journal of Restorative Medicine**, v. 4, n. 1, p. 50–59, dez. 2015. https://doi.org/10.14200/jrm.2015.4.0105.

GAO, Z.; DONG, K.; ZHANG, H. The Roles of CD73 in Cancer. **BioMed Research International**, v. 2014, p. 1–9, 2014. https://doi.org/10.1155/2014/460654.

GALLI, F. et al. Oxidative stress and reactive oxygen species. **Contrib Nephrol**, v. 149, p. 240-60, 2005.

GALLEY, H. Ascorbyl radical formation in patients with sepsis: Effect of ascorbate loading. **Free Radical Biology and Medicin**e, v. 20, n. 1, p. 139–143, 1996.

GASQUE P. P., BANDJEE, M. C. J. The immunology and inflammatory responses of human melanocytes in infectious diseases. **Journal of Infection**. https://doi.org/10.1016/j.jinf.2015.06.006

GEBREMESKEL, S., et al. The reversible P2Y12 inhibitor ticagrelor inhibits

metastasis and improves survival in mouse models of cancer: P2Y12 Inhibitor Ticagrelor Inhibits Metastasis. **International Journal of Cancer**, v. 136, n. 1, p. 234–240, jan. 2015. https://doi.org/10.1002/ijc.28947.

GHIRINGHELLI, F. et al. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1β–dependent adaptive immunity against tumors. **Nature Medicine**, v. 15, n. 10, p. 1170–1178, out. 2009. https://doi.org/10.1038/nm.2028.

GILL, J. G.; PISKOUNOVA, E.; MORRISON, S. J. Cancer, Oxidative Stress, and Metastasis. **Cold Spring Harbor Symposia on Quantitative Biology**, v. 81, p. 163–175, 2016.

GONDA T.A., TU, S., WANG T.C. Chronic inflammation, the tumor microenvironment and carcinogenesis. **Cell Cycle**, n. 8, p. 2005–2013, 2009.

GRIVENNIKOV, S. I.; GRETEN, F. R.; KARIN, M. Immunity, Inflammation, and Cancer. **Cell**, v. 140, n. 6, p. 883–899, 2011.

HANAHAN, D.; WEINBERG, R. A. Hallmarks of Cancer: The Next Generation. **Cell**, v. 144, n. 5, p. 646–674, mar. 2011. https://doi.org/10.1016/j.cell.2011.02.013.

HANAHAN, D.; WEINBERG, R. A. The Hallmarks of Cancer. **Cell**, v. 100, n. 1, p. 57–70, jan. 2000. https://doi.org/10.1016/S0092-8674(00)81683-9.

HAN, Y. H.; KEE, J. Y.; HONG, S. H. Rosmarinic Acid Activates AMPK to Inhibit Metastasis of Colorectal Cancer. **Frontiers in Pharmacology**, v. 9, p. 68, fev. 2018. https://doi.org/10.3389/fphar.2018.00068.

HAN, Y. et al. Rosmarinic inhibits cell proliferation, invasion and migration via up-regulating miR-506 and suppressing MMP2/16 expression in pancreatic cancer. **Biomedicine & Pharmacotherapy**, v. 115, p. 108878, jul. 2019. https://doi.org/10.1016/j.biopha.2019.108878.

HARTMAN, R. I.; LIN, J. Y. Cutaneous Melanoma-A Review in Detection, Staging, and Management. **Hematology/Oncology Clinics of North America**, v. 33, n. 1, p. 25–38, fev. 2019. https://doi.org/10.1016/j.hoc.2018.09.005.

HEVIA, M. J.; et al. Differential Effects of Purinergic Signaling in Gastric Cancer-Derived Cells Through P2Y and P2X Receptors. **Front Pharmacol**. jun. 2019; 10:612. https://doi.org/10.3389/fphar.2019.00612.

HITLE, M. et al. Rosmarinic Acid–Human Pharmacokinetics and Health Benefits. **Planta Medica**, p. a-1301-8648, 7 Dec. 2020. https://doi.org/10.1055/a-1301-8648.

HUSSAIN, T. et al. Oxidative Stress and Inflammation: What Polyphenols Can Do for Us? **Oxidative Medicine and Cellular Longevity**, v. 2016, p. 1–9, 2016.

HU, L-P.; et al. Targeting Purinergic Receptor P2Y2 Prevents the Growth of Pancreatic Ductal Adenocarcinoma by Inhibiting Cancer Cell Glycolysis. **Clin Cancer**

Res. 15 de feb. 2019; 25(4):1318–30. https://doi.org/10.1158/1078-0432.CCR-18-2297.

HOWELL, W. M. et al. Cytokine gene single nucleotide polymorphisms and susceptibility to and prognosis in cutaneous malignant melanoma. **European Journal of Immunogenetics**, v. 30, n. 6, p. 409–414, dez. 2003.

