UNIVERSIDADE FEDERAL DO PARANÁ

NATALIA MULINARI TURIN DE OLIVEIRA

EFEITO ANTITUMORAL DOS OLIGOSSACARÍDEOS EXTRAÍDOS DE VINHO TINTO NO MODELO DE CARCINOMA DE EHRLICH

CURITIBA

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Orientador(a): Profa. Dra. Alexandra Acco

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **NATALIA MULINARI TURIN DE OLIVEIRA** intitulada: **EFEITO ANTITUMORAL DOS OLIGOSSACARÍDEOS EXTRAÍDOS DE VINHO TINTO NO MODELO DE CARCINOMA DE EHRLICH**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua Aprovação no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 17 de Outubro de 2018.

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Esta dissertação tem os principais resultados apresentados na fora de artigo científico, sendo constituída, portanto, por introdução, um artigo científico e considerações finais. Este formato está de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná.

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“Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota”.

Madre Teresa de Calcutá

RESUMO

O câncer é uma das doenças com maior grau de mortalidade no mundo. As estimativas preveem um aumento significativo no número de novos casos a cada ano, sendo que até 2038 a previsão é que os casos de câncer devam aumentar em 70%. Estes dados alarmantes estão, muito provavelmente, associados ao estilo de vida adotado pela sociedade, como tabagismo, alcoolismo, dieta inadequada, sedentarismo e exposição prolongada às radiações solares, que predispõem ao desenvolvimento do câncer. As células neoplásicas se caracterizam por apresentar um crescimento desordenado, sem diferenciação, se proliferam rapidamente e tendem a ser muito agressivas. Assim, mesmo com os esforços gastos na busca por um tratamento potente e eficaz, as drogas antineoplásicas ainda exibem um baixo índice terapêutico e causam efeitos colaterais às células normais do organismo. Diante disso, pesquisadores continuam investigando um composto que seja ideal e seguro no combate ao câncer, reduzindo a progressão e a recorrência da doença e aumentando a sobrevida. Neste contexto, os oligossacarídeos merecem destaque, pois, por serem derivados dos polissacarídeos apresentam menor peso molecular e viscosidade e maior solubilidade em água, além de serem antioxidantes naturais, desempenhando funções biológicas valiosas em nível molecular, como, propriedades anti-inflamatória, antimicrobiana, hipocolesterolêmica, antitumoral e imunomoduladora, além de serem empregados na dieta como prebióticos. Recentemente foi demonstrado que uma fração de polissacarídeos extraídos de vinho tinto tem efeito antitumoral no tumor Walker-256 de ratos. Por isso, o presente estudo investigou os efeitos antineoplásicos e quimiopreventivos de oligossacarídeos extraídos do vinho tinto Cabernet Franc (Oligo) no carcinoma de Ehrlich e em linhagens de células tumorais mamárias *in vitro*, bem como os mecanismos de ação envolvidos. Após a inoculação subcutânea de 2 x 10^6 células tumorais de Ehrlich, camundongos fêmeas Swiss receberam Veículo (10 mL kg^{-1} de água destilada, via oral), como controle negativo, solução de Oligo (Oligo 9, 35 ou 70 mg kg^{-1}, via oral) ou Metotrexato (MTX 1,5 mg kg^{-1}, intraperitoneal, duas vezes por semana; controle positivo). Os protocolos posológicos duraram 21 dias (tratamento convencional) após a inoculação do tumor; ou 21 dias antes da inoculação do tumor, seguidos por mais 21 dias após a inoculação das células (tratamento quimiopreventivo). Análises de NMR e espectrometria de massa do Oligo indicaram uma complexa mistura de oligossacarídeos com grandes quantidades de xilose, ramnose e trealose. O Oligo reduziu o crescimento do tumor de Ehrlich em ambos os protocolos de tratamento, induziu intensa infiltração de células inflamatórias no tumor e aumentou a expressão gênica tumoral de *Hif1a*. Adicionalmente, o Oligo associado ao MTX (Oligo 9 ou 35 mg kg^{-1}, via oral + MTX 1,5 mg kg^{-1}, intraperitoneal) aumentou a eficácia do quimioterápico no controle do crescimento do tumor. No entanto, o Oligo não reduziu a viabilidade de células ascíticas de Ehrlich após 5 dias de tratamento, nem a viabilidade de células tumorais de mama MCF7, MDA-MB-231 e MDA-MB-436 após 24 e 48 h de tratamento em cultivo. O Oligo apresentou efeito antineoplásico contra células de Ehrlich, que foi dependente do tempo de tratamento e da resposta imunoinflamatória. Assim, o Oligo pode ser uma terapia adjuvante promissora para tumores sólidos.

Palavras-chaves: Oligossacarídeos do Cabernet Franc, carcinoma de Ehrlich, antitumoral, quimioprevenção, infiltração inflamatória, *Hif1α.*

ABSTRACT

Cancer is one of the diseases with the highest mortality rate in the world. Estimates indicate a significant increase in the number of new cases each year, and by 2038 the prediction is an increase of 70% in the disease. These alarming data are most likely related to the lifestyle adopted by society, such as smoking, alcoholism, inadequate diet, sedentary lifestyle and prolonged exposure to solar radiation, which predispose to the development of cancer. Neoplastic cells are characterized by disordered growth, without differentiation, proliferate quickly and tend to be very aggressive. Thus, even with the efforts expended in searching for a more effective treatment, the antineoplastic drugs still exhibit low therapeutic index and cause relevant toxic side effects to the normal cells. Researchers continue to investigate a compound that is both safe and effective against cancer, reducing the progression and recurrence of the disease and increasing the survival. In this context, the oligosaccharides deserve attention, since are polysaccharide derivatives with lower molecular weight and viscosity, have water solubility, and antioxidant activities. Thus, oligosaccharides present valuable biological functions at the molecular level, such as anti-inflammatory, antimicrobial, hypocholesterolemic, antitumor and immunomodulatory, besides being used in diets as prebiotics. It has been recently demonstrated that a fraction of polysaccharides extracted from red wine have an antitumor effect *in vivo*. The present study investigated the antineoplastic and chemopreventive effects of oligosaccharides extracted from Cabernet Franc red wine (Oligo) on Ehrlich carcinoma and on mammary tumor cell lineages *in vitro*, as well as the action mechanisms. After subcutaneous inoculation of 2 x 10^6 Ehrlich tumor cells, Swiss female mice received Vehicle (10 mL kg^{-1} distilled water, p.o.), as negative control, Oligo solution (Oligo 9, 35 or 70 mg kg^{-1}, p.o.) or Methotrexate (MTX 1.5 mg kg^{-1}, i.p., twice a week; positive control). The treatments lasted for 21 days (conventional treatment) after tumor inoculation, or 21 days before tumor inoculation followed for more 21 days after the cells inoculation (chemopreventive treatment). Oligo NMR and mass spectrometry analyzes indicated a complex mixture of oligosaccharides, and large amounts of xylose, rhamnose and trehalose. Oligo reduced the growth of the Ehrlich tumor in both protocols of treatment and induced intense inflammatory cells infiltration in the tumor. Additionally, Oligo associated with MTX (Oligo 9 or 35 mg kg^{-1}, p.o. + MTX 1.5 mg kg^{-1}, i.p.) increased the effectiveness of MTX in controlling the tumor growth. However, Oligo did not reduce the viability of Ehrlich ascitic cells after 5 days of treatment, and the viability of MCF7, MDA-MB-231 and MDA-MB-436 breast tumor cells after 24 and 48 h of treatment in culture. Oligo showed antineoplastic effect against Ehrlich cells that was dependent of the treatment time and the immune-inflammatory response. Thus, Oligo can be a promising adjuvant therapy for solid tumors.

Keywords: Cabernet Franc oligosaccharides, Ehrlich carcinoma, antitumor, chemoprevention, inflammatory infiltration, *Hif1α.*

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ARTIGO CIENTÍFICO

LISTA DE SIGLAS

ALT: Alanina aminotransferase/ alanine aminotransferase
ANOVA: Análise estatística de variância
AST: Aspartato aminotransferase
ATCC: American Type Culture Collection
Bax: Bcl-2 associada à proteína X
Bcl-2: Linfoma de células B2
CAPES: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CAT: Catalase
CEUA: Comissão de Ética no Uso de Animais
cDNA: Ácido desoxirribonucleico complementar
CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico
DNA: Ácido desoxirribonucleico
DMSO: Dimetilsulfóxido
EDTA: ácido etilenodiamino tetracético
ELISA: Enzimaimunoensaio
EGFR: Fator de crescimento de receptor epidérmico
ERs: Receptores de estrogênio
EROs: Espécies reativas de oxigênio
FBS: Soro fetal bovino
Gapdh: Gliceraldeíde-3-fosfato desidrogenase
GSH: Glutationa reduzida
G6PD: Glicose 6-fosfato desidrogenase
GPx: Glutationa peroxidase
HB: Hemoglobina
Ht: Hematócrito
HE: Hematoxilina e eosina
HER2: Receptor 2 do fator de crescimento epidérmico humano
HIF-1: Fator de hipóxia induzível - 1
HIF-2: Fator de hipóxia induzível - 2
H_2O_2: Peróxido de hidrogênio
Icam1: Molécula de adesão intercelular 1
IkBa: Inibidor NF-kappa-B alfa IL-1β: Interleucina 1 beta

IL-6: Interleucina 6

MLKL: Proteína quinase de domínio de linhagem mista

MPO: Mieloperoxidase

MRM: Monitoramento de múltiplas reações

mRNA: Ácido ribonucleico mensageiro

MTT: Brometo de 3-(4,5-dimetiltiazol-2-il) -2,5-difeniltetrazólio

MTX: Metotrexato

NAG: N-acetilglicosaminidase

NaOAc: Acetato de sódio

NF-κB: Fator nuclear Kappa B

NMR: Ressonância magnética nuclear

NO: Óxido nítrico

Nos2: Óxido nítrico sintase 2

Nrf2: Fator nuclear eritróide derivado 2

O2•-: Ânion superóxido

OH•: Radical hidroxila

OMS: Organização Mundial da Saúde

p53: Proteína 53

PBS: Salina tamponada com fosfato

PGE2: Prostaglandina E2

Pik3: Fosfatidilinositol-4,5-bisfosfato 3-quinase

PR: Receptores de progesterona

RBC: Glóbulos vermelhos

Relα Tx2: Subunidade do NF-κB ou RELA proto-oncogene

RIPK-1: Proteína quinase de interação ao receptor 1

RIPK-3: Proteína quinase de interação ao receptor 3

RNA: Ácido ribonucleico

RO•: Radical alcoxil

ROO•: Radical peroxil

ROOH•: Radical hidroperoxil

rpm: Rotação por minuto

Rplp0: Proteína ribossômica grande P0

RT-qPCR: Reação em cadeia da polimerase quantitativa em tempo real

SEM: Erro padrão da média

SOD: Superóxido dismutase

SFP: Fração solúvel do polissacarídeo de vinho tinto

sTIL: Linfócitos infiltrados no estroma

TILs: Linfócitos infiltrados do no tumor

TMB: Tetrametilbenzidina

TMSP: Ácido 3-trimetilsilil-2H4-propiônico

TNF-α: Fator de necrose tumoral alfa

UFPR: Universidade Federal do Paraná

VEGF: Fator de crescimento vascular epidérmico

LISTA DE ABREVIATURAS

μL: microlitro
μg: micrograma
cm^3: centímetro cúbico
dL: decilitro
eV: volt de elétron
Fig.: figura
g: grama
h: hora
Hz: hertz
i.p.: intraperitoneal
kg: quilograma
L: litro
mg: miligrama
min: minuto
mL: mililitro
mM: milimolar
m/z: relação massa carga
nm: nanômetro
Oligo(s): Oligossacarídeos extraídos do vinho tinto Cabernet Franc
p.o.: via oral
s: segundo
s.c.: subcutâneo
V: volt
Veh: Veículo
v/v: volume/volume
W: watt

SUMÁRIO

1. REVISÃO DA LITERATURA

1.1 CÂNCER E SUA INCIDÊNCIA

De acordo com o Instituto Nacional do Câncer (INCA), “o câncer é um conjunto de mais de 100 doenças que têm em comum o crescimento desordenado (maligno) de células que invadem os tecidos e órgãos, podendo espalhar-se (metástase) para outras regiões do corpo”. Este conjunto de doenças possui algumas características comuns, por outro lado, estas doenças são extremamente diferentes quanto à origem genética e histopatológica, a rapidez com que progridem, o grau da agressividade, o prognóstico, o tratamento e a resposta ao tratamento (SAITO, LANA, MEDRANO E CHAMMAS, 2016; INCA, 2018).

O câncer é uma das doenças com maior prevalência e com maior grau de mortalidade no mundo. Uma enorme mobilização da ciência mundial em busca de estratégias preventivas, de diagnósticos precoces e de tratamentos efetivos para o câncer, já avançou muito, mas ainda é insuficiente para a cura, levando à progressão ou recorrência da doença e à redução da sobrevida global (KRESO E DICK, 2014; JUNQUEIRA E CHAMMAS, 2018).

Com base em informações fornecidas, no ano de 2012, pelo programa Globocan/Iarc (*International Agency for Research on Cancer*) e pela Organização Mundial da Saúde (OMS), atribuiu-se o aparecimento de 14 milhões de novos casos de câncer em nível mundial, sendo que destes 8,2 milhões chegaram a óbito até o ano de 2015. Nos Estados Unidos, para o ano de 2018, a *American Cancer Society* estima 1,7 milhões de novos casos de câncer e aproximadamente 600 mil óbitos (AMERICAN CANCER SOCIETY, 2018), e no Brasil estima-se para os anos de 2018-2019 o surgimento de 640 mil novos casos, para cada ano (INCA, 2018). Ainda, a OMS estima que até o ano de 2038 os casos de câncer devem aumentar 70%, isso porque, apesar dos progressos na prevenção, tratamento e nos cuidados paliativos da doença, em muitos países o diagnóstico é tardio, o tratamento é caro ou inacessível e os serviços paliativos não estão disponíveis.

Em concordância com os dados acima, o câncer de mama é considerado uma das principais causas de mortalidade em mulheres, e é o segundo tipo de câncer mais comumente diagnosticado no mundo. Mais de 1,3 milhão de mulheres em todo o mundo são diagnosticadas com câncer de mama a cada ano. Esforços intensivos têm sido feitos para entender os mecanismos moleculares da patogênese do câncer de mama e sua aplicação a terapias antitumorais eficientes (BHATELIA, SINGH E SINGH, 2014).

1.2 CARCINOGÊNESE

As neoplasias malignas em sua maioria, são originárias das células normais do corpo, devido a um crescimento anormal e incontrolável, que resulta em uma rápida multiplicação dessas células. Com isso, as células neoplásicas proliferam-se rapidamente e perdem a sua capacidade de diferenciação, levando à malignidade (WONGTRAKOONGATE, 2015). A formação do tumor pode ser consequência de um evento químico, físico ou biológico, que altera de forma direta e irreversível o genoma celular. Estes eventos podem ser internos ou externos ao organismo, como a hipóxia (MAJMUNDAR et al., 2010, HUANG et al., 2014), o estresse oxidativo (GUPTA et al., 2014, SIES, 2018; KLAUNIG E WANG, 2018), o cigarro (ITOH et al., 2014; KOYANAGI et al., 2016), os agentes biológicos cancerígenos como aflatoxina (KEW, 2013; MAGNUSSEN E PARSEN, 2013), a radiação ultravioleta (MANCEBO E WANG, 2014; MOAN et al., 2014) e a radiação ionizante (DROOGER et al., 2015; DAS, 2014), os hábitos alimentares e o sedentarismo (MEHTA E SHIKE, 2014; MAHFOUZ et al., 2014); e uma inter-relação de fatores. Cerca de 60% dos casos de câncer estão associados com fatores externos.

A carcinogênese é o processo de formação do tumor que pode ser dividido em três estágios: iniciação, promoção e progressão (Figura 1). Durante o primeiro estágio, na iniciação, as células entram em contato com o agente carcinogênico chamado de iniciador, que provocará alteração, mudança ou mutações irreversíveis em seu DNA, muitas vezes após uma ligação covalente. Essas alterações genéticas podem resultar em desregulação das vias de

sinalização bioquímica associadas à proliferação celular, sobrevivência e diferenciação. Uma vez que a célula tenha sido afetada por um iniciador, ela torna-se suscetível à promoção. Ainda, quanto maior a exposição da célula ao iniciador, maior o risco de carcinogênese. A promoção é o segundo passo que ocorre nas células que já sofreram mutações pelos iniciadores. Ao contrário dos iniciadores, os promotores não se ligam covalentemente ao DNA da célula, mas muitos se ligam a receptores na superfície celular e afetam vias intracelulares que aumentam a proliferação celular ou diminuem a sinalização para morte celular. Há duas categorias de promotores: promotores específicos que interagem com receptores em células-alvo, e promotores não específicos, que alteram a expressão gênica sem envolver um receptor conhecido. O estágio de promoção é considerado um processo relativamente longo e reversível, no qual células pré-neoplásicas proliferantes se acumulam. Nesse momento, o processo pode ser alterado por agentes quimiopreventivos que afetarão as taxas de crescimento. Por fim, a progressão, refere-se ao estágio entre um tumor pré-maligno e o desenvolvimento do câncer invasivo. A progressão é a etapa final da transformação neoplásica, envolvendo um aumento rápido no tamanho do tumor, onde as células podem sofrer novas mutações com potencial invasivo e metastático. A metástase envolve a disseminação de células cancerígenas do local primário para outras partes do corpo através da corrente sanguínea ou do sistema linfático. Os agentes quimiopreventivos podem ser capazes de agir nas etapas da carcinogênese, inibindo a angiogênese, invasão e metástase. (LIU et al., 2015; SIDDIQUI et al., 2015; INCA, 2018).

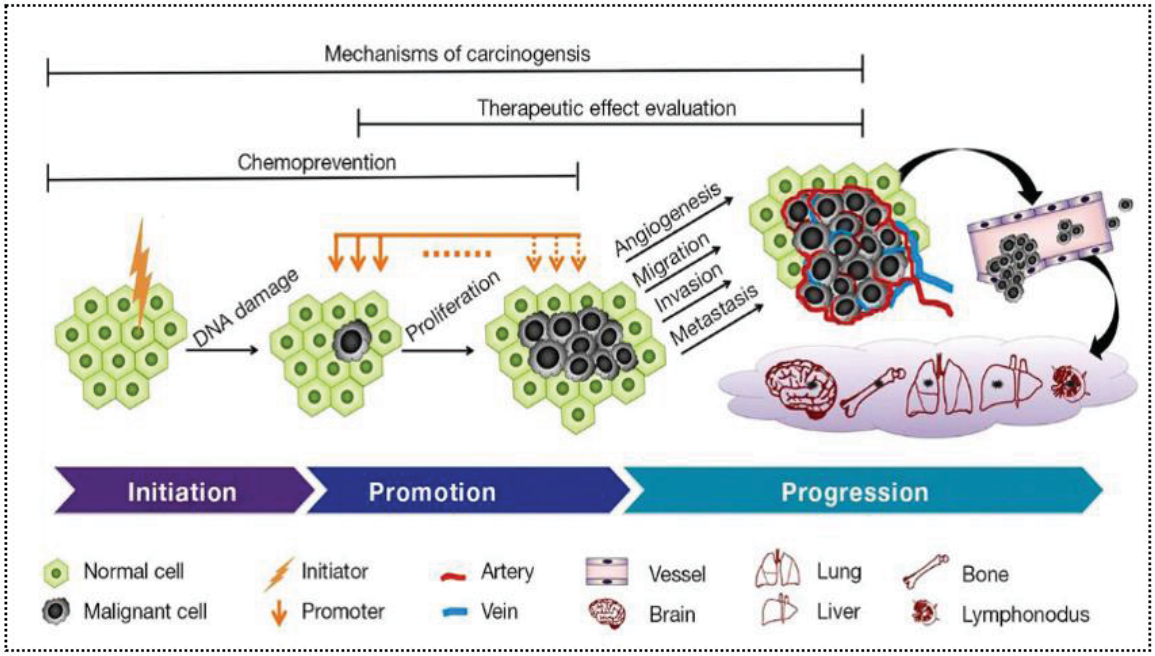

Figura 1: Ilustração das três etapas da carcinogênese. Processo trifásico da carcinogênese através da ação de carcinógenos e suas consequências, baseadas em modelos animais de cânceres primários. LIU et al., 2015.