HSU, Y.C.; et al. Emerging interactions between skin stem cells and their niches. **Nat. Med**. 2014, 20, 847–856.

HWANG, S.-Y., et al. Alternative options for skin cancer therapy via regulation of AKT and related signaling pathways. **IJMS**, v. 21, p. 6869, 2020. https://doi.org/10.3390/ijms21186869

IARC - International Agency of Research in Cancer (2020). **CANCER TODAY**. Disponível em: <http://gco.iarc.fr/today/home>. Acesso em maio 2021.

INCA - Instituto Nacional do Câncer (Brazil). **Estimative 2020: cancer incidence in Brazil.** [Brasília, DF]: Instituto Nacional do Câncer, 2018. Disponível em: <https://www.inca.gov.br/publicacoes/livros/estimativa-2020-incidencia-de-cancer-no-brasil>. Acesso em maio 2021.

INCA - Instituto Nacional do Câncer (Brazil). **Online mortality atlas**. (Brasília, DF): Instituto Nacional do Câncer, 2019. Disponível em: <https://mortalidade.inca.gov.br/MortalidadeWeb/pages/Modelo08/consultar.xhtml#panelResultado>. Acesso em: maio 2021.

INCA - Instituto Nacional do Câncer (Brazil). **Cancer of skin melanoma**. [Brasília, DF]: Instituto Nacional do Câncer, 2021. Disponível em: <https://www.inca.gov.br/tipos-de-cancer/cancer-de-pele-melanoma>. Acesso em maio 2021.

ISOLA, A.L.; EDDY, K.; CHEN, S. Biology, Therapy and Implications of Tumor Exosomes in the Progression of Melanoma. **Cancers**, v. 8, n. 110, 2016.

ITAKURA E, et al. IL-10 expression by primary tumor cells correlates with melanoma progression from radial to vertical growth phase and development of metastatic competence. **Mod. Pathol**, v. 24, p. 801–809, 2010.

JACOB, K. D. et al. Markers of oxidant stress that are clinically relevant in aging and age-related disease. **Mechanisms of Ageing and Development**, v. 134, n. 3–4, p. 139– 157, mar. 2013.

JANG, Y. G.; HWANG, K. A.; CHOI, K. C. Rosmarinic Acid, a Component of Rosemary Tea, Induced the Cell Cycle Arrest and Apoptosis through Modulation of HDAC2 Expression in Prostate Cancer Cell Lines. **Nutrients**, v. 10, n. 11, p. 1784, 16 Nov. 2018. https://doi.org/10.3390/nu10111784.

JENKINS, R. W. & FISHER, D. E. Treatment of advanced melanoma in 2020 and beyond. **J. Invest. Dermatol**., v. 141, p. 23–31, 2021.

JIANG, T. et al. Comprehensive evaluation of NT5E/CD73 expression and its prognostic significance in distinct types of cancers. **BMC Cancer**, v. 18, n. 1, p. 267, dez. 2018. https://doi.org/10.1186/s12885-018-4073-7.

JIN, B. et al. Detailed studies on the anticancer action of rosmarinic acid in human Hep-G2 liver carcinoma cells: evaluating its effects on cellular apoptosis, caspase activation and suppression of cell migration and invasion. **Journal of B.U.ON.: official journal of the Balkan Union of Oncology**, v. 25, n. 3, p. 1383–1389, jun. 2020.

JOO, Y. N.; et al. P2Y2R activation by nucleotides released from the highly metastatic breast cancer cell MDA-MB-231 contributes to pre-metastatic niche formation by mediating lysyl oxidase secretion, collagen crosslinking, and monocyte recruitment. **Oncotarget,** v. 5, p. 9322–9334, 2014. https://doi.org/10.18632/oncotarget.2427.

KAUNITZ, G.J., et al. Melanoma subtypes demonstrate distinct PD-L1 expression profiles. **Lab. Investig**. v. 97, p. 1063–1071, 2017.

KARTHIKKUMAR, V. et al. Rosmarinic acid inhibits DMH-induced cell proliferation in experimental rats. **Journal of Basic and Clinical Physiology and Pharmacology**, v. 26, n. 2, jan. 2015. https://doi.org/10.1515/jbcpp-2014-0044

KUDRYAVTSEVA, A. V. et al. Mitochondrial dysfunction and oxidative stress in aging and cancer. **Oncotarget,** v. 7, n. 29, p. 44879–44905, jun. 2016. https://doi.org/10.18632/oncotarget.9821.