Dentre as características que as células tumorais passam a apresentar, a mais significativa é o crescimento celular fora dos limites normais da célula, isto é, uma imortalidade**.** Essa capacidade pode ser explicada por: proliferação contínua, bloqueio dos genes supressores tumorais e resistência à morte celular (SAITO, LANA, MEDRANO E CHAMMAS, 2016).

Os genes supressores do tumor, são capazes de controlar a proliferação, a apoptose, a auto-renovação e a diferenciação celular. Contudo, esses genes podem sofrer alterações, como por exemplo, em TP53, resultando na desregulação do controle do ciclo celular (WONGTRAKOONGATE, 2015). Outros genes, como os proto-oncogenes, quando ativados passam a ser denominados de oncogenes, que quando aumentados tem a função de conduzir a um aumento na divisão celular e redução da diferenciação celular, favorecendo ao crescimento do tumor (CHIAL, 2008; LEE E MILLER, 2010; IURLARO et al., 2014). Assim, os mecanismos genéticos e epigenéticos envolvidos no câncer podem interagir através da ativação de oncogenes e do silenciamento de genes supressores de tumor, favorecendo o acúmulo de outras características do câncer de forma progressiva (Figura 2) (SAITO, LANA, MEDRANO E CHAMMAS, 2016).

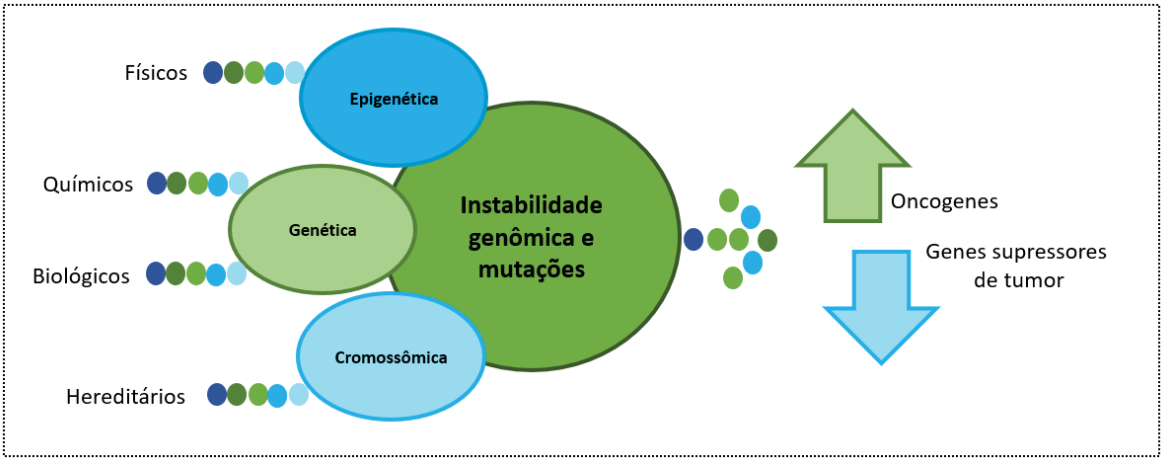

Figura 2: Instabilidade genômica e mutações. Neste diagrama se mostram as interações dos diferentes fatores que causam alterações em distintos níveis genéticos, que levam à instabilidade genômica traduzida em aumento da expressão de oncogenes e silenciamento de genes supressores de tumor. Modificado de SAITO, LANA, MEDRANO E CHAMMAS, 2016.

Para que as células cancerígenas continuem a se desenvolver, estas necessitam de oxigênio e nutrientes tais como, aminoácidos, lipídeos, lipoproteínas, glicose que são conduzidos pelos vasos sanguíneos e pela linfa (AL-ZOUGHBI et al., 2014). O microambiente tumoral está sempre em mudança e em algum momento o nível de oxigênio e os nutrientes necessários, tornam-se insuficientes para o crescimento do tumor. Assim, em resposta à redução da tensão de oxigênio, os fatores induzíveis por hipóxia, HIF1 e HIF2, são encontrados estabilizados e são ativados neste microambiente tumoral e atuam como reguladores transcricionais chave, para mediar a adaptação primária ao estresse hipóxico nas células tumorais. Há evidências que os HIFs estão envolvidos na regulação da angiogênese, sobrevivência celular, proliferação, apoptose, adesão e metabolismo, principalmente pela ativação transcricional de alvos à jusante, tais como fator de crescimento endotelial vascular (VEGF) (FEITELSON et al., 2015).

Ademais, a angiogênese tumoral pode ser mediada por citocinas como o fator de necrose tumoral alfa (TNF-α) e a interleucina-1 beta (IL-1β), as quais, expressam a enzima cicloxigenase 2 (COX-2), que consequentemente elevará a produção de prostaglandina E2 (PGE2) no tecido tumoral. A IL-1β e a PGE2 regulam os níveis de proteína HIF1α e ativam o VEGF, numa reação que é principalmente mediada via fator nuclear kB (NF-κB). Esta cascata de ativação do gene ilustra um exemplo importante do papel da inflamação no

desenvolvimento de tumores (AL-ZOUGHBI et al., 2014; HUANG, LI E ZHANG, 2014; FEITELSON et al., 2015).

A via do NF-κB é uma das importantes vias de sinalização no desenvolvimento do câncer que regulam uma variedade de genes celulares, como proliferação, sobrevivência, migração, inflamação, angiogênese, hipóxia e modulação das células imunes (PARK E HONG, 2016). Pode ser ativada por vários estímulos, como citocinas (TNF-α e IL-1β), fatores de crescimento (EGF), produtos bacterianos e virais (lipopolissacarídeos e RNA viral), raios ultravioletas e radiação ionizante, espécies reativas de oxigênio (EROs) e danos no DNA (BHATELIA, SINGH E SINGH, 2014). O NF-kB consiste em uma família de cinco fatores de transcrição NF-κB1/p105, NF-κB2/p100, RelA/ p65, RelB e c-Rel, que podem levar à ativação da via NF-κB (XIA, SHEN E VERMA, 2014). Esses estímulos também levam à ativação de outra proteína citoplasmática, o inibidor do complexo IkB cinase (IKK), que contém IKK1/IKKa, IKK2/IKKb e NEMO/IKKg. O complexo IKK ativado é responsável pela fosforilação do IkB, que leva à ubiquitinação e degradação do mesmo. Assim, a forma ativa do NF-κB transloca-se para o núcleo e ativa os genes-alvo. Portanto, a regulação cautelosa da via NF-κB é indispensável para a integridade celular e sua desregulação tem sido observada em muitas doenças, incluindo o câncer (LINGAPANN, 2018). O NF-κB é necessário para manter todas as células T e é conhecido por ser essencial para muitas das respostas dos linfócitos aos patógenos (GERONDAKIS E SIEBENLIST, 2010). Portanto, o NF-κB regula a proliferação e diferenciação de linfócitos T (YAMAMOTO E GAYNOR, 2001).

Estudos recentes têm mostrado que a resposta imune do hospedeiro está envolvida necessariamente com o crescimento, progressão e metástase do câncer. Esse desenvolvimento dos tumores malignos ocorre pela inter-relação entre as células cancerígenas e o microambiente tumoral, tais como a infiltração de células imunes. Há um interesse crescente no papel da resposta imune tumoral no prognóstico e na eficácia da terapia do câncer, em particular, o papel dos infiltrados linfocíticos. Variações dos subtipos de câncer de mama possuem um infiltrado imunológico que é detectado em até 75% dos tumores, sendo que até 20% dos tumores apresentam um infiltrado particularmente denso (ZHANG,

WANG E ZHANG, 2018). De acordo com o estudo de Demaria et al, a infiltração tumoral por linfócitos indica uma resposta imune celular antitumoral (DEMARIA et al., 2001).

Assim, o grau de infiltração linfocítica, especialmente, a presença de linfócitos em nichos de células tumorais (linfócitos infiltrados de tumor - TILs), os quais constituem populações predominantes de células imunes no microambiente tumoral, indicam uma resposta imune antitumoral e estão relacionados a um melhor prognóstico em muitos tipos de tumores sólidos (ZITVOGEL, KEPP E KROEMER, 2011; SALGADO et al., 2014; IBRAHIM et al., 2014). Além disso, Rakaee et al. (2018) já demonstraram que um aumento dos níveis de TIL no estroma (sTIL) é um fator prognóstico positivo independente, associado a um risco significativamente menor de progressão e mortalidade em pacientes com câncer de pulmão de células não pequenas. Outros estudos também mostraram que a maior expressão de TILs totais sugeriu um melhor prognóstico do câncer de mama (ZITVOGEL, KEPP E KROEMER, 2011; YU et al., 2016; STANTON, E DISIS, 2016) e poderiam contribuir para controlar o crescimento e a disseminação de alguns tumores.

Em paralelo, o estresse oxidativo é um dos fatores que favorece o desenvolvimento e a progressão tumoral (RANINGA et al., 2014). Define-se como estresse oxidativo o desequilíbrio entre a produção de espécies reativas de oxigênio (EROs) e de antioxidantes, resultando em uma toxicidade ao ambiente celular, danos a proteínas, DNA e aos lipídeos. As células inflamatórias podem levar ao quadro de estresse oxidativo, pois quando ativadas produzem EROs de forma excessiva. Ainda, estima-se que aproximadamente 20% dos cânceres ocorrem devido a doenças inflamatórias (MANTOVANI et al., 2004), associadas a estresse oxidativo.

No metabolismo normal os organismos aeróbicos produzem constantemente as EROs, que incluem radicais livres como ânion superóxido (O2–•), o hidroxil 6 (OH•), o alcoxil (RO•), o peroxil (ROO•) e o hidroperoxil (ROOH•), bem como não radicais, como o peróxido de hidrogênio (H_2O_2). Os antioxidantes atuam como defesa a esses radicais livres por serem capazes de competir com substratos oxidáveis e, consequentemente, inibirem ou atrasarem

o processo de oxidação. Essas moléculas podem ser não-enzimáticas como as vitaminas C e E, e enzimáticas como a superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx), glutationa redutase (GR) e a glicose 6-fosfato desidrogenase (G6PD) (DICHI et al., 2014). Há vários relatos que mostram que as moléculas antioxidantes estão diminuídas em certos tipos de câncer. Contudo, encontram-se aumentadas em tecidos tumorais, pois atuam na defesa do próprio tumor contra a ação das EROs, e com isso, as células tumorais aumentam sua proliferação, sobrevivência e resistência às drogas (RANINGA et al., 2014).

Outros processos celulares indispensáveis neste contexto são a apoptose e a necroptose. A apoptose é uma “morte natural programada”, isto é, ocorre naturalmente no organismo e é responsável por eliminar células mutadas que possam apresentar efeitos danosos ao funcionamento normal do ciclo celular e ainda desenvolver uma neoplasia. Esta via é extremamente importante para manter um equilíbrio fisiológico entre o crescimento e a morte celular, preservando a integridade do genoma ou induzindo as células mutadas à sobrevivência. O mecanismo deste processo é complexo e pode ocorrer por duas vias, a extrínseca (mediada por receptor) e a intrínseca (via mitocondrial), que são responsáveis por ativar as caspases, que levam à desintegração da cromatina e à fragmentação nuclear. Contudo, a desregulação dessas vias implica no desenvolvimento e/ou na resistência à terapia do câncer. Ainda, as células tumorais são capazes de desenvolver mecanismos que inibem o desenvolvimento desta morte celular programada, por modularem moléculas anti-apoptóticas ou inativarem componentes pró-apoptóticos (ZHAO et al., 2013; KOFF et al., 2015). Foi identificado um número considerável de genes capazes de influenciar a apoptose, sendo destaque a família *B-cell lymphoma* 2 (Bcl-2), que é dividida em proteínas anti-apoptóticas, como Bcl-2 e Bcl-xL, e pró-apoptóticas, como a Bax, Bad e Bid (PAROLIN E REASON, 2001; ZIMMER, 2007). O estresse oxidativo, por exemplo, estimula a apoptose por induzir a ativação das caspases, que ativam a família das proteínas Bcl-2 e modulam as quinases para levar a morte celular (RANINGA et al., 2014). Por fim, o desequilíbrio entre a ativação das proteínas pró- e anti-apoptóticas pode

aumentar a sobrevivência e a resistência dos tumores ao tratamento (JUIN et al., 2004).

A necroptose, outra via que vem sendo amplamente estudada, é um processo de necrose celular programada independente de caspases, e morfologicamente similar à necrose (DASGUPTA et al., 2017). A ativação ocorre por meio de fatores como TNF-α, que ativa a proteína quinase de interação ao receptor 1 (RIP-1), a qual promove a fosforilação da proteína quinase de interação ao receptor 3 (RIP-3), que, por sua vez, fosforila a proteína quinase de domínio de linhagem mista (MLKL), promovendo a trimerização, e translocando para a membrana plasmática, induzindo permeabilização da membrana necrótica, levando à morte celular (CAI et al., 2014). A indução tanto da apoptose quanto da necroptose é uma forma de promover a morte de células neoplásicas, sendo alvos de terapias contra o câncer.

1.3 TRATAMENTO ANTINEOPLÁSICO

As terapias anticâncer buscam a eliminação de células do tumor explorando as principais características que as diferenciam das células normais: dependência em proteínas oncogênicas, defeitos nos mecanismos de reparo de DNA, pontos de checagem de ciclo celular, controle das vias de apoptose e necroptose, e angiogênese. A combinação de esquemas terapêuticos para se evitar o surgimento de resistência têm se mostrado útil em casos específicos (SAITO, LANA, MEDRANO E CHAMMAS, 2016). Dentre as modalidades existentes para o tratamento do câncer está a cirurgia, a radioterapia, o transplante de medula óssea, a imunoterapia e a quimioterapia. Há muitos cânceres em que é necessária a combinação de mais de uma dessas modalidades de tratamento. A cirurgia é a ablação do tumor, desde que o tamanho do tumor não extrapole o total de tecido que é excisável e que ainda, seja compatível com a vida. A radioterapia utiliza a radiação para destruir e/ou impedir que o tumor possa expandir-se para outros tecidos. O transplante de medula óssea é utilizado em doenças malignas que afetam as células sanguíneas, tendo como objetivo a reconstituição de uma nova medula. A

imunoterapia promove a estimulação do sistema imunológico, por meio do uso de substâncias modificadoras da resposta biológica. Por fim, a quimioterapia é a terapia mais convencional, com a intenção de se utilizar drogas citotóxicas para inibir a proliferação de células malignas. Deste modo, os agentes antineoplásicos aplicados para este fim são os alquilantes, os antimetabólitos, os antibióticos antitumorais, os inibidores mitóticos, inibidores da topoisomerase, dentre outros (Tabela 1) (MERCK, 2015; INCA, 2018).

Os agentes alquilantes são efetivos ao combate de inúmeras formas de câncer, por se ligarem ao DNA e impedirem a sua replicação. Contudo, para que haja o efeito clínico, precisam ser combinados com outros agentes fase-específicos do ciclo celular (WORDING, PERISSINOTTI E MARINI, 2016; INCA, 2018). Os antimetabólitos impedem a multiplicação e a função das células normais, pois inibem a biossíntese dos componentes essenciais do DNA e RNA (BLACK & LIVINGSTON, 1990a; WORDING, PERISSINOTTI E MARINI, 2016; INCA, 2018). Os antibióticos antitumorais atuam inibindo a produção de ácidos nucleicos ou de proteínas. Por apresentarem anéis insaturados permitem a incorporação de excesso de elétrons e, consequentemente, a produção de EROs, causando a morte celular. Ainda, os inibidores mitóticos interrompem a divisão celular, pois paralisam a mitose na metáfase (BLACK & LIVINGSTON, 1990b; WORDING, PERISSINOTTI E MARINI, 2016; INCA, 2018). Os inibidores da topoisomerase afetam sobre as enzimas isomerases que atuam sobre a estrutura do DNA, interferindo na sua transcrição e replicação (WORDING, PERISSINOTTI E MARINI, 2016; INCA, 2018).

Apesar dessas terapias convencionais serem capazes de alterar a interação tumor/sistema imune favorecendo algum tipo de resposta antitumoral, nem todos os quimioterápicos conseguem produzir esse efeito. Recentes avanços na imunoterapia em vários tipos de câncer destacam o potencial da imunoterapia, em especial como componente da terapia adjuvante multi-modalidade. A eficácia na imunoterapia no câncer de mama pode ser explicada pela ação dos anticorpos monoclonais, já que seu efeito antitumoral pode ser alcançado atingindo funções de receptores (como agonistas ou antagonistas para subtipos específicos), modulando o sistema imune ou endereçando uma droga a um alvo específico. O Trastuzumab e o Ipilimumab são exemplos desses

anticorpos (ERNST E ANDERSON, 2015; SAITO, LANA, MEDRANO E CHAMMAS, 2016). Outro foco para o tratamento do câncer de mama é a terapia anti-hormônio, pois tanto os hormônios esteróides, como os estrogênios e a progesterona, possuem efeitos significativos no crescimento, diferenciação e função das células da mama e de outros tecidos. O tamoxifeno e o fulvestrant são exemplos dessa classe de drogas (ABDULKAREEM E ZURMI, 2012).

No entanto, as drogas antineoplásicas possuem efeito citotóxico tanto em células malignas quanto em células normais, levando a diversos efeitos colaterais, dentre eles náusea, vômito, hemorragia, fadiga, mielossupressão e alopecia (WORDEN, PERISSINOTTI E MARINI, 2016; JUNQUEIRA E CHAMMAS, 2018). Diante disso, pesquisas nesta área têm como foco a busca por medicamentos antineoplásicos que apresentem uma citotoxicidade mínima em células normais e que sejam capazes de transpor os processos de resistência celular, resultando em uma ação promissora e com potente eficácia na prevenção e no tratamento do câncer.

Tabela 1. Principais agentes de quimioterapia citotóxica.