KONG, H., et al. Differential expression of inflammasomes in lung cancer cell lines and tissues. **Tumor Biol,** v. 36, p. 7501–7513, 2015. https://doi.org/10.1007/s13277-015-3473-4

KRUG C., et al. Stability and activity of MCSP-specific chimeric antigen receptors (CARs) depend on the scFv antigen-binding domain and the protein backbone. **Cancer Immunology, Immunotherapy**, v. 64, n. 12, p. 1623–1635, 2015.

KRETZ M., et al. ATP release by B16 melanomas upregulate the expression of the ectonucleotidase CD39 on Treg. **J Invest Dermatol**, v. 129, 2009.

KOSZAŁKA, P. et al. Specific Activation of A3, A2A and A1 Adenosine Receptors in CD73-Knockout Mice Affects B16F10 Melanoma Growth, Neovascularization, Angiogenesis and Macrophage Infiltration. **PLOS ONE**, v. 11, n. 3, p. e0151420, 10 mar. 2016. https://doi.org/10.1371/journal.pone.0151420

KUMAR, V.; ABBAS, A. K.; ASTER, J. C. **Robbins patologia básica.** 9. ed. Rio de Janeiro: Elsevier, 2013.

LEDDEROSE, C.; et al. Cutting off the power: inhibition of leukemia cell growth by pausing basal ATP release and P2X receptor signaling? **Purinergic Signaling**, v. 12, n. 3, p. 439–451, 2016. https://doi.org/10.1007/s11302-016-9510-y.

LEMBO, S. et al. The Modulatory Effect of Ellagic Acid and Rosmarinic Acid on Ultraviolet-B-Induced Cytokine/Chemokine Gene Expression in Skin Keratinocyte (HaCaT) Cells. **BioMed Research International**, v. 2014, p. 1–8, 2014. https://doi.org/10.1155/2014/346793.

LE POOLE I.C., et al. A novel, antigen-presenting function of melanocytes and its possible relationship to hypopigmentary disorders. **Journal of immunology**, v. 151, n. 12, p. 7284-7292, dez. 1993.

LEAL, D. B. R. et al. Characterization of NTPDase (NTPDase1; Ectoapyrase; ecto-diphosphohydrolase; CD39; EC 3.6.1.5) activity in human lymphocytes. **Biochimica et Biophysica Acta - General Subjects**, v. 1721, n. 1–3, p. 9–15, 2005. https://doi.org/10.1016/j.bbagen.2004.09.006.

LEWANDOWSKA, A. M., et al. Environmental risk factors for cancer - review paper. **Ann Agric Environ Med**, 2019 Mar 22;26(1):1-7. doi:10.26444/aaem/94299

LI, Y. et al. Screening for the antiplatelet aggregation quality markers of Salvia yunnanensis based on an integrated approach. **Journal of Pharmaceutical and Biomedical Analysis**, v. 188, p. 113383, set. 2020. https://doi.org/10.1016/j.jpba.2020.113383.

LIU, V. et al. Pathologic characteristics of melanoma (2020). **UpToDate**. Available in: <https://www.uptodate.com/contents/pathologic-characteristics-of-melanoma?search=diagnos is%20of%20cutaneous%20melanoma&source=search_result&selectedTitle=2~150&u sage_ty pe=default&display_rank=2>. Acesso em maio de 2021.

LUKE, J. J. et al. Targeted agents and immunotherapies: optimizing outcomes in melanoma. **Nature Reviews. Clinical Oncology**, v. 14, n. 8, p. 463–482, ago. 2017. https://doi.org/10.1038/nrclinonc.2017.43.

LO, J. A., FISHER, D. E. The melanoma revolution: from UV carcinogenesis to a new era in therapeutics. **Science**. n. 346, p. 945–949, 2014.

LUO, Y. A. et al. Anticancer effects of rosmarinic acid in human oral cancer cells is mediated via endoplasmic reticulum stress, apoptosis, G2/M cell cycle arrest and inhibition of cell migration. **JBUON.**, v. 25, n. 2, p. 1245-1250, 2020. PMID: 32521932

LUO, C. et al. A Review of the Anti-Inflammatory Effects of Rosmarinic Acid on Inflammatory Diseases. **Frontiers in Pharmacology**, v. 11, fev. 2020. https://doi.org/10.3389/fphar.2020.00153.

LONGHI M.S.; et al. Biological functions of ecto-enzymes in regulating extracellular adenosine levels in neoplastic and inflammatory disease states. **J Mol Med (Berl)** 2013; 91: 165–172. https://doi.org/10.1007/s00109-012-0991-z.

LI, Y. et al. Screening for the antiplatelet aggregation quality markers of Salvia yunnanensis based on an integrated approach. **Journal of Pharmaceutical and**

Biomedical Analysis, v. 188, p. 113383, set. 2020. https://doi.org/10.1016/j.jpba.2020.113383.