Classe Farmacológica	Droga	Mecanismo de Ação	Indicadores Selecionados	Principais Efeitos Adversos
Agente Alquilante	Ciclofosfamida	Ligação cruzada de DNA e bloqueio da síntese e função do DNA	Câncer de mama e ovário	Urotoxicidade, alopecia, mielossupressão, náuseas e vômitos
Agente Alquilante	Isofosfamida	Inibição da síntese e função do DNA por metabólitos que formam ligações cruzadas	Câncer testicular e sarcomas	Urotoxicidade e mielossupressão
Antimetabólito	Metotrexato	Antifolato inibidor da síntese de DNA	Câncer de cabeça e pescoço	Mielossupressão, mucosite e diarreia
Antimetabólito	Mercaptopurina	Metabólitos que inibem a síntese de DNA e RNA	Leucemia linfoblástica aguda	Mielossupressão e toxicidade gastrointestinal
Antibiótico antitumoral	Doxorrubicina	Inibição da síntese e função do DNA por intercalar com pares de bases	Câncer de mama e linfoma não Hodgkin	Mielossupressão, cardiotoxicidade e alopecia
Antibiótico antitumoral	Mitomicina C	Inibição da síntese e função do DNA por alquilação do DNA e RNA	Câncer gástrico e pancreático	Mielossupressão e mucosite

Inibidores mitóticos	Vincristina	Inibição da polimerização da tubulina e induz quebra do microtúbulo na mitose	Leucemia linfoblástica aguda, neuroblastoma e linfoma de Hodgkin	Neurotoxicidade e mielossupressão
Inibidores mitóticos	Vimblastina	Inibição da polimerização da tubulina e induz quebra do microtúbulo na mitose	Tumor de células germinativas e linfoma de Hodgkin e não Hodgkin	Mielossupressão e sintomas gastrointestinais
Inibidores da topoisomerase	Topotecano	Inibição de topoisomerase II e bloqueio do desenrolamento do DNA	Carcinoma metastático de ovário	Alopecia e mielossupressão
Inibidores da topoisomerase	Etoposídeo	Inibição de topoisomerase I e bloqueio do desenrolamento do DNA	Tumores de células germinativas e pulmão de células não pequenas	Alopecia, mielossupressão e segunda neoplasia

Muitos agentes não atuam diretamente sobre a inibição de processos do DNA, podendo ser dependentes de ativação pela célula tumoral. Uma vez que a quimioterapia citotóxica afeta células que se dividem rapidamente, toxicidade na pele e nas mucosas, mielossupressão e alopecia são efeitos bastante frequentes. Adaptado de SAITO, LANA, MEDRANO E CHAMMAS, 2016.

1.3.1 Oligossacarídeos

As pesquisas científicas vêm progredindo na compreensão do processo carcinogênico em nível celular e molecular. Isso levou ao desenvolvimento de uma nova abordagem para a prevenção do câncer, denominada “quimioprevenção”, que tem como objetivo bloquear, inibir ou reverter o desenvolvimento e progressão de células pré-cancerosas através do uso de nutrientes não citotóxicos e/ou agentes farmacológicos. Assim, busca-se componentes dietéticos, produtos naturais, ou seus análogos sintéticos com potencial ação quimiopreventiva do câncer. Com isso, agentes na forma de alimentos funcionais ou nutracêuticos tornaram-se alvo dos cientistas (LI et al., 2013).

Diante disso, os oligossacarídeos merecem destaque, pois são derivados da despolimerização de polissacarídeos e possuem uma cadeia menor de monossacarídeos, consequentemente apresentam menor peso molecular. Além disso, são menos viscosos e mais solúveis em água, sendo capazes de desempenhar funções biológicas valiosas em nível molecular (ZOU et al., 2016). Em comparação com polissacarídeo, um oligossacarídeo é mais fácil de

preparar em grande escala e com controle de qualidade (LI et al., 2013). Os oligossacarídeos podem ser encontrados em frutas, vegetais, leite e mel, na forma de dissacarídeos, como a lactose e a sacarose; ou como a maltose e a celobiose, que são obtidos a partir de hidrólise química ou enzimática do amido e celulose, respectivamente (HIRAYAMA, 2002; ALMEIDA, PASTORE, 2004). Com isso, os oligossacarídeos têm sido amplamente utilizados na indústria alimentícia por atuarem como substituinte de açúcares em alimentos de baixo teor calórico, com a intenção de aumentar o consumo de fibras , visto que, as fibras auxiliam o bom funcionamento do processo digestivo, evitando assim o aparecimento de doenças crônicas como a obesidade, aterosclerose, hipertensão, osteoporose e o desenvolvimento de carcinomas (MATTOS, MARTINS, 2000; OKU, NAKAMURA, 2002). Vários estudos vêm sendo feito com oligossacarídeos mostrando sua atividade biológica e aplicação terapêutica. Alguns desses estudos estão comentados a seguir.

Os fruto-oligossacarídeos e os xilo-oligossacarídeos atuam como prebióticos, que são resistentes à ação das enzimas salivares e intestinais, estimulam a proliferação de bactérias intestinais benéficas, as quais produzem ácidos orgânicos, e contribuem para a diminuição do pH. Também apresentam benefícios à saúde pela sua atividade antiadesiva (HOLZAPFEL, SCHILLINGER, 2002; SHOAF et al., 2006). Além disso, ambos são utilizados no tratamento do câncer de cólon, contudo, os xilo-oligossacarídeos têm sido descritos como mais eficientes do que os fruto-oligossacarídeos (HSU et al., 2004).

Outra função dos oligossacarídeos, como as ciclodextrinas, é a atuação como carreadores de pequenas moléculas como proteínas, peptídeos e ácidos nucléicos, podendo ser úteis na indústria farmacêutica. Com isso, nanopartículas deste material servem como proteção, transporte e entrega das drogas, sendo rapidamente eliminadas da corrente sanguínea após a administração intravenosa (LEMARCHAND et al., 2006).

Os gluco-oligossacarídeos bioativos derivados das glucanas estão envolvidos na estimulação da resposta anti-inflamatória mediada pelas células do sistema imune, por induzirem mediadores pró- e anti-inflamatórios, como o

TNF-α e a IL-1. O mecanismo de ação antitumoral está principalmente relacionado com a ativação das células "*natural killer*" (NK) (PANG et al., 2005).

Os oligossacarídeos derivados da quitosana já foram relatados por apresentarem aplicações potenciais em alimentos (DU, WANG, YUAN, WEI, E HU, 2009), na indústria farmacêutica (BERGER, REIST, MAYER, FELT E GURNY, 2004), na agricultura e no meio ambiente (CRINI, 2005). Ainda, por serem antioxidantes naturais (XIE, XU, E LIU, 2001), possuem propriedades anti-inflamatória, antimicrobiana (CHOI et al., 2001), hipocolesterolêmica (MUZZARELLI et al., 2006), imunoestimulante (FENG, ZHAO, E YU, 2004) e antitumoral (SALAH et al., 2013). Além disso, não são tóxicos e alergênicos para os tecidos vivos (DASH, CHIELLINI, OTTENBRITE, E CHIELLINI, 2011). Outro estudo mostrou *in vivo,* a ação quimiopreventiva de oligossacarídeos derivados da quitosana contra o câncer de colon retal em ratos Fisher 344 (VERGHESE et al., 2005). Verifica-se que os oligossacarídeos exercem atividade anticâncer bloqueando a carcinogênese, aumentando a eficácia dos agentes quimioterápicos, ou exibindo diretamente o efeito de citotoxicidade.

Apesar desses relatos sobre as funções dos oligossacarídeos, ainda não se tem estudos direcionados aos oligossacarídeos extraídos de vinho tinto. Nosso grupo recentemente realizou estudos mostrando os efeitos antitumorais *in vivo* da fração solúvel de polissacarídeos extraídos de vinho tinto no carcinossarcoma Walker-256 (STIPP et al., 2017). Sabe-se que os carboidratos presentes no vinho são compostos principalmente de monossacarídeos, álcoois de açúcar, ácidos de açúcar e dissacarídeos. Alguns deles são constituintes naturais, enquanto outros são formados em fermentação. No vinho Cabernet Franc foi encontrada uma diversidade de oligossacarídeos, com alta quantidade de xilose, ramnose e trealose. De acordo com a literatura, a L-ramnose é um constituinte de muitos glicosídeos e polissacarídeos, enquanto a D-xilose é um componente importante da madeira, que é apenas fermentável por certos microrganismos, como *Lactobacilli*, *Torula* e *Monilia*. Dos dissacarídeos presentes no vinho, a trealose tem sido relatada como a principal, formada pela atividade metabólica da levedura, enquanto a sacarose está presente em baixos níveis (RUIZ-MATUTE, SANZ, MORENO-ARRIBAS E MARTÍNEZ-CASTRO, 2009).

Nosso trabalho foi delineado para avaliar o efeito antitumoral de oligossacarídeos extraídos do vinho tinto Cabernet Franc, utilizando modelos tumorais *in vitro* e *in vivo*, todos com células originárias de tumor mamário.

1.4 MODELO TUMORAL DE EHRLICH

O tumor de Ehrlich tem origem epitelial e é uma neoplasia maligna. Foi descoberto em 1886 por Paul Ehrlich, através do aparecimento espontâneo no tecido mamário de camundongos fêmeas. No entanto, foi apenas em 1905, que os pesquisadores Ehrlich e Apolant testaram o tumor experimentalmente, pelo transplante de tecidos tumorais de camundongo para camundongo por via subcutânea (OZASLAN et al., 2011). Em 1932, os pesquisadores Loewenthal e Jahn desenvolveram uma variante especial do carcinoma de Ehrlich. Eles observaram que após a inoculação de uma suspensão de células carcinogênicas de Ehrlich na cavidade peritoneal de camundongos, não só obtiveram tumores na forma sólida, mas também um fluído ascítico que continha muitas células neoplásicas, sendo facilmente transmissível a outros animais. Após esta descoberta, esse modelo tumoral passou a ser conhecido como “tumor ascítico de Ehrlich” (KLEIN, 1950; MIRANDA-VILELA et al., 2011).

Quando a inoculação ocorre pela via intraperitoneal de camundongos o tumor de Ehrlich cresce na forma ascítica, aumentando a agressividade tumoral através de passagens repetidas; e quando é inoculado por via subcutânea desenvolve-se na forma de tumor sólido (BAILLIF, 1954). As principais características deste tumor são: origem hiperdiplóide, rápido crescimento denotando elevado grau de malignidade, não induz caquexia, não regride, tem elevada capacidade transplantável, espécie-específico, e permite avaliação *in vivo* em um período mais curto (BAILLIF, 1954; KLEIN, 1950; OZASLAN et al., 2011).

O carcinoma de Ehrlich é indiferenciado e tem uma taxa acelerada de crescimento, se assemelhando a tumores humanos que são sensíveis à quimioterapia (OZASLAN et al., 2011). Entende-se assim que o tumor de Ehrlich

é um bom modelo para estudar a patogenia e a terapia de tumores sólidos em humanos. Estudos anteriores demonstraram que houve desenvolvimento do tumor sólido em 100% dos camundongos em até 14 dias após a inoculação subcutânea no membro pélvico destes animais (ABDIN et al., 2014; BASSIONY et al., 2014), e estudos realizados em nosso laboratório confirmaram esses resultados (ADAMI et al., 2018; CORSO et al., submetido em 2018). Foi relatado também crescimento de tumor sólido após inoculação subcutânea na região da cabeça, na região dorsal e nas patas (NASCIMENTO et al., 2006; MIRANDA-VILELA et al., 2011; PEREIRA et al., 2013; DE FATIMA PEREIRA et al., 2014; MIRANDA-VILELA et al., 2014).

Além disso, após a inoculação das células tumorais de Ehrlich, ocorrem alterações morfológicas e metabólicas no hospedeiro, diminuição de síntese de DNA e RNA, perda de nucleotídeos intracelulares, declínio da concentração de ATP, diminuição da síntese de proteínas, aumento dos triglicerídeos, ésteres de colesterol e ácidos graxos livres (OZASLAN et al., 2011). No tecido tumoral tem sido demonstrado que há aumento da concentração de TNF-α e aumento da concentração de malondialdeído (SEGURA et al., 2001; KABEL et al., 2013).

Este modelo experimental de neoplasia já está padronizado no Laboratório de Farmacologia & Metabolismo da UFPR, visto que possui a vantagem da utilização de camundongos como espécie de estudo, que requerem menor quantidade de fármaco/composto administrado em relação a ratos. Com o tumor de Ehrlich avaliou-se o efeito antineoplásico dos oligossacarídeos do vinho Cabernet Franc utilizando técnicas farmacológicas, bioquímicas e de biologia celular *in vivo*, bem como se investigou os possíveis mecanismos de ação envolvidos. Em paralelo, as linhagens celulares MCF7, MDA-MB-231 e MDA-MB-436 foram utilizadas em cultivo para avaliar o efeito dos oligossacarídeos sobre células de tumor mamário de origem humana. De acordo com a literatura, as células MCF-7 são uma linhagem celular que representa um subtipo chamado luminal A, que possui positividade para receptores de estrogênio (REs) e progesterona (RP) apresentam-se pouco agressivas e não invasivas, e não possuem potencial metastático (COMŞA, CÎMPEAN, E RAICA, 2015). Já as células MDA-MB-231 e MDA-MB-436 são subtipos triplas-negativas, altamente agressivas, invasivas e pouco diferenciadas, e não

apresentam a expressão dos receptores REs e RP e também do fator de crescimento epidérmico humano (HER2), (KARGIONOVA et al., 2015; LECONET et al., 2017).

2 JUSTIFICATIVA

De acordo com as estimativas, os casos de câncer tendem a aumentar consideravelmente a cada ano em todo o mundo, principalmente por conta do estilo de vida adotado pela população. O câncer de mama se enquadra como o segundo tipo de câncer mais diagnosticado mundialmente. Por isso as pesquisas buscam um tratamento que seja minimamente citotóxico às células normais do organismo, ao mesmo tempo que seja altamente eficaz na prevenção e/ou na cura câncer desta doença, diminuindo assim sua reincidência.

Diante disso, os produtos naturais estão sendo amplamente estudados tanto na prevenção como no tratamento, podendo ser associado a uma droga já existente, melhorando a eficácia de terapias e diminuindo os efeitos colaterais. Sabendo disso, e de acordo com a literatura, os oligossacarídeos já demonstraram sua potencial atividade anticancerígena, porém ainda não se tem nenhum estudo mostrando especificamente a ação antitumoral de oligossacarídeos extraídos do vinho tinto. Nossa hipótese é que estes oligossacarídeos possuem atividade antineoplásica, cujos mecanismos carecem de investigação. A proposta deste trabalho é avaliar o efeito antineoplásico e os possíveis mecanismos dos oligossacarídeos do vinho Cabernet Franc, em modelos de estudo *in vitro* e *in vivo*.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar o efeito antineoplásico de oligossacarídeos extraídos do vinho tinto Cabernet Franc (Oligos) em modelos de estudos *in vivo* e *in vitro* em células tumorais de origem mamária.

3.2 OBJETIVOS ESPECÍFICOS

- ✓ Avaliar a viabilidade de células tumorais de mama (MCF7, MDA-MB-231 e MDA-MB-436) tratadas com os Oligos *in vitro;*
- ✓ Avaliar se o uso dos Oligos por um curto período (5 dias) diminui a viabilidade das células tumorais no modelo ascítico de Ehrlich *in vivo*;
- ✓ Avaliar se o uso dos Oligos por um período de 21 dias (tratamento convencional) diminui o desenvolvimento do tumor sólido de Ehrlich *in vivo*;
- ✓ Avaliar se o tratamento prolongado com Oligos (42 dias), iniciando anteriormente à inoculação tumoral (quimioprevenção), diminui o crescimento tumoral;
- ✓ Avaliar se a associação dos Oligos com um quimioterápico de uso corrente, o metotrexato (MTX), é mais eficiente para reduzir o desenvolvimento tumoral;
- ✓ Investigar os possíveis mecanismos de ação antitumoral dos Oligos, avaliando alguns parâmetros de vias de desenvolvimento tumoral, como inflamação, apoptose, necroptose, ciclo celular e angiogênese.

4. ARTIGO CIENTÍFICO

Antineoplastic effects of oligosaccharides from red wine in Ehrlich tumor-bearing mice are mediated by immune-inflammatory responses

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Abstract

A fraction of polysaccharides that are extracted from red wine has been reported to have antitumor effects *in vivo*. The present study investigated the antineoplastic and chemopreventive effects of oligosaccharides that were extracted from Cabernet Franc red wine (Oligo) on Ehrlich carcinoma and mammary tumor cell lineages *in vitro*. Female Swiss mice were subcutaneously inoculated with 2×10^6 Ehrlich tumor cells and then received vehicle (10 mL kg^{-1} distilled water, p.o.; negative control), Oligo solution (9, 35, or 70 mg kg^{-1}, p.o.), or methotrexate (MTX; 1.5 mg kg^{-1}, i.p., twice weekly; positive control). The treatments were administered for 21 days (conventional treatment) after tumor inoculation or administered for 21 days before tumor inoculation and then for 21 days after tumor inoculation (chemopreventive treatment). The Oligo compound, characterized by NMR and MicrOTOF-Q II-MS analysis, had a complex mixture of oligosaccharides. Oligo reduced the growth of Ehrlich tumors in both treatment protocols and induced intense inflammatory cell infiltration in the tumor. Additionally, Oligo treatment with MTX (9 or 35 mg kg^{-1} Oligo, p.o., + 1.5 mg kg^{-1} MTX, i.p.) increased the effectiveness of MTX in controlling tumor growth. Oligo did not reduce the viability of ascitic Ehrlich cells after 5 days of treatment or the viability of MCF7, MDA-MB-231, and MDA-MB-436 breast tumor cells after 24 and 48 h of treatment in culture. Overall, Oligo exerted an antineoplastic effect against Ehrlich cells that depended on the treatment time and immune-inflammatory response. Thus, Oligo may be a promising adjuvant therapy for solid tumors.

Keywords: Cabernet Franc oligosaccharides, Ehrlich carcinoma, antitumor, chemoprevention, inflammatory infiltration, *Hif1a*.

4.1 INTRODUCTION

Cancer has a high mortality rate worldwide. Breast cancer is the second most commonly diagnosed type of cancer. More than 1.3 million women worldwide are diagnosed with breast cancer each year. Intensive efforts have been made to understand the molecular mechanisms of the pathogenesis of breast cancer and its application to efficient antitumor therapies (Bhatelia, Singh, & Singh, 2014). Despite the numerous anticancer drugs that are available, these treatments still have low efficacy and high toxicity, leading to cancer progression, cancer recurrence, and a reduction of survival. Therefore, research has focused on searching for new antineoplastic compounds that have minimal cytotoxicity in normal cells and are capable of circumventing cellular resistance processes (Yang, Lee, & Yen, 2000; Kreso & Dick, 2014).

Several chemopreventive agents are present in natural products, such as food and vegetables, and have great potential to safely and effectively prevent carcinogenesis (Sporn, 1976; Wattenberg, 1966). For example, chitosan-derived oligosaccharides are a type of antioxidant with antiinflammatory, antimicrobial, hypocholesterolemic, immunostimulant, and antitumor properties (Choi et al., 2001; Muzzarelli et al., 2006; Feng, Zhao, & Yu, 2004; Salah et al., 2013). Another study reported the chemopreventive effects of chitosan-derived oligosaccharides on rectal cancer (Nam, Kim, & Shon, 2007). Oligosaccharides are non-toxic and non-allergenic to living tissues (Dash, Chiellini, Ottenbrite, &

Chiellini, 2011). Oligosaccharides have applications in the food, pharmaceutical, and agricultural industries (Du, Wang, Yuan, Wei, & Hu, 2009; Berger et al., 2004; Crini, 2005).