LUNKES, G. I., et al. Enzymes that hydrolyze adenine nucleotides in diabetes and associated pathologies. **Thromb Res**, v. 109, p. 189– 194, 2003. doi: 10.1016/s0049-3848(03)00178-6

MAIBACH F, et al. Tumor-Infiltrating Lymphocytes and Their Prognostic Value in Cutaneous Melanoma. **Front. Immunol.** v. 11, p. 2105, 2020. https://doi.org/10.3389/fimmu.2020.02105

MÂNICA, A. et al. The signaling effects of ATP on melanoma-like skin cancer. **Cellular Signalling**, v. 59, p. 122–130, jul. 2019. https://doi.org/10.1016/j.cellsig.2019.03.021.

MANICA, A. et al. High levels of extracellular ATP lead to chronic inflammatory response in melanoma patients. **Journal of Cellular Biochemistry**, v. 119, n. 5, p. 3980–3988, mai.2018. https://doi.org/10.1002/jcb.26551.

MALDONADO P.A., et al. Nucleotide degrading enzymes in platelets from uterine cervical neoplasia patients treated with conization or radiotherapy. **Biomed Pharmacother,** v.64, n. 7, p. 499– 504, 2010. https://doi.org/10.1016/j.biopha.2010.02.007.

MARINHO, S. et al. Anti-Inflammatory Effects of Rosmarinic Acid-Loaded Nanovesicles in Acute Colitis through Modulation of NLRP3 Inflammasome. **Biomolecules**, v. 11, n. 2, p. 162, 26 Jan. 2021. https://doi.org/10.3390/biom11020162.

MARKOVIC, S.N., et al. Malignant melanoma in the 21st century, Part 1: Epidemiology, risk factors, screening, prevention, and diagnosis. **Mayo Clinic Proceedings**, v. 82, p. 364-380, 2007. https://doi.org/10.4065/82.3.364

MERIGHI, S. et al. Adenosine Receptors as Mediators of Both Cell Proliferation and Cell Death of Cultured Human Melanoma Cells. **Journal of Investigative Dermatology**, v. 119, n. 4, p. 923–933, out. 2002. https://doi.org/10.1046/j.1523-1747.2002.00111.x.

MESSEHA, S. S. et al. Rosmarinic acid-induced apoptosis and cell cycle arrest in triple-negative breast cancer cells. **European Journal of Pharmacology**, v. 885, p. 173419, out. 2020. https://doi.org/10.1016/j.ejphar.2020.173419.

MICHIELIN, O., et al. Cutaneous melanoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. **Ann Oncol**, v. 30, n. 12, p.1884–1901, 2019. https://doi.org/10.1093/annonc/ mdz411

MICKSCHE M., et al. Production of polypeptide regulatory factors by human melanoma cells. **In Vivo**, v. 8, n. 5, p. 859-65, 1994.

MULDER, K. C.; LIMA, L. A.; MIRANDA, V. J. Current scenario of peptide-based drugs: the key roles of cationic antitumor and antiviral peptides. **Frontiers in Microbiology,** v. 4, p. 321, 2013.

MOLNÁR E. L., et al. Biosynthesis of interleukin-6, an autocrine growth factor for melanoma, is regulated by melanoma-derived histamine. seminars in **CANCER BIOLOGY**, v. 10, p. 25–28, 2000. https://doi.org/10.1006/scbi.2000.0304.

MOREIRA, A. et al. Current Melanoma Treatments: Where Do We Stand? Cancers, v. 13, n. 2, p. 221, 9 jan. 2021. https://doi.org/10.3390/cancers13020221.

MONTEIRO, I. et al. CD73 expression and clinical significance in human metastatic melanoma. **Oncotarget**, v. 9, n. 42, p. 26659–26669, 1 jun. 2018. https://doi.org/10.18632/oncotarget.25426.

MURATA, M. Inflammation and cancer. **Environmental Health and Preventive Medicine**, v. 23, 2018.

MULLER, A. G. et al. Delivery of natural phenolic compounds for the potential treatment of lung cancer. **DARU Journal of Pharmaceutical Sciences**, v. 27, n. 1, p. 433-449, 21 mai. de 2019. https://doi.org/10.1007/s40199-019-00267-2.

NAMIKAWA, K.; YAMAZAKI, N. Targeted Therapy and Immunotherapy for Melanoma in Japan. **Current Treatment Options in Oncology**, v. 20, n. 1, p. 7, 2019. https://doi.org/10.1007 / s11864-019-0607-8.

NGUYEN, A. V.; SOULIKA, A. M. The Dynamics of the Skin's Immune System. **International Journal of Molecular Sciences**, v. 20, n. 8, abr. 2019. https://doi.org/10.3390/ijms20081811

NUNES, S. et al. Therapeutic and nutraceutical potential of rosmarinic acid-Cytoprotective properties and pharmacokinetic profile. **Critical Reviews in Food Science and Nutrition**, v. 57, n. 9, p. 1799–1806, jun. 2017. https://doi.org/10.1080/10408398.2015.1006768.

NOEL, B. et al. Role of natural compounds in preventing and treating breast cancer. **Frontiers in bioscience (Scholar edition)**, v. 12, p. 137-160, mar. 2020.

OBRADOR, E. et al. Oxidative stress and antioxidants in the pathophysiology of malignant melanoma. **Biological Chemistry**, v. 400, n. 5, p. 589-612, 24 abr. 2019.

OCHIENG, J. et al. The impact of low-dose carcinogens and environmental disruptors on tissue invasion and metastasis. **Carcinogenesis**, v. 36, p. 128-159, jun. 2015.

OUYANG, L. et al. Plant natural products: from traditional compounds to new emerging drugs in cancer therapy. **Cell Proliferation**, v. 47, n. 6, p. 506, dez. 2014.

OĞUZ, A. et al. Investigation of antioxidant effects of rosmarinic acid on liver, lung and kidney in rats: a biochemical and histopathological study. **Folia Morphologica**, v. 79, n. 2, p. 288–295, 2020. https://doi.org/10.5603/FM.a2019.0087.

OLIVARES, A. et al. Effect of Rosmarinic Acid and Ionizing Radiation on Glutathione in Melanoma B16F10 Cells: A Translational Opportunity. **Antioxidants**, v. 9, n. 12, p. 1291, dez. 2020. https://doi.org/10.3390/antiox9121291.

OZTURK, H.; et al. Protective effects of Rosmarinic acid against renal ischaemia/reperfusion injury in rats. **J Pak Med Assoc**, v. 64, n.3, p. 260-265, Mar. 2014. PMID: 24864596.

PADDOCK, L. E. et al. Skin self-examination and long-term melanoma survival. **Melanoma Research**, v. 26, n. 4, p. 401–408, ago. 2016. https://doi.org/10.1097/CMR.0000000000000255.

PAULSON, K. G. et al. Age-Specific Incidence of Melanoma in the United States. **JAMA Dermatology**, v. 156, n. 1, p. 57–64, jan. 2020. https://doi.org/10.1001/jamadermatol.2019.3353.

PASQUALI, S. et al. Systemic treatments for metastatic cutaneous melanoma. **The Cochrane Database of Systematic Reviews**, v. 2018, n. 2, p. CD011123, fev. 2018. https://doi.org/10.1002/14651858.CD011123.pub2.

PELINSON, L. P. et al. Antiproliferative and apoptotic effects of caffeic acid on SK-Mel-28 human melanoma cancer cells. **Molecular Biology Reports**, v. 46, n. 2, p. 2085–2092, abr. 2019. https://doi.org/10.1007/s11033-019-04658-1.

PETERSEN, M. Rosmarinic acid: new aspects. **Phytochemistry Reviews**, v. 12, n. 1, p. 207–227, mar. 2013. https://doi.org/10.1007/s11101-013-9282-8.

PFAFFENZELLER, M. S.; FRANCIOSI, M. L. M.; CARDOSO, A. M. Purinergic signaling and tumor microenvironment in cervical cancer. **Purinergic Signalling**, v. 16, n. 1, p. 123–135, mar. 2020. https://doi.org/10.1007/s11302-020-09693-3.

PILLA, C., et al. ATP diphosphohydrolase activity (apyrase, EC 3.6.1.5) in human blood platelets. **Platelets**, v. 7, p. 225–230, 1996. https://doi.org/10.3109/09537109609023582

PISKOUNOVA, E.; et al. Oxidative stress inhibits distant metastasis by human melanoma cells. **Nature**, v. 527, p. 186–191, 2015.

PISOSCHI, A. M.; POP, A. The role of antioxidants in the chemistry of oxidative stress: A review. **European Journal of Medicinal Chemistry**, v. 97, p. 55–74, jun. 2015.

PIZZINO, G. et al. Oxidative Stress: Harms and Benefits for Human Health. **Oxidative Medicine and Cellular Longevity**, v. 2017, 2017. https://doi.org/10.1155/2017/8416763.

PRAMANIK, K. C. et al. Caffeic acid phenethyl ester suppresses melanoma tumor growth by inhibiting PI3K/AKT/XIAP pathway. **Carcinogenesis**, v. 34, n. 9, p. 2061–2070, set. 2013. https://doi.org/10.1093/carcin/bgt154.

PUBCHEM. **National Center for Biotechnology Information**. Chemical Information about Rosmarinic acid (RA). Disponível em: <https://pubchem.ncbi.nlm.nih.gov>. Acesso em: maio de 2021.