Despite these reports of the effects of oligosaccharides, no studies have yet evaluated the Oligo. Previous studies have shown that red wine has health benefits that are attributable to its components, such as polyphenol resveratrol that is present in grape bark and has been shown to have antioxidant activity and protective effects against several tumors (Jang et al., 1997; Singh, Liu, & Ahmad, 2015). Furthermore, our group recently conducted a study that showed the *in vivo* antitumor effects of a soluble fraction of polysaccharides that were extracted from red wine on Walker-256 carcinosarcoma, the cells of which have a mammary origin (Stipp et al., 2017). Thus, the present study was designed to evaluate the antitumor effects of Oligo on mammary tumor cells and the possible mechanism of action.

To test the hypothesis that Oligo inhibits mammary tumor cell development, different experimental strategies were used: *in vitro* study of human MCF7, MDA-MB-231, and MDA-MB-436 breast adenocarcinoma cell lineages, *in vivo* study of effects of Oligo on ascitic Ehrlich tumors in mice, and *in vivo* study of a solid Ehrlich carcinoma model in mice using conventional and chemopreventive treatment protocols. All of these cell lineages have a mammary origin. The results indicated that Oligo has treatment time-dependent antineoplastic effects *in vivo* that are mediated by immune-inflammatory regulation.

4.2 MATERIAL AND METHODS

4.2.1 Chemicals

N-acetylglucosamine, p-nitrophenol, sodium nitrite, and tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypan blue, absolute ethanol and methanol, trichloroacetic acid, acetic acid, ascorbic acid, *N,N*-dimethylformamide, formaldehyde, hydrogen peroxide, citric acid, sodium acetate, sodium chloride, potassium chloride, sodium phosphate, dibasic sodium phosphate, monobasic potassium phosphate, dibasic potassium phosphate, hematoxilin, aluminium potassium phosphate, mercuric oxide, eosin, sulphanilamide, phosphoric acid, naphthylethylenadiamide, Tween 20, Triton X-100, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Vetec (Rio de Janeiro, Brazil). Aspartate (AST), alanine transaminase (ALT), and urea kits were purchased from Kovalent (São Paulo, Brazil). TriZol, MTT, and primers were obtained from Invitrogen-ThermoFisher (Waltham, MA, USA). The High Capacity cDNA Reverse Transcription Kit and SYBR Green PCR Master Mix were obtained from Applied Biosystems-ThermoFisher (Waltham, MA, USA). RPMI1640 and fetal bovine serum were obtained from Gibco-ThermoFisher (Waltham, MA, USA). Dimethylsulfoxide (DMSO) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

4.2.2 Oligosaccharides preparation

The oligosaccharides were obtained from Cabernet Franc red wine. The wine was concentrated under reduced pressure, and then polysaccharides were precipitated with cold ethanol (3 vol) following centrifugation at 8000 rotations per minute at 4ºC for 20 min. The ethanolic supernatant was collected and concentrated under reduced pressure. The latter underwent organic partitioning

(v/v) with ethyl acetate and n-butanol to remove phenolic compounds. The water-soluble phase was chromatographed with activated charcoal and celite (1:1) as the stationary phase; as the mobile phase, an H_2O:ethanol gradient was used, starting with 100:0 to 95:05, 90:10, 85:15, and 80:20 (Morales, Sanz, Olano, & Corzo, 2006; Soares, 2017). The oligosaccharides were collected in ethanolic fractions. These fractions were concentrated under reduced pressure and freeze dried. The fraction was reconstituted in distillate water before the administration.

4.2.2.1 Nuclear magnetic resonance (NMR) spectroscopy

Oligo (20 mg) was solubilized in 500 µL of D2O using 20 µL of 3-trimethylsilyl-2H4-propionic acid sodium salt (TMSP; 1 mg/mL) as a reference. The NMR analyses were performed using a 600 MHz Bruker Avance III NMR spectrometer that was equipped with an inverse 5 mm probe head (QXI) at 303 K. The 1D ^{1}H NMR spectrum was collected after 90º pulse calibration for each sample. ^{1}H and ^{13}C chemical shifts were determined by 2D NMR. 2D $^{1}H/^{13}C$ multiplicity-edited HSQC was performed by correlation via double inept transfer with decoupling during acquisition using sensitivity improvement trim pulses as compiled in the pulse program hsqcedetgpsisp2.2 using 6993 Hz (^{1}H) and 24900 Hz (^{13}C) widths and a recycle delay of 1.080 s. 2D correlation maps were recorded using quadrature detection in the indirect dimension and 32 scans per series of 1024 $\times$ 320 W data points, with zero filling in F1 (2048) prior to Fourier transformation (Sassaki et al., 2014). The analyses were performed using Topspin 3.5 software (Bruker).

4.2.2.2 Mass spectrometry analysis / MicrOTOF-Q II MS analysis

Oligo (250 µg) were solubilized in methanol:water (v/v, 70:30) with LiCl (1 mM), and mass spectrometry (MS) was performed through direct infusion in a high-resolution MicrOTOF-Q IITM mass spectrometer (Bruker Daltonics, Billerica, MA, USA) that was equipped with an orthogonal electrospray ionization source. The selected mass range for each spectrum was 300-1700 m/z, with acquisition of one spectrum per second. The capillary voltage was 4500 V, and the endplate offset was 500 V. The nebulizer gas flow was 0.6 bar. The drying gas was nitrogen at a flow rate of 6.0 L/min at 180ºC. The equipment was calibrated with sodium formiate (10 mM), with a calibration score of 99%. The volumetric flow of the sample at the infusion source was 180 µL/h. Fragmentation was obtained with a collision energy that ranged from 10.0 eV to 37.1 eV. The analysis was performed using DataAnalysis 4.1 software (Bruker).

4.2.3 Cell culture

All of the cell lines that were used in this study were obtained from the *American Type Culture Collection (ATCC).* MCF7, MDA-MB-231, and MDA-MB-436 human breast adenocarcinoma cells were chosen based on their features. MCF7 cells are positive for estrogen receptors (ERs) and progesterone receptors (PRs). MCF-7 cells are a poorly aggressive and non-invasive cell line, with particularly low metastatic potential (Comşa, Cîmpean, & Raica, 2015). MDA-MB-231 and MDA-MB-436 cells are triple-negative, defined by the absence of ER, PR, and human epidermal growth factor receptor 2 (HER2) gene expression subtypes (Leconet et al., 2017; Kargionova et al., 2015). The cell lines were cultured in 1640 RPMI medium (Gibco) supplemented with 10% fetal bovine

serum (FBS; Gibco) and 1% penicillin/streptomycin. The cells were maintained at 37ºC in a 5% CO_2 humidified atmosphere.

4.2.3.1 MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was used to determine the cytotoxicity of Oligo (Riss et al., 2013). Human MCF-7, MDA-MB-231, and MDA-MB-436 breast cancer cells were cultured in 96-well plates at a density of 5×10^3 cells/well. Oligo was diluted in supplemented culture medium and added at different concentrations (4, 12, 38, 120, and 350 μg mL^{-1}), with untreated wells as control. The cells were incubated for 24 or 48 h. Two concentrations of MTX (45 and 363 μg mL^{-1}) were used as a positive control with 48 h of incubation. Afterward, 100 μL of 0.5 mg mL^{-1} MTT was added to each well, and the plates were incubated for an additional 4 h at 37ºC. The resulting violet formazan precipitate was solubilized by adding 100 μL of DMSO. The plates were then immediately read at 570 nm using an iMark Microplate Absorbance Reader (Bio-Rad, Hercules, CA, USA).

4.2.4 Preparation of Ehrlich cells for ascitic and solid tumor models

All of the procedures were reviewed and approved by the institutional Ethical Committee for Animal Care (CEUA; authorization no. 1062). Groups of female Swiss mice, weighing 28-35 g, were housed under conditions of controlled temperature (20°C ± 2°C) and a 12 h/12 h light/dark cycle and received food and water *ad libitum*. The animals were obtained from the vivarium of the Federal University of Parana (Curitiba, Brazil).

To maintain ascitic Ehrlich tumors, 0.2 mL of the cells were weekly transplanted by intraperitoneal (i.p.) injections of 2×10^6 cells/mice. After three or four passages, 100 µL of the cells was carefully and aseptically collected from the peritoneum and mixed in 1 mL of phosphate-buffered saline (PBS; 16.5 mM phosphate, 137 mM NaCl, and 2.7 mM KCl, pH 7.4) and 0.5 M ethylenediaminetetraacetic acid (pH 8.0). Cell viability was assessed using the Trypan blue dye exclusion method (≥ 98%; Philips, 1973; de Fátima Pereira, Mara da Costa, Cristina Magalhães Santos, Carmo Horta Pinto, & Rodrigues Da Silva, 2014; El-Sisi, Sokar, Salem, & Abu Risha, 2015). For solid tumor growth, 0.2 mL of the Ehrlich ascitic content (2×10^6 cells/mice) was subcutaneously (s.c.) inoculated in the right pelvic member of the mice (Bassiony et al., 2014).

4.2.5 Experimental design

The animals were divided into the following groups: (*i*) naive (no tumor) and treated with vehicle (10 mL kg^{-1} distilled water, p.o.), (*ii*) tumor-bearing and treated with vehicle (10 mL kg^{-1} distilled water, p.o.; negative control), (*iii*) tumor-bearing and treated with Oligo solution (9, 35, or 70 mg kg^{-1}, p.o. or i.p.), and (*iv*) tumor-bearing and treated with MTX (1.5 mg kg^{-1}, i.p., twice weekly; positive control). Furthermore, some tumor-bearing mice were treated with Oligo combined with MTX (9 or 35 mg kg^{-1} Oligo, p.o., + 1.5 mg kg^{-1} MTX, i.p.). The doses of Oligo (35 and 70 mg kg^{-1}) and MTX were based on previous studies (Stipp et al., 2017; Adami et al., 2018). For the longest treatment time, the dose of 9 mg kg^{-1} Oligo was used, equivalent to one-quarter of the regular treatment dose (35 mg kg^{-1}).

Three different protocols were employed (Fig. 1): (*i*) ascitic tumors (treatments started 1 day after intraperitoneal inoculation [day 1] and continued for 5 days, for a total of 5 days of treatment), (*ii*) conventional treatment of solid tumors (treatments started 1 day after subcutaneous tumor inoculation [day 1] and continued until day 21, for a total of 21 days of treatment), and (*iii*) chemopreventive protocol in solid tumors (treatment started 21 days before subcutaneous tumor inoculation [day 0] and continued for 21 days after inoculation, for a total of 42 days of treatment). In all of the treatment protocols, the animals were weighed daily. In protocols (*ii*) and (*iii*) above, the tumor volume was also assessed daily with a pachymeter. The solid tumor mass became palpable around day 7 after inoculation and started to be measured in two dimensions (height and width) until the last day. Tumor volume was calculated as the following: $V(cm^3) = \left(\frac{4\pi}{3}\right).a^2.(\frac{b}{2})$, where *a* is the smallest tumor diameter, and *b* is the largest tumor diameter (in centimeters; Mizzuno et al., 1999).

On the last day of treatment in protocols (*ii*) and (*iii*) above, the animals were fasted for 16 h with free access to water. The next day, they were anesthetized (i.p. injection of 80 mg kg^{-1} ketamine hydrochloride and 10 mg kg^{-1} xylazine) for biological material collection and subsequent euthanasia. Blood was collected from the inferior cava vein for hematological and plasma biochemical analysis (Adami et al., 2018). Tumors were collected and measured with a pachymeter in three dimensions (height, depth, and width) to confirm the tumor volume that was measured during treatment. Subsequently, these tumors and the livers were collected, weighed, fragmented, and properly stored for further analysis.

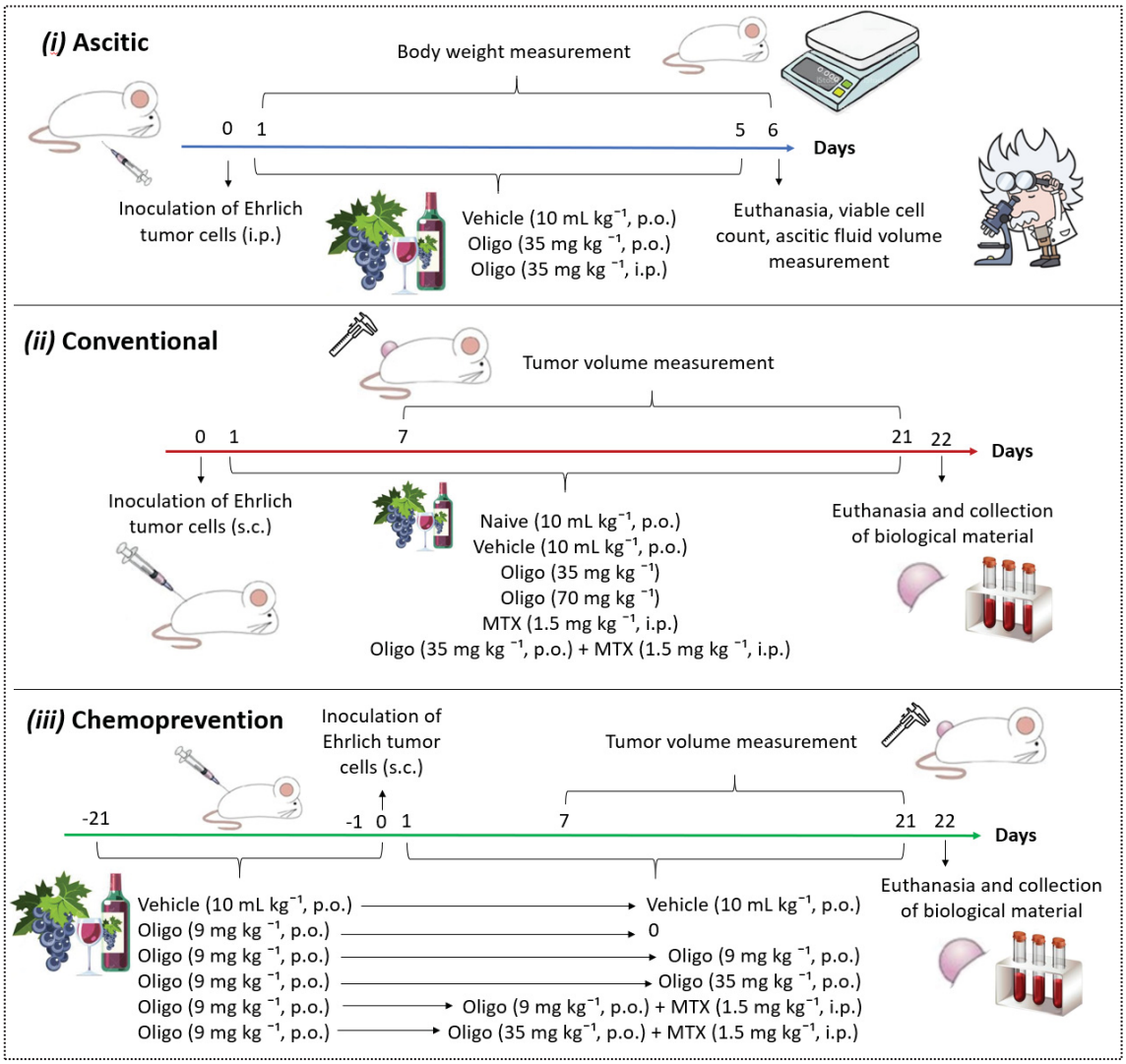


Figure 1. Experimental design of Ehrlich tumor models in naive mice and mice treated with vehicle, red wine oligosaccharides (Oligo), or methotrexate (MTX). p.o.: oral; i.p., intraperitoneal; s.c., subcutaneous.

4.2.6 Hematological and biochemical parameters

Blood samples that were collected at the end of treatment were used to analyze red blood cells (RBCs), hemoglobin (Hb), hematocrit (Ht), and leukocytes (lymphocytes, monocytes, and granulocytes) using a BC2800-Vet automated device. The samples were then centrifuged at 1344 × *g* for 5 min. Plasma was separated and used to measure glucose, ALT, AST, creatinine, total protein,

albumin, and globulin levels. These parameters were analyzed using an automated system (Mindray BS-200).

4.2.7 Histopathological analysis

Fragments of tumor tissue were collected and fixed in ALFAC solution (85% ethanol, 10% formaldehyde, and 5% glacial acetic acid) at room temperature for 16 h. The samples were then dehydrated in ethanol, clarified in xylene, and embedded in paraffin. Tissues were sectioned at 5 μm thickness and stained with hematoxylin and eosin for analysis under an optical microscopy.

The following parameters were analyzed in tumor sections: coagulative necrosis, inflammatory infiltration, apoptosis, and histological characteristics. The following classification of necrosis was used: 0 (< 5% of tissue), I (5-25% of tissue), II (26-50% of tissue), III (51-75% of tissue), and IV (> 75% of tissue; Alves de Souza et al., 2017). According to Salgado et al., the classification of the percentage of stroma tumor-infiltrating lymphocytes (sTILs) in the slices of tumor tissue was the following: mild (0-10%), moderate (20-40%), and intense (50-90%).

4.2.8 Inflammatory parameters

4.2.8.1 Determination of nitrite levels and quantification of cytokines

Inflammatory parameters were measured in tumor tissue. The tumor samples were homogenized in PBS buffer (pH 7.2) and centrifuged at 9,000 × *g* at 4ºC. The supernatants were used to measure nitric oxide (NO) and cytokine levels. Nitrite levels were measured at 540 nm using Griess solution (0.1% N-1-naphthyl-tilediamine and 1% sulfanilamide in 5% H_3PO_4). Sodium nitrite was

used as the standard to calculate the amount of nitrite in the incubation medium (Green et al., 1982). The concentrations of cytokines (tumor necrosis factor-α [TNF-α] and interleukin-6 [IL-6]) were determined using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions.

4.2.8.2 Determination of myeloperoxidase and N-acetylglucosaminidase levels

The centrifuged tumor pellets were resuspended and homogenized in 1.0 mL of 0.1% Triton X-100 saline and centrifuged at 11,000 × *g* at 4°C for 10 min. The supernatants were used to measure myeloperoxidase (MPO) and N-acetylglucosaminidase (NAG) levels. For the measurement of MPO, 30 μL of the supernatant was added to 200 μL of peroxide solution. The reaction started with 18.4 μL of 3,3,5,5'-tetramethylbenzidine (TMB; 18.4 mM) in 8% aqueous dimethylformamide. The reaction was incubated for 3 min at 37°C and stopped with the addition of 30 μL of NaOAc. Absorbance was read at 620 nm (Bradley, Priebat, Christensen, & Rothstein, 1982).