RAFFAGHELLO, L., et al. The P2X7 receptor sustains the growth of human neuroblastoma cells through a substance P-dependent mechanism. **Cancer Res**., v. 66, p. 907–914, 2006.

RADZIEJEWSKA, I., SUPRUNIUK, K., BIELAWSKA, A. Anti-cancer effect of combined action of anti-MUC1 and rosmarinic acid in AGS gastric cancer cells. **Eur J Pharmacol**, v. 902, jul. 2021. https://doi.org/10.1016/j.ejphar.2021.174119.

RAMOS-CASALS, M. et al. Immune-related adverse events of checkpoint inhibitors. **Nature Reviews. Disease Primers**, v. 6, n. 1, p. 38, mai. 2020. https://doi.org/10.1038/s41572-020-0160-6.

RASTRELLI, M. et al. Melanoma: epidemiology, risk factors, pathogenesis, diagnosis and classification. **In Vivo (Athens, Greece)**, v. 28, n. 6, p. 1005–1011, dez. 2014.

RAZAVI, A., et al. Chimeric antigen receptor T-cell therapy for melanoma, **Expert Review of Clinical Immunology**, v.17, n. 3, p. 209-223, 2021. https://doi.org/10.1080/1744666X.2021.1880895

REBECCA, V. W.; SOMASUNDARAM, R.; HERLYN, M. Pre-clinical modeling of cutaneous melanoma. **Nature Communications**, v. 11, n. 1, p. 2858, dez. 2020. https://doi.org/10.1038/s41467-020-15546-9.

REUTER, S. et al. Oxidative stress, inflammation, and cancer: How are they linked? **Free radical biology & medicine**, v. 49, n. 11, p. 1603–1616, dez. 2010.

RIBEIRO, L. R., et al. **Genética – Mutagênese Ambiental.** Universidade Luterana do Brasil (ULBRA), 2006.

ROCHA, J. et al. Anti-inflammatory Effect of Rosmarinic Acid and an Extract of *Rosmarinus officinalis* in Rat Models of Local and Systemic Inflammation. **Basic & Clinical Pharmacology & Toxicology**, v. 116, n. 5, p. 398–413, mai. 2015. https://doi.org/10.1111/bcpt.12335.

RONCATI, L., et al. Adoptive cell transfer (ACT) of autologous tumor-infiltrating lymphocytes (TILs) to treat malignant melanoma: the dawn of a chimeric antigen receptor T (CAR-T) cell therapy from autologous donor: CAR-T cell therapy in malignant melanoma. **International Journal of Dermatology**, v. 59, 2020. https://doi.org/10.1111/ijd.14945.

ROSENBERG S.A.., RESTIFO N.P.J.S. Adoptive cell transfer as personalized immunotherapy for human cancer. **Science** (New York, N.Y.), v. 348, n. 6230, p. 62–68, 2015.

RUZSNAVSZKY, O. et al. UV-B induced alteration in purinergic receptors and

signaling on HaCaT keratinocytes. **Journal of Photochemistry and Photobiology B: Biology**, v. 105, n. 1, p. 113–118, out. 2011. https://doi.org/10.1016/j.jphotobiol.2011.07.009.

SADEJ, R.; SPYCHALA, J.; SKLADANOWSKI, A. C. Expression of ecto-5′-nucleotidase (eN, CD73) in cell lines from various stages of human melanoma. **Melanoma Research**, v. 16, n. 3, p. 213–222, jun. 2006. https://doi.org/10.1097/01.cmr.0000215030.69823.11

SADEJ, R.; SKLADANOWSKI, A. C. Dual, enzymatic and non-enzymatic, function of ecto-5'-nucleotidase (eN, CD73) in migration and invasion of A375 melanoma cells. **Acta Biochimica Polonica**, v. 59, n. 4, p. 647–652, 2012. PMID: 23162807.

SCHMATZ, R. **Efeitos do resveratrol, do suco de uva e do vinho tinto nos biomarcadores de estresse oxidativo e na atividade de ectoenzimas em ratos diabéticos**. 2011. Tese (Doutorado em Ciências Biológicas: Bioquímica Toxicológica) - Universidade Federal de Santa Maria, Santa Maria - RS, 2011.

SEVGI, K.; TEPE, B.; SARIKURKCU, C. Antioxidant and DNA damage protection potentials of selected phenolic acids. **Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association**, v. 77, p. 12–21, Mar. 2015. https://doi.org/10.1016/j.fct.2014.12.006.

SAHA, S. K. et al. Correlation between Oxidative Stress, Nutrition, and Cancer Initiation. **International Journal of Molecular Sciences**, v. 18, n. 7, jul. 2017.

SAMPLE, A.; HE, Y.Y. Mechanisms and prevention of UV-induced melanoma. **Photodermatology, photoimmunology & photomedicine**, v. 34, n. 1, p. 13–24, jan. 2018. https://doi.org/10.1111/phpp.12329.

SANSOM, D.M. CD28, CTLA-4 and their ligands: Who does what and to whom? **Immunology**, v. 101, p. 169–177, 2000.

SCHADENDORF, D. et al. Melanoma. **The Lancet**, v. 392, n. 10151, p. 971–984, set. 2018. https://doi.org/10.1016/S0140-6736(18)31559-9

SHIRSHIN, E.A.; et al. Two-photon autofluorescence lifetime imaging of human skin papillary dermis in vivo: Assessment of blood capillaries and structural proteins localization. **Sci. Rep**., v. 7, p. 1171, 2017.

SIEGEL, R.; NAISHADHAM, D.; JEMAL, A. Cancer statistics, 2013. **CA: A Cancer Journal for Clinicians**, v. 63, n. 1, p. 11–30, jan. 2013. https://doi.org/10.3322/caac.21166.

SILVA, A. A. DA; GONÇALVES, R. C. Espécies reativas do oxigênio e as doenças respiratórias em grandes animais. **Ciência Rural**, v. 40, n. 4, p. 994–1002, mar. 2010.

SILVA, G.B. da, et al. Oxidative stress in Hashimoto's thyroiditis: possible adjuvant therapies to attenuate deleterious effects. **Mol Cell Biochem** (2022). https://doi.org/10.1007/s11010-022-04564-4

SILVA, G. B. da, et al. Novel possibility for cutaneous melanoma treatment by means of rosmarinic acid action on purinergic signaling. **Purinergic Signalling** 18, 61–81, 2022. https://doi.org/10.1007/s11302-021-09821-7

SINGH, N. et al. Inflammation and Cancer. **Annals of African Medicine**, v. 18, n. 3, p. 121–126, 2019.

SOENGAS, M.S.; LOWE, S.W. Apoptosis and melanoma chemoresistance. **Oncogene**, v. 22, p. 3138-3151, nov. 2003. https://doi.org/10.1038/sj.onc.1206454

STAGG, J.; SMYTH, M. J. Extracellular adenosine triphosphate and adenosine in cancer. **Oncogene**, v. 29, n. 39, p. 5346–5358, sept. 2010. https://doi.org/10.1038/onc.2010.292.

SWARUP, V. et al. Antiviral and Anti-Inflammatory Effects of Rosmarinic Acid in an Experimental Murine Model of Japanese Encephalitis. **Antimicrobial Agents and Chemotherapy**, v. 51, n. 9, p. 3367–3370, sep. 2007. https://doi.org/10.1128/AAC.00041-07.

SWETTER, S; GELLER, A. C. Melanoma: clinical features and diagnosis. **UpToDate**. 2020. Disponível em: <https://www.uptodate.com/contents/melanoma-clinical-features-and-diagnosis>. Acesso em: maio 2021.

TORTORA, G. J., et al. **Princípios de Anatomia Humana**. 14º Ed, Rio de Janeiro: Guanabara Koogan, 2019.

TRAN K.Q., et al. Minimally cultured tumorinfiltrating lymphocytes display optimal characteristics for adoptive cell therapy. **J Immunother.,** v. 31, p. 742–751, 2008.

VANHARANTA, S., MASSAGUÉ, J. Origins of metastatic traits. **Cancer Cell**, v. 24, p. 410-421, 2013. https://doi.org/10.1016/j.ccr.2013.09.007

VISOVATTI, S. H.; et al. Increased CD39 nucleotidase activity on microparticles from patients with idiopathic pulmonary arterial hypertension. **PLOS One**, v. 7, p. 408-429, 2012. https://doi.org/10.1371/journal.pone.0040829.

VOLKOVOVA, K. et al. Associations between environmental factors and incidence of cutaneous melanoma. Review. **Environmental Health**, v. 11, n. Suppl 1, p. S12, 28 jun. 2012. https://doi.org/10.1186/1476-069X-11-S1-S12.

WANG, L. et al. Rosmarinic acid inhibits proliferation and invasion of hepatocellular carcinoma cells SMMC 7721 via PI3K/AKT/mTOR signal pathway. **Biomedicine & Pharmacotherapy**, v. 120, p. 109443, dez. 2019. https://doi.org/10.1016/j.biopha.2019.109443.

WEBER, J.S., et al. Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. **Lancet Oncol.**, v. 16, n. 4, p. 375-384, abr. 2015. https://doi.org/10.1016/S1470-2045(15)70076-8.

WEI, Y. et al. Rosmarinic Acid Mitigates Lipopolysaccharide-Induced Neuroinflammatory Responses through the Inhibition of TLR4 and CD14 Expression and NF-κB and NLRP3 Inflammasome Activation. **Inflammation**, v. 41, n. 2, p. 732–740, mar. 2018. https://doi.org/10.1007/s10753-017-0728-9.