The activity of NAG is based on the hydrolysis of p-nitrophenyl-*N*-acetyl-β-D-glucosamine (substrate) to *N*-acetyl-β-D-gluscosamine, which releases p-nitrophenol. The supernatant (100 μL) was first added to a 2.24 mM solution of NAG (substrate) in citrate buffer (39 mM, pH 4.5). The reaction was then incubated for 60 min at 37°C and interrupted with 100 μL of glycine buffer (200 mM, pH 10.4) in each well. Absorbance was read at 405 nm (Bailey, 1988).

4.2.9 Gene quantification by real-time quantitative polymerase chain reaction

In the tumor samples, the expression of target genes for apoptosis, necroptosis, angiogenesis, inflammation, and cell proliferation was evaluated.

RNA was isolated using TriZol reagent (Invitrogen), and 2 µg of this RNA was used to perform complementary DNA synthesis (cDNA) using the High Capacity cDNA reverse transcriptase kit (Applied Biosystems) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (RT-qPCR) was performed using SYBR Green PCR Master Mix according to the manufacturer's protocol with StepOne Plus equipment (Applied Biosystems). In all of the reactions, the samples were diluted 1:5, and the primer concentration was 800 nM in a volume of 25 µL. For the calibration curve, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and ribosomal protein large P0 (*Rplp0*) were used as housekeeper control genes. For amplification, the following specific primers were used: Bcl-2-associated X protein (*Bax*), B-cell lymphoma 2 (*Bcl-2*), *caspase 8*, *cyclin D1*, vascular endothelial growth factor (*Vegf*), hypoxia-inducible factor 1α (*Hif1a*), intercellular adhesion molecule 1 (*Icam1)*, nitric oxide synthase 2 (*Nos2*), nuclear factor erythroid derived 2 (*Nrf2*), phosphatidylinositol-4,5-bisphosphate 3-kinase (*Pik3*), receptor-interacting serine-threonine kinase 3 (*Ripk3*), receptor-interacting serine-threonine kinase 1 (*Ripk1*), and the nuclear factor-κB (NF-κB) subunits *Rela Tx2* and *IkBa*. The primer sequences are shown in Supplementary Table S1. The final data are expressed as relative expression using *Rplp0* as the control gene according to the ΔCt method (Livak & Schmittgen, 2001).

4.2.10 Statistical analysis

The statistical analysis was performed using GraphPad Prism 6.0 software. The data are expressed as mean ± standard error of the mean (SEM) and were analyzed using *t*-tests or one- or two-way analysis of variance (ANOVA)

followed by the Newman Keuls or Bonferroni *post hoc* test as appropriate. Values of $p < 0.05$ were considered statistically significant.

4.3 RESULTS

4.3.1 Characterization of oligosaccharide fraction

4.3.1.1 Oligosaccharide chromatography and characterization

The ethanolic supernatant was concentrated, yielding 117.78 g. This syrup was applied to the chromatographic column and eluted with the H_2O:EtOH gradient. At the end of this step, the yield of oligosaccharides was 2.55% of the initial mass.

4.3.1.2 Oligo NMR spectroscopy

The 1D ^1H NMR spectrum (Suppl. Fig. S1) showed diverse signals in the anomeric region, C2-C6 region, and C6 deoxy and acetyl region, demonstrating the diversity of oligosaccharides that were present in the mixture compared with the polysaccharides that were identified by Bezerra et al. (2018). Signals of phenolic compounds were not detected in the region of aromatic carbons (δ 7.00-8.00 ppm), showing that the phenolic compounds were removed after the partition step with organic solvents.

The oligosaccharide signals in the anomeric region (δ 90.0/4.20-115.0/5.80 ppm) of the 2D $^1H/^{13}C$ multiplicity-edited HSQC correlation map are shown in Fig. 2. The diversity of signals in the region of reducing terminals (93.8/4.56-99.60/5.46 ppm) corroborates the presence of oligosaccharides in the mixture. Signals of residual-free monosaccharides were attributed to D-Glc*p* (α: δ 94.8/5.23 and δ 94.5/5.44; β: δ 98.7/4.65 and δ 98.56/4.68), D-Gal*p* (α: δ

95.3/5.25; β: δ 99.2/4.57), D-Xyl*p* (α: δ 94.90/5.19; β: δ 99.07/4.58), and D-Gal*p*A (α: δ 94.85/5.32; β: δ 98.70/4.61; Delgobo, Gorin, Jones, & Iacomini, 1998; Delgobo, Gorin, Tischer, & Iacomini, 1999; Simas et al., 2004; Sassaki et al., 2014).

Sucrose was detected, reflected by the presence of signals at δ C1/H1: 94.9/5.41, C2/H2: 74.2/3.53, C3/H3: 74.9/3.75, C4/H4: 72.4/3.48, and C5/H5: 74.9/3.51. Trehalose was confirmed at δ C1/H1: 96.00/5.19, C2/H2: 73.8/3.66, C3/H3: 75.2/3.85, C4/H4: 72.4/3.46, and C5/H5: 74.9/3.81 (Wishart et al., 2018). The major components in the oligosaccharide mixture were identified by the examination of anomeric linkages, which were attributed to β-D-Gal*p* signals at δ 105.0/4.40-107.20/4.80 ppm and α-D-Man*p* signals at δ 102.6/4.95-105.1/5.25 ppm (Kobayashi et al., 1995; Delgobo, Gorin, Jones, & Iacomini, 1998; Vinogradov, Petersen, & Bock, 1998; Delgobo, Gorin, Tischer, & Iacomini, 1999; Nascimento et al., 2013; Shakhmatov, Atukmaev, & Makarova, 2016; Stipp et al., 2017; Bezerra et al., 2018; Cordeiro Caillot et al., 2018; John, Yang, Liu, Jiang, & Yang, 2018). The signals at δ 102.3/5.40 and δ 100.7/4.95 corresponded to →6)α-D-Glc*p*(1→4 and α-D-Glc*p*(1→6, respectively. These signals, together with reducing terminals of D-Glc*p*, suggest the presence of Panosyl oligosaccharides, and the remaining signals of α-D-glucose pyranose units at δ 101.3/4.93 and δ 100.9/4.92 suggest the presence of isomaltotriose (Taniguchi & Honnda, 2009; Dobruchowska et al., 2012; Dobruchowska et al., 2013). We also observed α-L-arabinofuranosyl oligosaccharide, reflected by the presence of signals at δ 109.1/4.98-112.3/5.28 ppm. Signals of α-L-Rha*p*, β-D-Xyl*p*, α-D-Gal*p*A, and β-D-Glc*p*A were observed at a lower intensity compared with previous reports (Fig. 2; Ray & Lahaye, 1995; Delgobo, Gorin, Jones, & Iacomini, 1998; Delgobo, Gorin,

Tischer, & Iacomini, 1999; Nascimento et al., 2013; Shakhmatov, Atukmaev, & Makarova, 2016; Stipp et al., 2017; Bezerra et al., 2018; Cordeiro Caillot et al., 2018; John, Yang, Liu, Jiang, & Yang, 2018). The C1/H1 signals at δ 104.6/4.49, C4/H4 signals at δ 85.20/3.24, and *O*-methyl CH_3 signals at δ 62.7/2.48 suggested the presence of 4-*O*-metyl-β-glucuronic acid (Shakhmatov, Atukmaev, & Makarova, 2016).

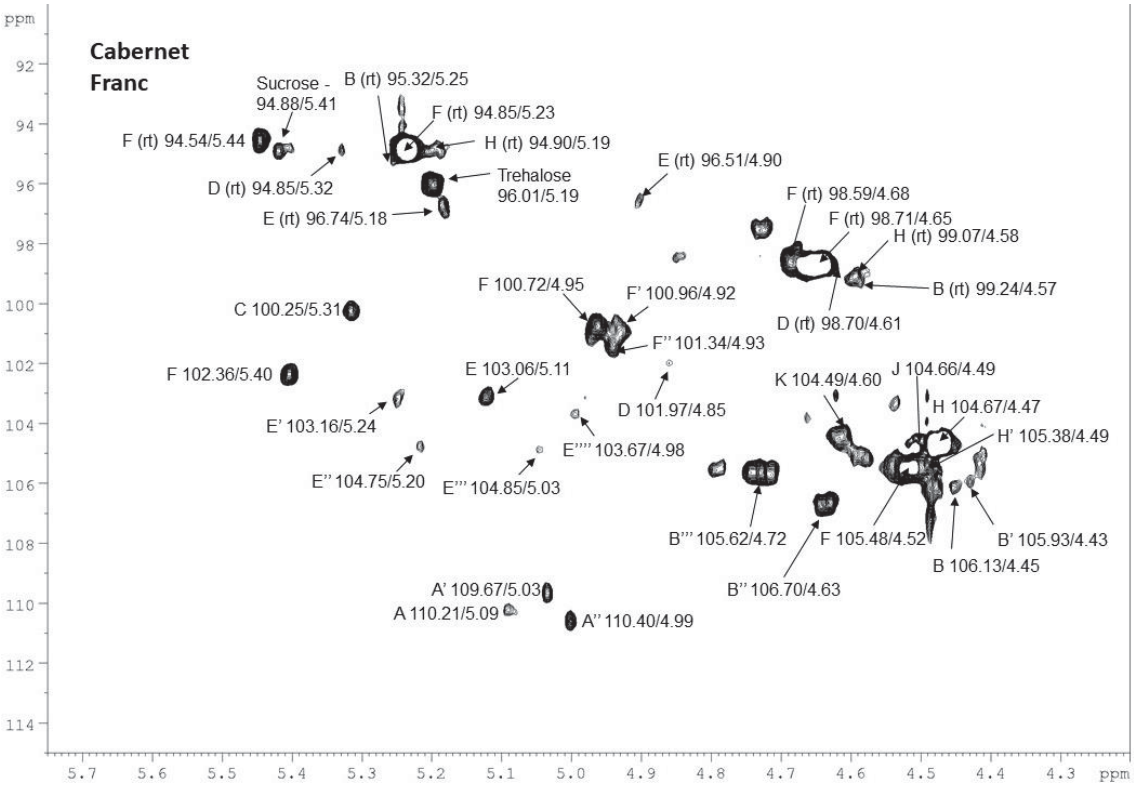


Figure 2. Cabernet Franc oligosaccharide signals in the anomeric region of the 2D $^{1}H/^{13}C$ multiplicity-edited HSQC correlation map. (**A**) L-Araf. (**B**) D-Galp. (**C**) L-Rhap. (**D**) D-GalpA. (**E**) D-Manp. (**F**) D-Glcp. (**H**) D-Xylp. (**J**) 4-*O*-Me-β-GlcpA. (**K**) D-GlcpA. rt, reducing terminal.

4.3.1.3 Mass spectrometry analyses of Oligo

The main peaks that were found in the mass spectra and their fragmentation by multiple reaction monitoring (MRM) suggested the presence of

structures that are shown in Table 1. The *m/z* values were obtained through adduction with lithium ions. The *m/z* values and their fragmentations were consistent with the presence of disaccharides, trisaccharides, and tetrasaccharides (Sassaki & Souza, 2013).

The 351.15 *m/z* was the main peak in the spectrum. This ion and the mass fragments are associated with hexose disaccharides (Suppl. Fig. S2). The 215.10 *m/z* resulted from fragmentation 2,5A1, with the loss of one water molecule. The 189.08 *m/z* originated through B1/Y1 fragmentation, in which 189.08 is the Y ion. We observed the presence of a B ion at a lower quantity at *m/z* 169.08 when it was adducted with one lithium ion. We could not determine, based only on the ESI-MS analyses, which of the hexoses formed the disaccharide (351.15 *m/z*) or tetrasaccharide (695.27 *m/z*) or where the glycosidic linkage occurred. The trisaccharide (513.19 *m/z*) was compatible with the presence of panose and isomaltotriose (Dobruchowska et al., 2012; Dobruchowska et al., 2013; Wishart et al., 2018). The presence of sucrose and trehalose was confirmed by the mass of 349.13 *m/z* (Wishart et al., 2018), thus corroborating the NMR signals at δ C1/H1: 94.8/5.40 and C1/H1: 96.01/5.19, respectively.

Table 1. NMR and MS data on the presence of Oligo.

m/z	Proposed structure from MS lithium adducts and NMR signals
305.13	*[Rha*p*-Xyl*p*•Li]$^+$ α-L-Rha*p* non-reducing end N1: (δ 100.2/5.31); α-D-Xyl*p* reducing end N1: (δ 94.9/5.19); β-D-Xyl*p* reducing end N1: (δ 99.1/4.58)
349.13	*[Suc•Li]$^+$ or [Tre•Li]$^+$ sucrose α-D-Glc*p* N1: (δ 94.8/5.41) or Trehalose α-D-Glc*p* N1: (δ 96.0/5.19)
351.14	[Hex_2•Li]$^+$
423.20	*[Ara*f*-Xylp_2•Li]$^+$ α-L-Ara*f* non-reducing end N1: (δ 110.4/4.99); β-D-Xyl*p* non-reducing end N1: (δ 104.7/4.47); α-D-Xyl*p* reducing end N1: (δ 94.9/5.19); β-D-Xyl*p* reducing end N1: (δ 99.1/4.58)
513.19	*[α-D-Glcp_3•Li]$^+$ panose α-D-Glc*p* non-reducing end N1: (δ 100.7/4.95); α-D-Glc*p* non-reducing end N1: (δ 102.36/5.40); α-D-Glc*p* reducing end N1: (δ 94.85/5.23); β-D-Glc*p* reducing end N1: (δ 98.7/4.65) or Isomaltotriose α-D-Glc*p* non-reducing end N1: (δ 101.3/4.93); α-D-Glc*p* non-reducing end N1: (δ 100.9/4.92); α-D-Glc*p* reducing end N1: (δ 94.8/5.23); β-D-Glc*p* reducing end N1: (δ 98.7/4.65).
695.27	[Gal*p*A-Hex_3•Li]$^+$

*Deduced from 2D NMR HSQC. The MS data on oligosaccharides were obtained after

$NaBH_4$ reduction and cation exchange to form lithium adducts.

4.3.2 Oligo is non-cytotoxic to mammary cells in vitro

Cell viability was evaluated in human breast tumor cell lineages using the Oligo at different concentrations (4-350 μg mL^{-1}). After 24 and 48 h of incubation, the cell viability of MCF7, MDA-MB-231, and MDA-MB-436 cells was unaffected by the Oligo treatment (Fig. 3), indicating that the Oligo was non-cytotoxic *per se*. In contrast, both concentrations of MTX significantly reduced the viability of all cell lineages.

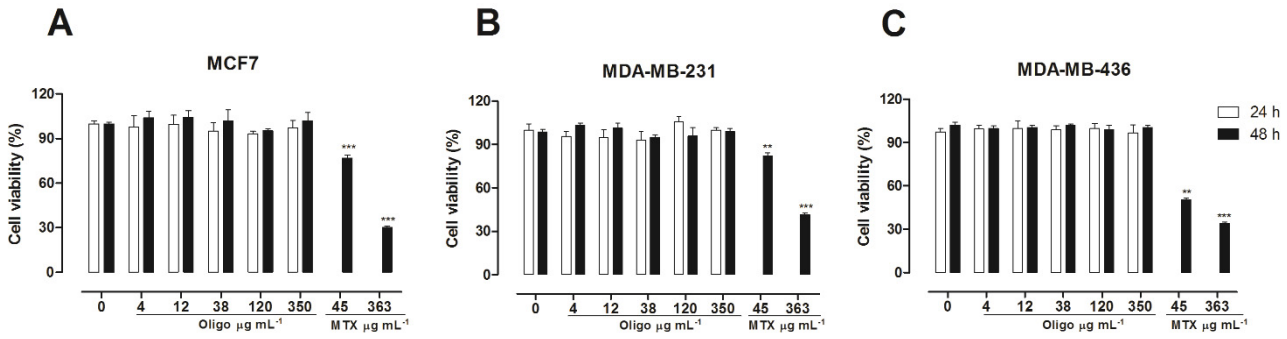


Figure 3. MTT assay in (A) MCF-7, (B) MDA-MB-231, and (C) MDA-MB-436 cell lineages. Oligo was diluted in supplemented culture medium and added at different concentrations (4, 12, 38, 120, and 350 μg mL^{-1}), with untreated wells (0) as the negative control and MTX as the positive control. The cells were incubated for 24 or 48 h. The results are expressed as mean ± SEM (n = 3). The data were analyzed using one-way ANOVA followed by the Newman Keuls *post hoc* test. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with negative control.

4.3.3. Effect of Oligo on Ehrlich ascitic tumors—protocol (i)

After the *in vitro* assay with mammary tumor cells, we investigated whether short *in vivo* treatment with Oligo (5 days) inhibits the development of the ascitic Ehrlich tumor model. The group of mice that were treated orally with 35 mg kg^{-1} Oligo but not intraperitoneally presented an increase in cell viability compared

with vehicle (Fig. 4A). The ascitic fluid volume (Fig. 4B) and body weight gain (Fig. 4C) did not differ significantly between groups.

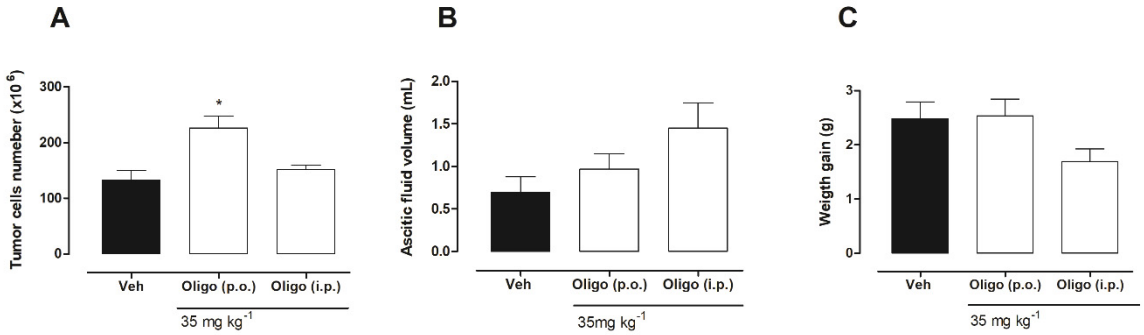


Figure 4. Effect of Oligo on (A) cell viability, (B) ascitic fluid volume, and (C) animal weight gain. The animals were orally treatment with vehicle (10 mL kg^{-1}) or Oligo (35 mg kg^{-1}) or intraperitonially with Oligo (35 mg kg^{-1}) for 5 days. The data are expressed as mean ± SEM (*n* = 5-6/group) and were analyzed using one-way ANOVA followed by the Newman Keuls *post hoc* test. **p* < 0.05, compared with vehicle group.

4.3.4 Effects of conventional Oligo treatment on Ehrlich solid tumors—protocol (ii)

4.3.4.1 Oligo treatment reduced Ehrlich tumor development

In this experiment, we tested the effect of Oligo on Ehrlich solid tumors using oral treatment. Oligo (70 mg kg^{-1}) inhibited tumor development only on the last day (-28.8%) of treatment, whereas 35 mg kg^{-1} Oligo inhibited tumor development from day 16 to day 21 (-52.4% and -53.5%, respectively) compared with the vehicle group. Treatment with MTX prevented tumor development from day 12 to day 21 (-80.6% and -64.5%, respectively; Fig. 5A, B). Oligo (35 mg kg^{-1}) decreased tumor volume by 39% in three dimensions (Fig. 5C) and decreased the tumor weight by 54.4% compared with the vehicle group (Fig. 5D) on the last day of the experiment. Based on these results, the next analyses of this protocol

used only the 35 mg kg^{-1} dose of Oligo, which was the most effective against solid tumor development.