WILSON, M. A.; SCHUCHTER, L. M. Chemotherapy for Melanoma. In: KAUFMAN, H. L.; MEHNERT, J. M. (Eds.). Melanoma. **Cancer Treatment and Research. Cham**: Springer International Publishing, 2016. v. 167, p. 209–229. https://doi.org/10.1007/978-3-319-22539-5_8.

WHITE, N. et al. P2Y purinergic receptors regulate the growth of human melanomas. **Cancer Letters**, v. 224, n. 1, p. 81–91, jun. 2005.
WEINBERG, Robert A. **A Biologia do Câncer**. Porto Alegre: Artmed, 2008.

WHITTON, B. et al. Vacuolar ATPase as a potential therapeutic target and mediator of treatment resistance in cancer. **Cancer Medicine**, v. 7, n. 8, p. 3800–3811, aug. 2018. https://doi.org/10.1002/cam4.1594.

YANG, K. et al. Rosmarinic acid inhibits migration, invasion, and p38/AP-1 signaling via miR-1225-5p in colorectal cancer cells. **Journal of Receptors and Signal Transduction**, p. 1–10, ago. 2020. https://doi.org/10.1080/10799893.2020.1808674. https://doi.org/10.1016/j.canlet.2004.11.027.

YAHFOUFI, N. et al. The Immunomodulatory and Anti-Inflammatory Role of Polyphenols. **Nutrients**, v. 10, n. 11, nov. 2018. https://doi.org/10.3390/nu10111618.

YEGUTKIN, G. G. Nucleotide-and nucleoside-converting ectoenzymes: important modulators of purinergic signalling cascade. **Biochimica et Biophysica Acta (BBA) – Molecular Cell Research**, v. 1783, p. 673-694, 2008. https://doi.org/10.1016/j.bbamcr.2008.01.024.

XIE, R., et al. The P2Y2 nucleotide receptor mediates the proliferation and migration of human hepatocellular carcinoma cells induced by ATP. **J Biol Chem**, v. 289: p. 19137–19149, 2014. https://doi.org/10.1074/jbc.M113.540047.

XU, Y. et al. Anti-invasion effect of rosmarinic acid via the extracellular signal-regulated kinase and oxidation–reduction pathway in Ls174-T cells. **Journal of Cellular Biochemistry**, v. 111, n. 2, p. 370–379, 2010. https://doi.org/10.1002/jcb.22708.

ZANINI, D. et al., 2012. Lung cancer alters the hydrolysis of nucleotides and nucleosides in platelets. **Biomedicine & Pharmacotherapy,** v. 66, n. 1, p. 40-45, 2012. http://dx.doi.org/10.1016/j.biopha.2011.09.003

ZANINI, D. et al. Ectoenzymes and cholinesterase activity and biomarkers of oxidative stress in patients with lung cancer. **Molecular and Cellular Biochemistry**, v. 374, n. 1–2, p. 137–148, fev. 2013. https://doi.org/10.1007/s11010-012-1513-6.

ZANINI, D. et al. ADA activity is decreased in lymphocytes from patients with advanced stage of lung cancer. **Medical Oncology**, v. 36, n. 9, p. 78, set. 2019. https://doi.org/10.1007/s12032-019-1301-1.

ZHANG, Y. et al. Anticancer effects of Rosmarinic acid in OVCAR-3 ovarian cancer cells are mediated via induction of apoptosis, suppression of cell migration and modulation of lncRNA MALAT-1 expression. **Journal of B.U.ON.: official journal of the Balkan Union of Oncology**, v. 23, n. 3, p. 763–768, jun. 2018.

ZHANG, Y. et al. Highly-expressed P2X7 receptor promotes growth and metastasis of human HOS/MNNG osteosarcoma cells via PI3K/Akt/GSK3β/β-catenin and mTOR/HIF1α/VEGF signaling. **Int. J. Cancer**. p. 1068-1082, ago. 2019. https://doi.org/10.1002/ijc.32207.

ZHANG, J., et al. Does Pyroptosis Play a Role in Inflammasome-Related Disorders? **IJMS**, v. 23, p. 10453, 2022. https://doi.org/10.3390/ijms231810453

ZIMMERMAN, H; et al. Ectonucleotidases, molecular properties and functional impact. **An R Acad Nac Farm**, v.73, p. 537-566, 2007.

ZOU, J. et al. Discovery of a Novel ERp57 Inhibitor as Antiplatelet Agent from Danshen *(Salvia miltiorrhiza)*. **Evidence-Based Complementary and Alternative Medicine**, v. 2018, p. 1–9, 2018. https://doi.org/10.1155/2018/9387568.