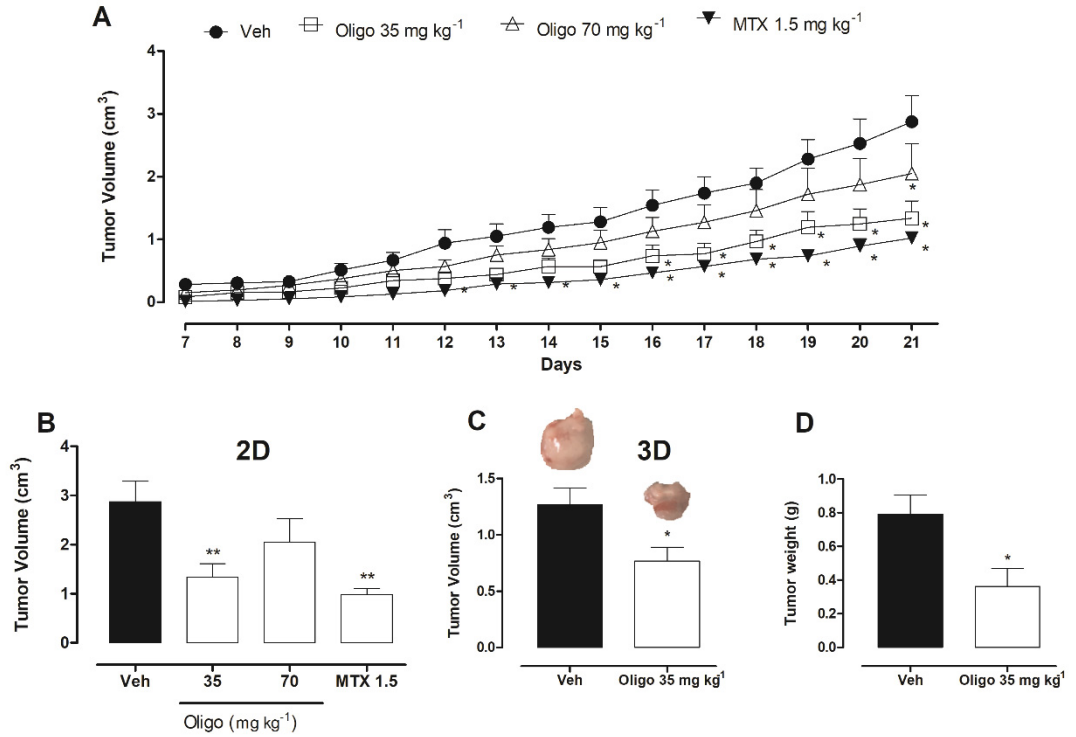


Figure 5. Oligo treatment reduced tumor development. The animals were treated with vehicle (10 mL kg^{-1}, p.o.), Oligo (35 or 70 mg kg^{-1}, p.o.), or MTX (1.5 mg kg^{-1}, i.p.) for 21 days. (**A**) Tumor development during 21 days of experiment. (**B**) Tumor volume in two dimensions (2D). (**C**) Tumor volume in three dimensions (3D). (**D**) Tumor weight on day 21. The results are expressed as mean ± SEM (*n* = 10-15/group) and were analyzed using two-way (A) or one-way (B) ANOVA followed by the Bonferroni or Newman Keuls *post hoc* test, respectively, and by *t*-tests (C, D). *p < 0.05, **p < 0.01, compared with vehicle group.

4.3.4.2 Oligo conventional treatment slightly modified hematological and biochemical parameters

The groups did not present significant differences in white blood cells, but red cells decreased in animals that received vehicle, Oligo, and MTX compared with the naive group. These alterations were related to presence of the tumor instead of the treatment. In plasma, the vehicle- and Oligo-treated groups

exhibited an increase in plasma levels of ALT, total protein, and albumin. The three tumor-bearing groups exhibited significant increases in AST levels compared with the naive group (Table 2).

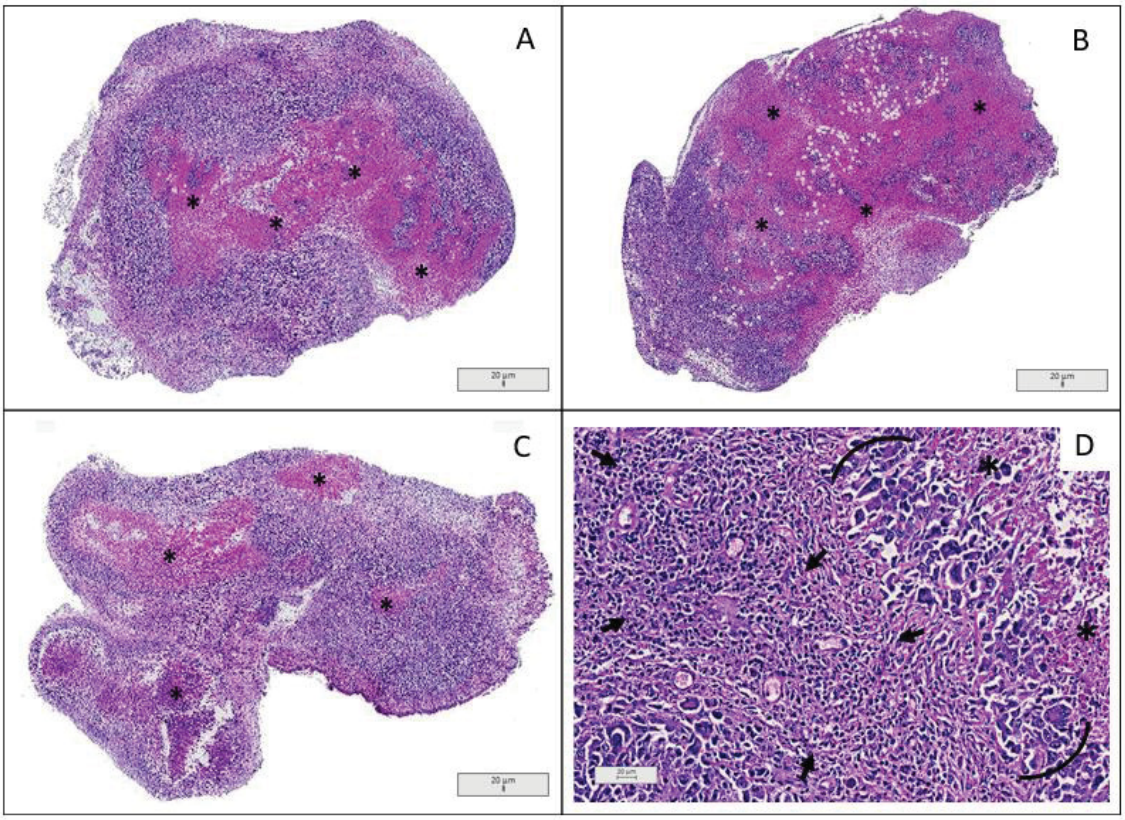
Table 2. Hematological and biochemical effects of Oligo conventional treatment on Ehrlich tumors.

	Experimental Group			
Parameter	**Naive**	**Vehicle**	**35 mg kg^{-1} Oligo**	**1.5 mg kg^{-1} MTX**
White blood cells ($\times 10^3$ µL^{-1})	4.77 ± 1.57	6.77 ± 2.22	7.03 ± 2.29	6.34 ± 2.43
Lymphocytes ($\times 10^3$ µL^{-1})	3.27 ± 1.27	4.27 ± 1.49	4.72 ± 1.45	4.27 ± 1.64
Monocytes ($\times 10^3$ µL^{-1})	0.24 ± 0.11	0.38 ± 0.24	0.30 ± 0.18	0.34 ± 0.15
Granulocytes ($\times 10^3$ µL^{-1})	1.25 ± 0.46	2.12 ± 0.89	2.17 ± 0.63	1.96 ± 0.38
Red blood cells (x 10^6 µL^{-1})	9.35 ± 0.51	7.27 ± 1.02**	7.75 ± 1.05*	6.68 ± 2.15**
Hemoglobin (g dL^{-1})	12.59 ± 0.64	10.00 ± 1.50*	10.90 ± 1.58	9.86 ± 3.18**
Hematocrit (%)	40.67 ± 2.40	30.94 ± 4.78**	32.51± 4.65*	28.76 ± 9.62**
Glucose (mg dL^{-1})	91.47 ± 44.21	121.6 ± 36.29	111.3 ± 35.93	124.7 ± 49.56
Alanine aminotransferase (U L^{-1})	30.31 ± 4.34	70.62 ± 28.14***	65.53 ± 17.59**	37.93 ± 6.07
Aspartate aminotransferase (U L^{-1})	75.73 ± 5.31	296.5 ± 69.73***	295.4 ± 54.72***	228.3 ± 33.83***
Creatinine (mg dL^{-1})	0.25 ± 0.11	0.36 ± 0.15	0.20 ± 0.16	0.42 ± 0.09
Total protein (g dL^{-1})	4.57 ± 0.17	6.25 ± 0.96***	6.72 ± 0.62***	5.22 ± 0.41
Albumin (g dL^{-1})	1.65 ± 0.15	2.92 ± 0.90**	3.91 ± 0.36***	2.18 ± 0.65
Globulin (mg dL^{-1})	2.91 ± 0.15	3.33 ± 0.34	3.00 ± 0.41	3.04 ± 0.29

Animals without tumors (naive) or with tumors were treated for 21 days with vehicle (10 mL kg^{-1}, p.o.), Oligo (35 mg kg^{-1}, p.o.), or MTX (1.5 mg kg^{-1}, i.p.). The data are expressed as mean ± SEM (n = 7-10/group) and were analyzed using one-way ANOVA followed by the Newman Keuls *post hoc* test. *p < 0.05, **p < 0.01, ***p < 0.001, compared with naive group.

4.3.4.3 Oligo treatment increased inflammatory cell infiltration in tumor tissue

The tumors in the vehicle and 35 mg kg^{-1} Oligo groups presented moderate necrosis (grade II), whereas tumors in the MTX group presented more coagulative necrosis (grade IV). Furthermore, in the 35 mg kg^{-1} Oligo group, sTILs were more expressive (50-90% of tumor area) compared with the other groups (0-10%; Fig. 6).



	Vehicle	Oligo 35 mg kg^{-1}	MTX 1.5 mg kg^{-1}
Necrosis	II	II	IV
sTILs	Mild	Intense	Mild

Figure 6. Histological features on Ehrlich tumor tissue in mice after treatment with vehicle or 35 mg kg^{-1} Oligo. The animals were orally treated with vehicle (10 mL kg^{-1}) or 35 mg kg^{-1} Oligo or intraperitoneally with 1.5 mg kg^{-1} MTX for 21 days. (**A**) Vehicle group. (**B**) MTX group. (**C, D**) Oligo group. Parentheses, black arrows, and asterisks indicate viable Ehrlich cells, sTILs, and coagulative necrosis area, respectively. The samples were observed under an optical microscope at 20× magnification (scale bar = 20 μm). Scores of necrosis: 0 (< 5% of tissue), I (5-25% of tissue), II (26-50% of tissue), III (51-75% of tissue), and IV (> 75% of tissue). sTILs, stroma tumor-infiltrating lymphocytes. Classification of sTILs: mild (0-10%), moderate (20-40%), and intense (50-90%).

4.3.4.4 Oligo treatment did not modify inflammatory parameters in tumor tissue

The Oligo treatment induced the intense migration of sTILs to tumor tissue. Inflammatory parameters were then analyzed in tumor tissue. The treatment did not induce significant variations in tumor inflammatory parameters compared with the vehicle group (Fig. 7).

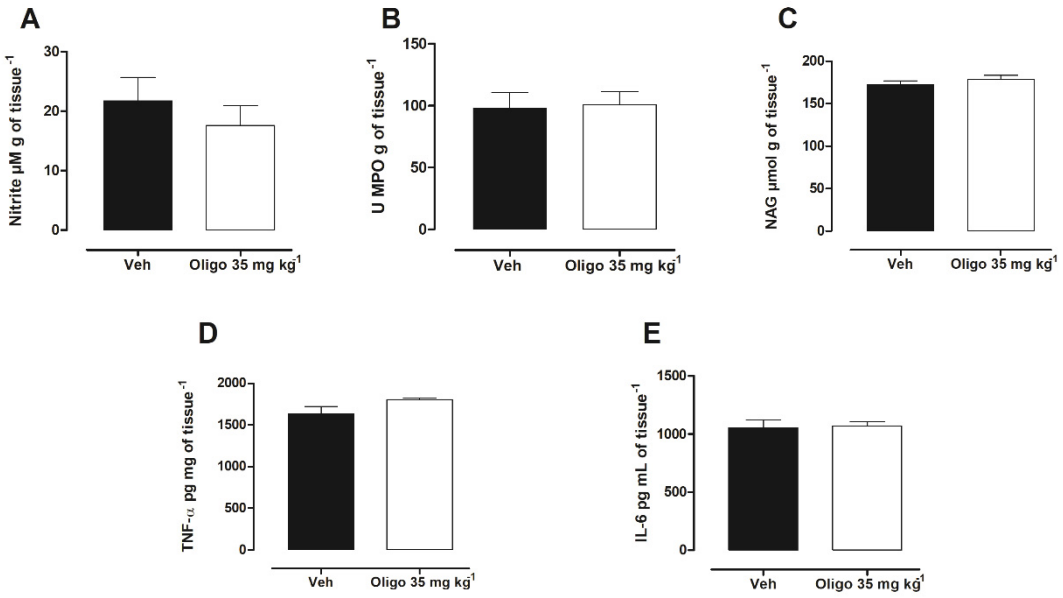


Figure 7. Effects of Oligo treatment on inflammatory parameters in tumor tissue. (**A**) Tumor nitrite oxide (NO) levels. (**B**) Tumor myeloperoxidase (MPO) activity. (**C**) Tumor *N*-acetylglucosaminidase (NAG) activity. (**D**) Tumor necrosis factor-α (TNF-α). (**E**) Interleukin 6 (IL-6) levels. The animals were orally treated with vehicle (10 mL kg^{-1}) or Oligo (35 mg kg^{-1}) for 21 days. The data are expressed as mean ± SEM and were analyzed using *t*-tests. (A-C) n = 12/group. (D, E) n = 6/group. *$p < 0.05$, compared with vehicle group.

4.3.4.5 Oligo treatment altered gene expression of *Hif1a* in tumor tissue

Treatment with Oligo increased the expression of *Hif1a* (by 500%) in tumor tissue compared with the vehicle group (Fig. 8A). The other genes that were analyzed (i.e., genes related to apoptosis, necroptosis, and the cell cycle) were unaltered by Oligo. Representative *IkBa*, *Rela Tx2*, and *Icam1* expression is shown in Fig. 8.

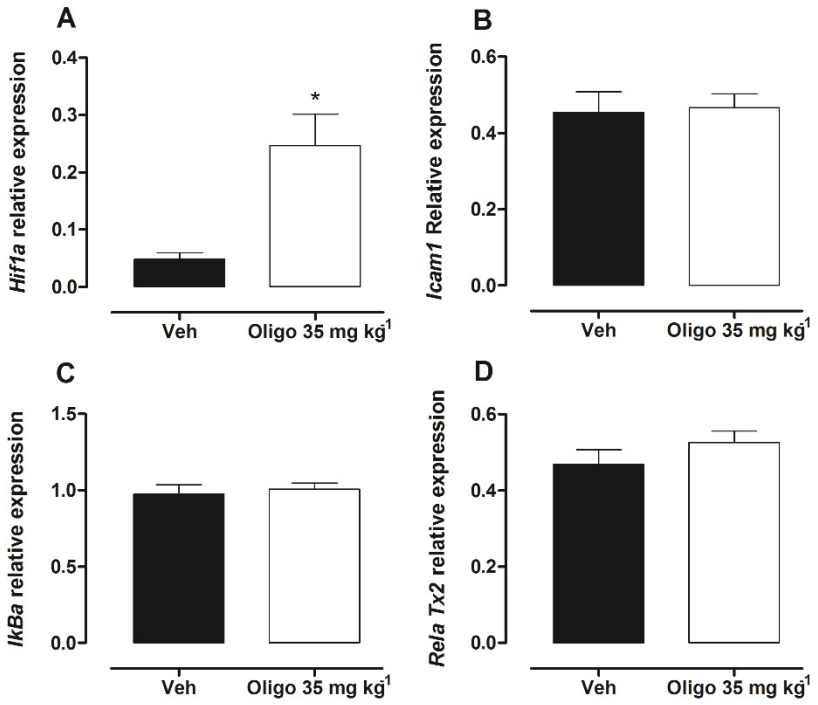


Figure 8. Gene expression of (A) *Hif1a*, (*B*) *Icam1*, (*C*) *IkBa*, and (D) *Rela Tx2* in tumor tissue from mice that were orally treated with vehicle (Veh) or Oligo (35 mg kg^{-1}) for 21 days. The data are expressed as mean ± SEM (*n* = 3-5/group) and represent expression relative to the *Rplp0* reference gene. The data were compared using *t*-tests. **p* < 0.05, compared with vehicle group.

4.3.5 Effects of Oligo chemopreventive treatment on Ehrlich solid tumors—protocol (iii)

4.3.5.1 Oligo chemopreventive protocol reduced tumor development

Considering that the conventional treatment reduced Ehrlich solid tumor growth, we investigated the chemopreventive potential of Oligo, tested at a lower dose (9 mg kg^{-1}) with a longer treatment period (42 days). Treatment with vehicle only after tumor cell inoculation failed to prevent tumor development, whereas 9 and 35 mg kg^{-1} Oligo significantly inhibited tumor development beginning on day 14 (62.4%) and day 13 (76.4%), respectively, until the last day of treatment (69.5% and 77.9%, respectively) compared with the vehicle group (Fig. 9A, B).

The group that received 9 mg kg^{-1} Oligo before tumor inoculation and did not receive treatment after tumor inoculation (i.e., 9 mg kg^{-1} Oligo before tumor inoculation + 0 mg kg^{-1} after tumor inoculation) exhibited a reduction of tumor volume compared with the vehicle group but only on the last day (Fig. 9A). Tumor weight decreased in all of the Oligo groups compared with the vehicle group, but this decrease did not reach statistical significance (Fig. 9C).

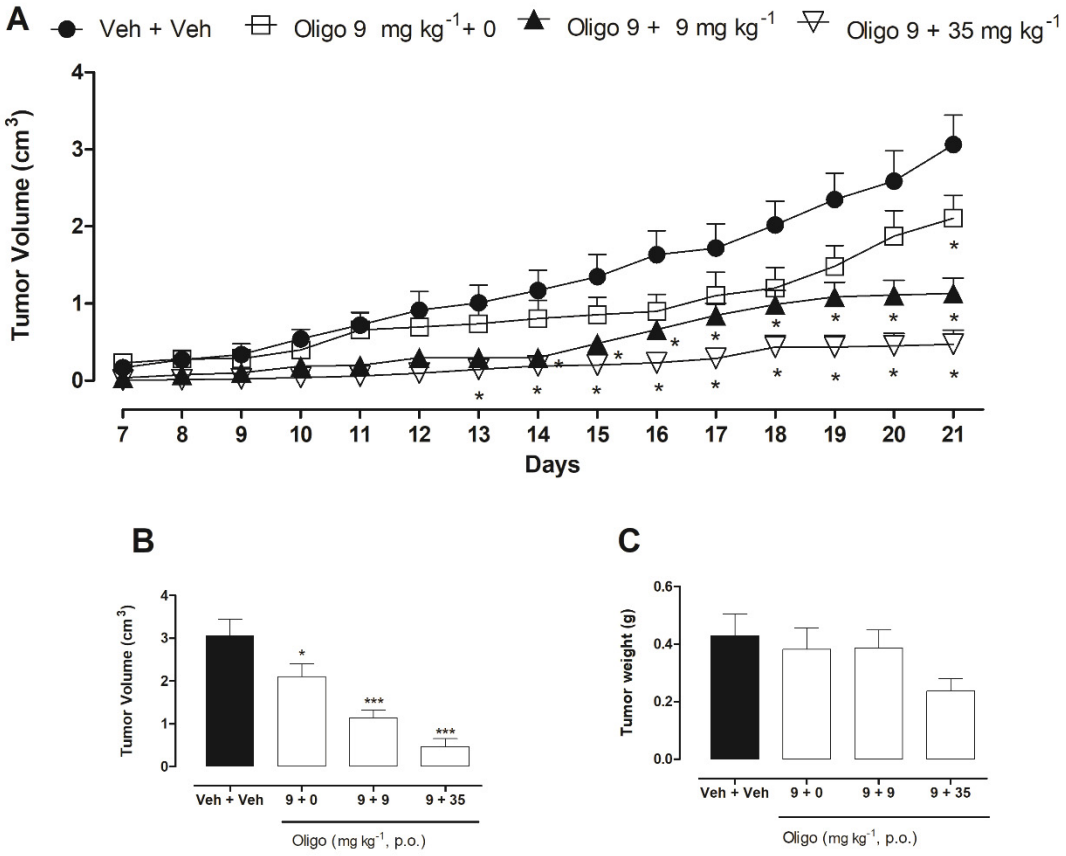


Figure 9. Effect of Oligo chemopreventive protocol on Ehrlich solid tumor (A, B) volume and (C) weight. The animals were orally treated with vehicle (10 mL kg^{-1}) or Oligo (9 mg kg^{-1}) for 21 days before tumor inoculation, and this treatment continued for 21 days after tumor inoculation with vehicle (10 mL kg^{-1}) or Oligo (9 and 35 mg kg^{-1}). One group was not treated. The results are expressed as mean ± SEM (n = 7-10/group) and were analyzed using (A) two-way or (B, C) one-way ANOVA followed by the Bonferroni or Newman Keuls *post hoc* test, respectively. *p < 0.05, **p < 0.01, ***p < 0.001, compared with vehicle group.

4.3.5.2 Oligo chemopreventive treatment altered hematological and biochemical parameters

The animals that received long treatment with Oligo (9 + 9 mg kg^{-1} and 9 + 35 mg kg^{-1}) exhibited a reduction of the total white blood cell count, with a more expressive reduction of lymphocytes and monocytes, compared with the vehicle group, indicating the modulation of immune cells by long Oligo treatment. Other hematological parameters, such as granulocytes, red blood cells, hemoglobin, and hematocrit, were unaltered by the longer treatment with Oligo (Table 3). The most pronounced plasma results of long treatment with Oligo was the increase in globulin levels, whereas the other parameters were not significantly different (Table 3).

Table 3. Hematological and biochemical parameters in Ehrlich tumor-bearing mice treated with the chemopreventive Oligo treatment protocol.

	Experimental Group			
Parameter	**Veh + Veh**	**9 + 0 mg kg^{-1}**	**9 + 9 mg kg^{-1}**	**9 + 35 mg kg^{-1}**
White blood cells ($\times 10^3$ μL^{-1})	7.01 ± 2.38	6.05 ± 1.56	4.25 ± 1.12***	3.37 ± 0.89***
Lymphocytes ($\times 10^3$ μL^{-1})	4.98 ± 1.10	3.40 ± 1.88*	2.68 ± 0.98**	2.15 ± 0.60**
Monocytes ($\times 10^3$ μL^{-1})	0.32 ± 0.17	0.26 ± 0.08	0.18 ± 0.09	0.12 ± 0.04*
Granulocytes ($\times 10^3$ μL^{-1})	1.40 ± 0.90	1.48 ± 0.50	1.18 ± 0.53	1.08 ± 0.29
Red blood cells (x 10^6 μL^{-1})	7.78 ± 0.61	7.03 ± 1.76	8.57 ± 0.60	8.91 ± 0.41
Hemoglobin (g dL^{-1})	10.78 ± 1.02	9.64 ± 2.92	11.30 ± 0.80	11.60 ± 0.52
Hematocrit (%)	32.28 ± 3.05	29.60 ± 7.85	37.36 ± 1.83	38.24 ± 2.31
Glucose (mg dL^{-1})	127.10 ± 63.76	73.56 ± 33.15	171.40 ± 80.88	152.60 ± 51.93
Alanine aminotransferase (U L^{-1})	47.96 ± 4.50	46.84 ± 9.79	69.41 ± 21.46	72.57 ± 32.82
Aspartate aminotransferase (U L^{-1})	265.30 ± 41.69	269.10 ± 74.0	264.90 ± 65.23	251.60 ± 73.49
Creatinine (mg dL^{-1})	0.34 ± 0.089	0.34 ± 0.11	0.32 ± 0.07	0.40 ± 0.22
Total protein (g dL^{-1})	4.52 ± 0.54	5.50 ± 0.62*	5.04 ± 0.48	4.62 ± 0.56
Albumin (g dL^{-1})	2.74 ± 0.49	2.58 ± 0.78	2.80 ± 0.37	2.77 ± 0.44
Globulin (mg dL^{-1})	1.78 ± 0.27	2.92 ± 0.31***	2.24 ± 0.22*	1.85 ± 0.29

The animals were chemopreventively treated with vehicle (10 mL kg^{-1}) or Oligo (9 mg kg^{-1}) for 21 days before tumor inoculation. After tumor inoculation, the animals were orally treated for a further 21 days with vehicle (10 mL kg^{-1}) or Oligo (9 and 35 mg kg^{-1}). One group was not treated. The data are expressed as mean ± SEM (n = 5-7/group) and were analyzed using one-away ANOVA followed by the Newman Keuls *post hoc* test. *p < 0.05, **p < 0.01, ***p < 0.001, compared with vehicle group.

4.3.6 Effects of conventional and chemopreventive treatment with Oligo combined with MTX

4.3.6.1 Treatment with Oligo combined with MTX further reduced tumor development

Considering that Oligo treatment reduced Ehrlich tumor development in both protocols (i.e., conventional and chemopreventive), we investigated whether its combination with MTX would further reduce tumor development. The combination of 35 mg kg^{-1} Oligo and 1.5 mg kg^{-1} MTX in the conventional treatment protocol significantly reduced tumor development from day 13 until day 21 compared with vehicle (Fig. 10A). The tumor volume decreased by ~50% when Oligo was combined with MTX compared with MTX treatment alone (Fig. 10B).

We also tested the combination of Oligo and MTX in the chemopreventive treatment protocol, which replicated the above results. The group that was treated with 9 + 35 mg kg^{-1} Oligo combined with 1.5 mg kg^{-1} MTX exhibited greater inhibition of tumor development compared with the group that received 9 + 9 mg kg^{-1} Oligo combined with 1.5 mg kg^{-1} MTX and compared with the vehicle group (Fig. 10C). The tumor volume decreased by ~50% when Oligo was administered before tumor cell inoculation and then administered with MTX compared with MTX alone (Fig. 10D).

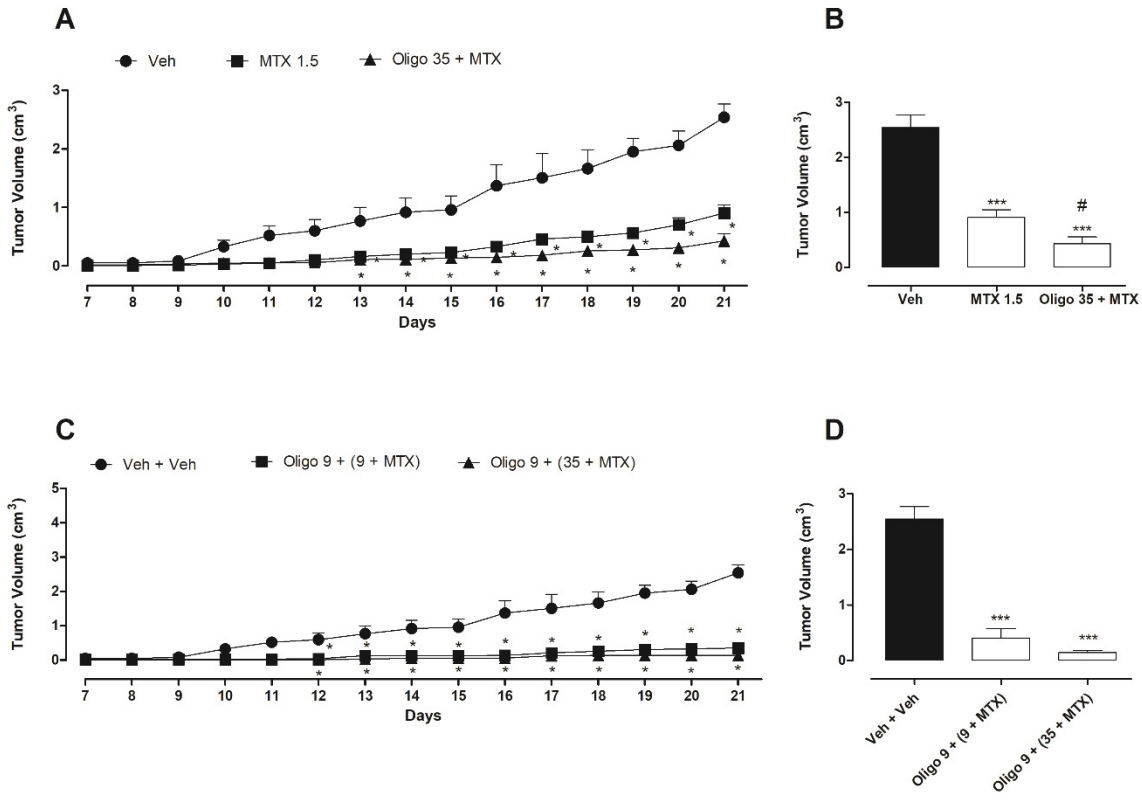


Figure 10. Effect of conventional and chemopreventive treatment protocols of Oligo combined with MTX on the development of tumor volume. (**A, B**) The animals were treated with vehicle (10 mL kg^{-1}), MTX (1.5 mg kg^{-1}), or a combination of Oligo (35 mg kg^{-1}) and MTX (1.5 mg kg^{-1}). The treatment continued orally for 21 days, once daily, in the vehicle and Oligo groups, and MTX was administered intraperitoneally every 4 days. (**C, D**) The animals were treated with vehicle (10 mL kg^{-1}) or Oligo (9 mg kg^{-1}) for 21 days before tumor inoculation. After tumor inoculation, the animals were treated for 21 days with vehicle (10 mL kg^{-1}), a combination of Oligo (9 mg kg^{-1}) and MTX (1.5 mg kg^{-1}), or a combination of Oligo (35 mg kg^{-1}) and MTX (1.5 mg kg^{-1}). The treatment was administered orally, once daily, in the vehicle and Oligo groups, and MTX was administered intraperitoneally every 4 days. The data are expressed as mean ± SEM (n = 5-7/group) and were analyzed using two-way (A, C) or one-way (B, D) ANOVA followed by the Bonferroni or Newman Keuls *post hoc* test, respectively, or by *t*-tests (B). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with vehicle group; $^{\#}p < 0.05$, compared with MTX group.

4.3.6.2 Oligo combined with MTX altered hematological and biochemical parameters

The two groups that were treated for a longer time with Oligo combined with MTX exhibited significant decreases in total leukocyte counts, lymphocytes, and monocyte counts compared with the vehicle group (Table 4). These results are consistent with those that are shown in Table 3.

Table 4. Hematological parameters in Ehrlich tumor-bearing mice treated with Oligo combined with MTX.

	Experimental Group		
Parameter	**Veh + Veh**	**9 + 9 Oligo + MTX**	**9 + 35 Oligo + MTX**
White blood cells ($\times 10^3$ μL^{-1})	6.70 ± 2.09	3.40 ± 1.82**	2.84 ± 1.08**
Lymphocytes ($\times 10^3$ μL^{-1})	4.98 ± 1.10	2.64 ± 1.21***	1.98 ± 0.56***
Monocytes ($\times 10^3$ μL^{-1})	0.32 ± 0.17	0.14 ± 0.05*	0.08 ± 0.08**
Granulocytes ($\times 10^3$ μL^{-1})	1.40 ± 0.90	1.04 ± 0.42	0.77 ± 0.46
Red blood cells (x 10^6 μL^{-1})	7.78 ± 0.61	8.22 ± 1.03	8.45 ± 0.53
Hemoglobin (g dL^{-1})	10.78 ± 1.02	10.91 ± 1.51	10.73 ± 0.55
Hematocrit (%)	32.28 ± 3.05	35.71 ± 4.71	36.40 ± 1.81

The animals were treated with the chemopreventive protocol with vehicle (10 mL kg^{-1}) or Oligo (9 mg kg^{-1}) for 21 days before tumor inoculation. After tumor inoculation, the animals were treated for a further 21 days with vehicle (10 mL kg^{-1}), a combination of Oligo (9 mg kg^{-1}) and MTX (1.5 mg kg^{-1}), or a combination of Oligo (35 mg kg^{-1}) and MTX (1.5 mg kg^{-1}). The treatment was administered orally, once daily, in the vehicle and Oligo groups, and MTX was administered intraperitoneally every 4 days. The data are expressed as mean ± SEM (n = 5-7/group) and were analyzed using one-way ANOVA followed by the Newman Keuls *post hoc* test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with vehicle group.

4.4. DISCUSSION

The present results showed the *in vivo* antineoplastic effects of Oligo, which contained diverse oligosaccharides and high amounts of xylose, rhamnose, and trehalose. Consistent with the literature, the carbohydrates that are present in wine are mainly composed of monosaccharides, sugar alcohols, sugar acids, and disaccharides. Some of these are natural constituents, whereas others are formed during various stages of the wine-making process through the

degradation of polysaccharide structures that originate from the grape berry cell wall (Bordiga et al., 2012). L-rhamnose is a constituent that consists of many glycosides and polysaccharides. D-xylose is only fermentable by certain microorganisms, such as *Lactobacilli*, *Torula*, and *Monilia*. Trehalose has been reported to be the main disaccharide in wine, which is formed by the metabolic activity of yeast, whereas sucrose is present at low levels (Ruiz-Matute, Sanz, Moreno-Arribas, & Martínez-Castro, 2009), which is consistent with the present results.

The antineoplastic effects of Oligo that were observed in the present study corroborate previous studies that reported the effectiveness of other oligosaccharides as antitumor and chemopreventive agents against ovarian, colonic, and prostate cancer cells and astroglioma (Salah et al., 2013; Nam, Kim, & Shon, 2007; Mattaveewong et al., 2016). Chitosan oligosaccharides have been shown to exert chemopreventive effects against HT-29 human colon cancer cells *in vitro* (Nam, Kim, & Shon, 2007). Li et al. (2013), showed that apple oligosaccharides concentration- and time-dependently decreased the cellular viability of HT-29 cells. According to Huang et al. (2018), the combined use of oligogalacturonides with cisplatin exerted a synergistic inhibitory effect on A549 human lung carcinoma cell growth. However, in this study it was not possible to observe the *in vitro* antineoplastic effect of Oligo against breast cancer cell lines in 24 and 48 h (Fig. 3). Instead, we observed an *in vivo* antitumor effect of Oligo (Fig. 5, 9). Our group previously reported the antitumor effect of polysaccharides that were extracted from Cabernet Franc red wine in Walker-256 tumor-bearing rats (Stipp et al., 2017). Thus, both studies by our group found antineoplastic effects of red wine compounds beyond resveratrol, which is considered the main

compound with health benefits in this beverage (Xu et al., 2015; Carter, D'Orazio, & Pearson, 2014).

The effect of Oligo clearly depended on the treatment time. The short treatment (5 days) did not reduce the viability of ascitic Ehrlich cells, whereas the conventional (21-day) and chemopreventive (42-day) treatment protocols reduced the development of Ehrlich solid tumors, with the highest inhibition with the longest treatment time. Chemoprevention was performed with a lower dose of Oligo (9 mg kg^{-1}) compared with the dose that was applied in the conventional treatment (35 mg kg^{-1}) because the chemopreventive protocol was two-times longer than the conventional therapy. In both protocols, the antineoplastic mechanism of action of Oligo appeared to involve modulation of the inflammatory response that is mediated by immune cells. The phenomenon of sTILs occurred in both protocols of Oligo treatment, thus reinforcing the immunomodulatory mechanism of Oligo, which has already been reported for polysaccharides. For example, polysaccharides from mushrooms activate the innate immune system and exert antitumor activity by accelerating the defense mechanisms of the host by T cells and other immune cells (Meng, Liang, & Luo, 2016). Several studies have shown that the host immune response is essential for cancer growth, progression, and metastasis, and the intensity of the tumoral immune response influences the effectiveness of cancer therapy. sTILs indicate an antitumor immune response and are related to a better prognosis in many solid tumor types (Salgado et al., 2014; Zitvogel, Kepp, & Kroemer, 2011; Ibrahim, Al-Foheidi, Al-Mansour, & Kazkaz, 2014). Rakaee et al. (2018), reported that an increase in sTILs levels is an independent positive prognosis that is associated with a significantly lower risk of progression and lower overall mortality in non-small-cell

lung cancer patients. Other studies also showed that the greater presence of total sTILs suggests a better breast cancer prognosis (Zitvogel, Kepp, & Kroemer, 2011; Yu et al., 2016; Stanton & Disis, 2016). Therefore, the intense sTILs that was observed in tumor tissue in animals that were treated with Oligo may at least partially explain the antitumor effect of Oligo. Interestingly, the modulation of immune cells was also observed in blood in Oligo-treated mice. A longer treatment time was associated more pronounced immune cell regulation. After 42 days of oral Oligo administration (chemopreventive protocol), a significant reduction of white blood cells, lymphocytes, and monocytes was observed (Table 3, 4).

The regulatory effect of Oligo on inflammatory cells may partially explain the absence of Oligo's effect on the viability of cultured breast cancer lineages (Fig. 3) because those cells were not present in the culture medium. Another possibility that may explain the minimum *in vitro* effect of Oligo is the culture treatment time (24 and 48 h), which was shorter than the effective treatment time *in vivo* (21 and 42 days). Our *in vitro* results are similar to Bezerra et al. (2018), who tested polysaccharides from red and white wines in RAW 264.7 macrophages. Their results showed that cell viability was not significantly influenced by polysaccharide fractions at concentrations of 0.1-100 μg mL^{-1} cultured for 24 h. These concentrations are within the range of Oligo concentrations that were applied herein (4-350 μg mL^{-1}).

To investigate the influence of Oligo on other parameters that are related to inflammation in tumor tissue, we measured MPO and NAG enzymes, indications of the presence of neutrophils and macrophages, respectively. Treatment with 35 mg kg^{-1} Oligo did not alter the activity of either enzyme in either

the conventional and chemopreventive treatment protocol, with no effect of the Oligo + MTX combination (Fig. 7; Suppl. Fig. S3, S4). These results confirm that the cells that infiltrated tumor tissue were predominantly lymphocytes, notably sTILs. Additionally, the gene expression of *IkBa* and *Rela Tx2*, both subunits of NF-κB, was evaluated to determine the involvement of this pathway in Oligo's effects. NF-κB regulates the proliferation and differentiation of T lymphocytes and the expression of several genes that are involved in inflammation, cell proliferation, cell survival, and innate immunity (Yamamoto & Gaynor, 2001; Courtois & Gilmore, 2006). Oligo treatment did not alter the expression of NF-κB subunits. This may explain the absence of effects on IL-6 and TNF-α levels in tumor tissue in Oligo-treated mice because these cytokines are regulated by NF-κB signaling.

Hif1a gene expression was induced by Oligo. The activation of HIF-1 transcription factor is the most recognized pathway that is activated by cells in a hypoxic microenvironment (Masoud & Li, 2015). Activated HIF-1 plays a crucial role in adaptive responses of tumor cells to changes in oxygen through the transcriptional activation of over 100 downstream genes that regulate vital biological processes (Masoud & Li, 2015). However, the increase in *Hif1a* expression that was induced by Oligo was not accompanied by an increase in the expression of *Vegf*, which suggests that Oligo did not interfere with the angiogenesis pathway that depends on *Hif1a*. Cell survival, metabolism, and proliferation can also depend on *Hif1a* (Masoud & Li, 2015), and the induction of *Hif1a* regulating the glutathione system was already reported in mammary cell lineages treated with the chemotherapeutic agents paclitaxel and carboplatin (Lu et al., 2015; Lu et al., 2017).

Considering the antineoplastic effect of Oligo, we investigated whether Oligo augments the effects of traditional drugs for cancer treatment, such as MTX. Of all of the tested protocols (Fig. 1), the best inhibitory effect on tumor development was observed with the combination of Oligo 9 (mg kg^{-1}) and MTX (35 mg kg^{-1}), in which the low dose of Oligo (9 mg kg^{-1}) was administered orally for 21 days before tumor cell inoculation, followed by 35 mg kg^{-1} Oligo (p.o.) together with MTX (i.p.) for the next 21 days (Fig. 10). MTX is known to have side effects in mice and humans, such as effects on the gastrointestinal system (e.g., nausea, vomiting, stomatitis, ulcers, and diarrhea), bone marrow (e.g., leukopenia, anemia, and thrombocytopenia), liver (e.g., increases in transaminases, hepatitis, fibrosis, and cirrhosis), lungs (e.g., pneumonitis and fibrosis), pregnancy (e.g., abortion and teratogenic effects), and several other side effects (e.g., alopecia, anaphylaxis, oligospermia, photosensitivity, and reactivation sunburn; Wood & Wu, 2015). MTX is used in numerous protocols to treat breast, skin, head, and neck cancer. It interferes with the growth of body cells that reproduce quickly, such as cancer cells, bone marrow cells, and skin cells. However, the clinical application of this drug is limited by its dose-related toxic effects. Its pharmacokinetic properties, mainly its short half-life, and rapid diffusion throughout the body result in essentially uniform tissue distribution (de Fátima Pereira, Mara da Costa, Cristina Magalhães Santos, Carmo Horta Pinto, & Rodrigues Da Silva, 2014). Therefore, Oligo may be a promising drug for use in cancer therapy in combination with MTX. We suggest that a lower dose of MTX can be used with Oligo to increase its effectiveness and decrease its side effects. This hypothesis needs to be tested because Oligo has potential to be an adjuvant of chemotherapy in solid tumors, mainly mammary tumors. Additionally, Oligo

treatment did not produce adverse effects, in which the elevations of ALT, AST, total protein, and albumin levels were also observed in the vehicle group (Table 2). Thus, these alterations were related to the presence of the tumor rather than Oligo treatment.

In conclusion, the present data showed that Oligo has antitumor effects against Ehrlich solid tumors in mice. These effects were evident in several treatment protocols, including treatment that began after tumor cell inoculation and chemopreventive treatment that began before tumor inoculation. In both treatment regimens at the tested doses, Oligo induced intense inflammatory cell infiltration in the tumor. Oligo also increased the effectiveness of MTX in controlling tumor growth. Thus, Oligo may be a promising adjuvant therapy for solid tumors.

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Conflict of Interest

The authors declare no conflict of interest.

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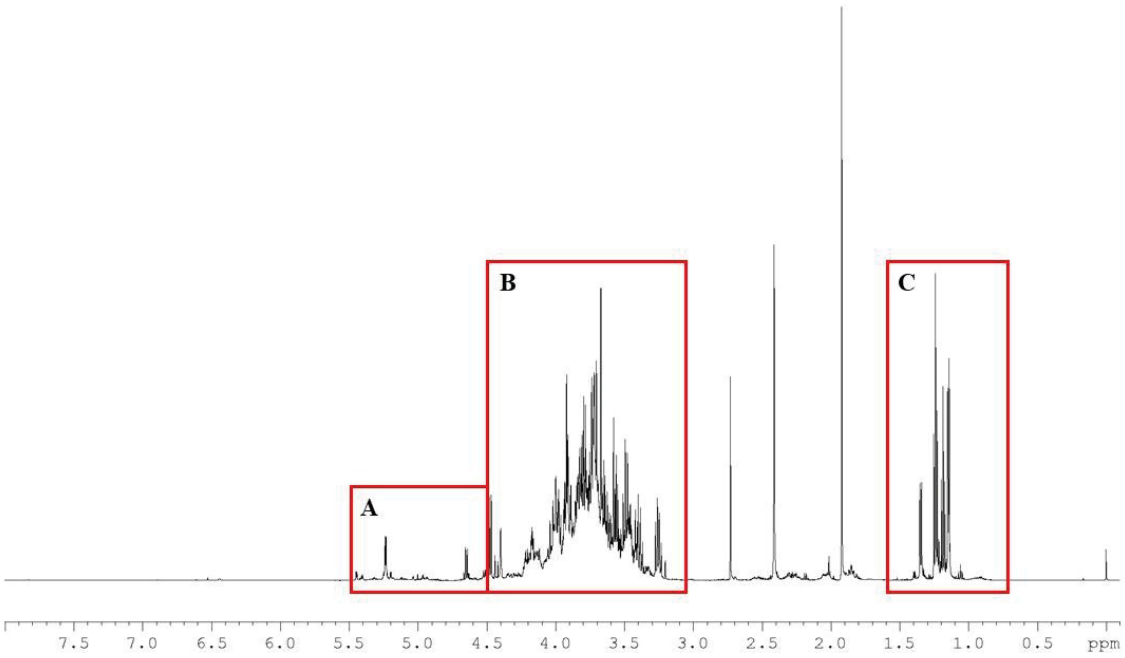
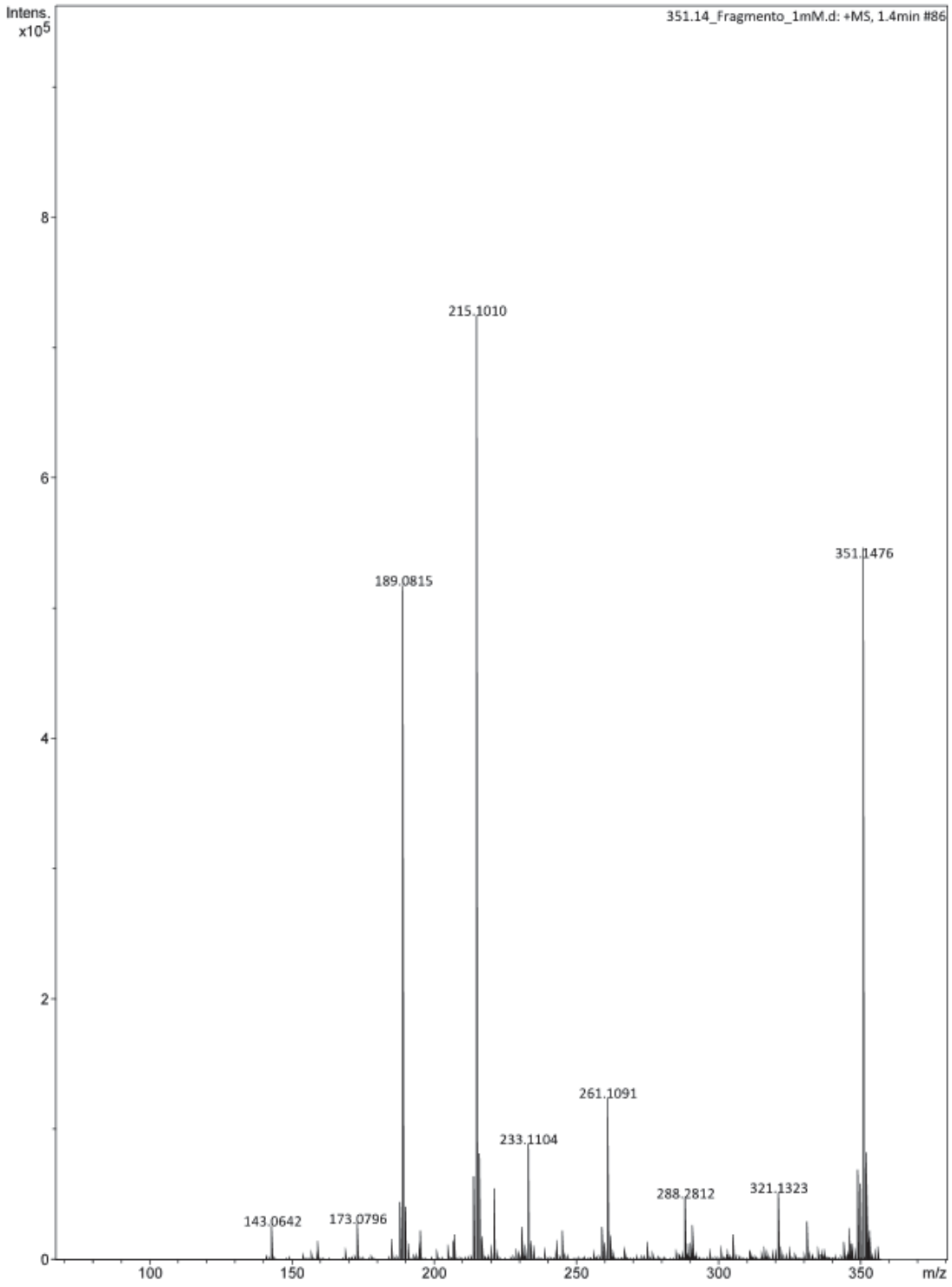
4.6 SUPPLEMENTARY MATERIAL

Supplementary Table 1. Sequence of primers used for genes expression of mice tumor tissue in real-time quantitative PCR.

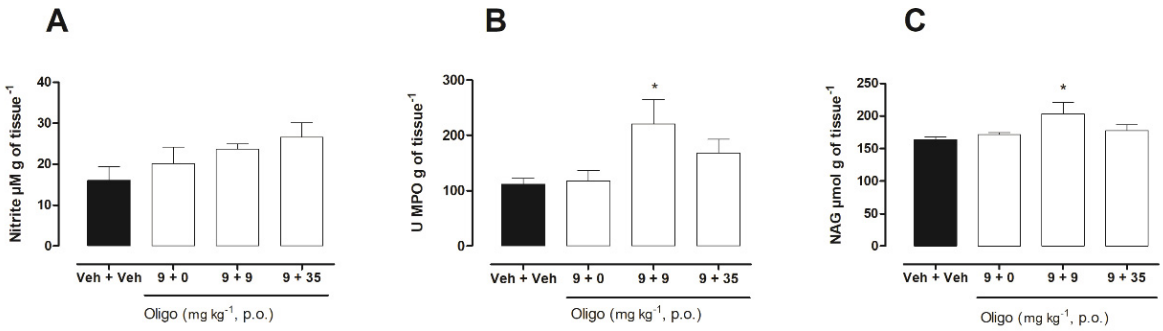
Target	Forward primer (5'→ 3')	Reverse primer (5'→ 3')
Bax	GCCTCCTCTCCTACT TC	CCTCAGCCCATCTTCTT
Bcl-2	CACTTGCCACTGTAGAGA	GCTTCACTGCCTCCTT
Caspase-8	CCAGGAAAAGATTTGTGTCTA	GGCCTTCCTGAGTACTGTCACCTG
Cyclin D1	AGAAGTGCGAAGAGGAG	GGATAGAGTTGTCAGTGTAGAT
Vegf	ACTGGACCCTGGCTTTACTGCT	TGATCCGCATGATCTGCATGGTG
Gapdh	GGTGAAGCAGGCATCT	TGTTGAAGTCGCAGGAG
Rplp0	CGACCTGGAAGTCCAACTAC	ACTTGCTGCATCTGCTTG
Icam-1	CAATTTCTCATGCCGCACAG	AGCTGGAAGATCGAAAGTCCG
Nos2	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGGTTCG
Nrf2	GTGGATCCGCCAGCTACTCCCA	TGGGGATATCCAGGGCAAGCGA
Pik3	CCTGCTCCGTAGTGGTA	TTCATCGCCTCTGTTGTG
Ripk3	CCAGAGGCCACTTGTGTAGCG	CGCTTTAGAAGCCTTCAGGTTGAC
Ripk1	TCATCTAGCGGGAGGTTGGA	ATGCCCAGTAGCTTCACCAC
Rela_Tx2	ACCTGGAGCAAGCCATTAGC	GAGGCGCACTGCATTC
IkBa	GCT ACC CGA GAGCGAGGAT	GCCTCCAAACACACAGTCATCAT
Hif1a	ACCTTCATCGGAAACTCCAAAG	CTGTTAGGCTGGGAAAGTTAGG



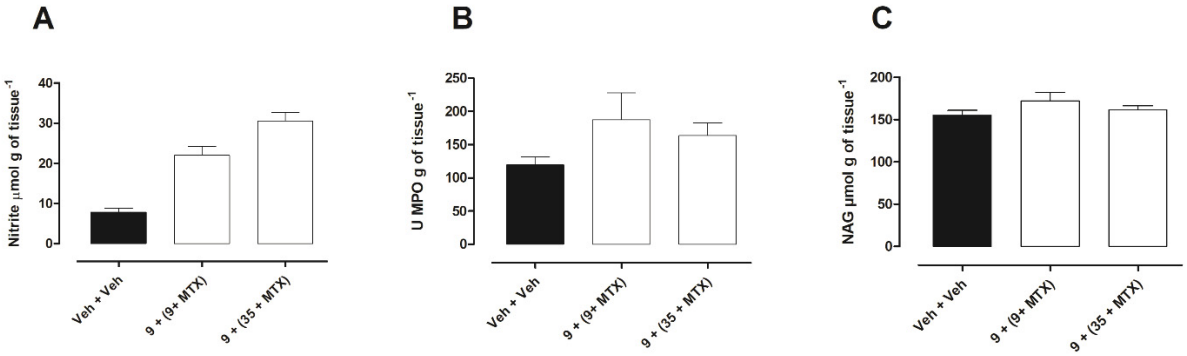
Supplementary figure 1: Cabernet Franc oligosaccharides 1D ^{1}H spectrum NMR. A: Anomeric region; B: C2-C6 region; C: C6 deoxy and acetyl region.



Supplementary figure 2: Mass spectrum of cation exchange Li adducts from reduced oligosaccharides. Product ions formed in low-energy CID MS/MS spectrum of 351.15 m/z ion, MRM fragments are consistent with [hex2•Li]+. The m/z at 215.10 [M -2,5A1 -H2O•Li]+ and m/z at 189.08 [M-B1•Li]+ and m/z at 169.08 [M-Y0•Li]+.



Supplementary Figure 3: Effect of Oligo chemoprevention treatment on inflammatory parameters in tumor tissue. A: Nitrite oxide (NO); **B:** Myeloperoxidase (MPO); **C:** N-acetylglucosaminidase (NAG)**.** Animals were chemopreventively treated with Vehicle (10 mL kg^{-1}) or Oligo (9 mg kg^{-1}) by gavage for 21 days before inoculation of tumor. After tumor cells inoculation the mice were orally treated for a further 21 days with Vehicle (10 mL kg^{-1}) or Oligo (9 and 35 mg kg^{-1}), and one group was not treated (0). The results are expressed as mean ± S.E.M. (n = 6-10/group) and analyzed by one-way ANOVA followed by Newman Keuls test, respectively. ***** $p < 0.05$ when compared to Vehicle group.



Supplementary Figure 4: Effect of chemoprevention treatment of Oligo associated with MTX on inflammatory parameters in tumor tissue. A: Nitrite oxide (NO); **B:** Myeloperoxidase (MPO); **C:** N-acetylglucosaminidase (NAG)**.** Animals were treated with Vehicle (10 mL kg^{-1}) or Oligo (9 mg kg^{-1}) for 21 days before tumor inoculation and continued for 21 days with vehicle (10 mL kg^{-1}) or association of Oligo (9 mg $kg^{-1}$and 35 mg kg^{-1}) with MTX (1.5 mg kg^{-1}) after tumor inoculation. The treatment was orally, once a day, for the groups Vehicle and Oligo, and intraperitoneally every 4 days for MTX. Results are expressed as mean ± S.E.M. (n = 5-7/group) and analyzed by one-way ANOVA followed by Newman Keuls test.

5 CONSIDERAÇÕES FINAIS

No presente trabalho os estudos de NMR e espectrometria de massa identificaram uma complexa composição dos oligossacarídeos extraídos do vinho tinto Cabernet Franc, com grande quantidade de hexose, xilose, ramnose e trealose. A avaliação da atividade biológica dos Oligos evidenciou seu efeito antitumoral *in vivo*, no modelo de carcinoma de Ehrlich. Claramente este efeito antitumoral foi dependente do tempo de tratamento. O tratamento curto (5 dias) não reduziu a viabilidade das células ascíticas de Ehrlich, enquanto os tratamentos convencional (21 dias) e quimiopreventivo (42 dias) reduziram o desenvolvimento do tumor sólido de Ehrlich, atingindo a maior inibição com o protocolo mais longo de tratamento. Nesses dois tratamentos, nas doses testadas, os Oligos induziram uma intensa infiltração de células inflamatórias no tumor. Além disso, os Oligos aumentaram a eficácia do MTX no controle do crescimento do tumor. Interessantemente, a dose diária oral de 35 mg kg^{-1} foi a mais eficaz em comparação com a dose mais elevada (70 mg kg^{-1}).

Assim, os Oligos parecem ser promissores como terapia adjuvante no tratamento de tumores sólidos, especialmente de tumores mamários. Outros estudos em diferentes modelos e linhagens celulares, abordando diferente posologia e parâmetros farmacocinéticos, farmacodinâmicos e toxicológicos, devem ser encorajados.

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