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DEPARTAMENTO DE BIOQUÍMICA PROFESSOR TUISKON DICK
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

FRANCIANE BRACKMANN MENDES

α-BISABOLOL E ÓXIDO DE BISABOLOL A: ATIVIDADE ANTITUMORAL EM LINHAGENS CELULARES DE CÂNCERES DO SISTEMA NERVOSO CENTRAL

Porto Alegre, março de 2014

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Dissertação de Mestrado apresentada como requisito parcial para a obtenção do título de Mestre.

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Porto Alegre, março de 2014

“-Há milhões e milhões de anos que as flores produzem espinhos. Há milhões e milhões de anos que apesar disso, os carneiros as comem. E não será importante procurar saber por que elas perdem tanto tempo produzindo espinhos inúteis? Não terá importância a guerra dos carneiros e das flores?”

O pequeno príncipe

Antonie de Saint-Exupéry

Dedicatória

À minha mãe, Rosicler Luzia Brackmann, pelo exemplo,

honestidade e amor incondicional.

Agradecimentos

A Universidade Federal do Rio Grande do Sul e ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica pela oportunidade de fazer o mestrado.

Ao CNPq pela bolsa concedida e apoio financeiro.

As duas professoras que me iniciaram na pesquisa de fato e que abriram suas portas para mim quando eu estava apenas no início da faculdade: Profa. Dra. Vera Lúcia Eifler-Lima e Janice Carneiro Coelho

A Profa Ana Battastini não apenas pela orientação, contribuições e ideias, mas também, e principalmente, por ser um exemplo incrível de postura, conduta, ética e profissionalismo. Pelos puxões de orelha e pela cobrança que fazem com que aprendamos não apenas como alunos de pós-graduação, mas como profissional e como pessoa.

As minhas amigas Ana, Anelise, Fernanda, Kamila, Karine, Laura e Raissa por serem tão pacientes, amorosas e dedicadas, por sempre me manterem conectada ao mundo real, por serem minha sanidade, por todas as nossas discussões políticas e filosóficas, mas principalmente pelas intermináveis horas de risadas.

As minhas fiéis escudeiras durante a faculdade toda, Aline, Débora, Giovana, Jamila, Paula e Valeska, que são as pessoas que mais me entendem no mundo e que escutaram pacientemente minhas inseguranças, reclamações e teorias durante esses 7 anos de convivência.

Aos meus amados colegas de laboratório Angélica, Fabrício, Fabrícia, Letícia, Liliana, Carolina, Elisa, Fabiana, Mariana por fazerem o laboratório 22 um lugar prazeroso de se trabalhar, ambiente fantástico de se conviver e pela grande amizade que cresceu entre nós. Agradeço também aos ex colegas Cristiane e Elizandra pelos ensinamentos passados e à Manoella pelo companheirismo e risadas sem fim.

Ao Matheus, meu amor e companheiro, que foi sempre muito paciente e me apoiou frente à minha escolha e durante todas as crises existenciais desde então. Agradeço também a sua família que sempre me recebeu e tratou muito bem.

A minha família como um todo: avós, tios, primos por fazerem de conta que entendiam que o mestrado foi também meu trabalho por dois anos. Em especial, a minha Dinda Téia, que é minha segunda mãe, minha base, meu exemplo de força e coragem.

A minha mãe Rosicler. Ela que é sempre a primeira a me criticar, a me mostrar que estou errada, mas que ao mesmo tempo é capaz de enfrentar um exército inteiro pra proteger minha felicidade. Obrigada por ser essa mulher fantástica, por me mostrar o quanto o estudo e a qualificação são importantes, por me ensinar que não devo desistir nunca, por todos os conselhos, pelo apoio e

incentivo incondicionais, por ser meu exemplo, por ser essa supermãe, por toda dedicação e por me amar tanto, com tanta força.

Resumo

Diversas neoplasias podem atingir o sistema nervoso central e dentre elas, os tumores cerebrais são os mais prevalentes. Dentre todos os tumores cerebrais, dois merecem especial atenção: gliomas e meduloblastomas, por serem os mais recorrentes em adultos e crianças, respectivamente. O alfa-bisabolol é um pequeno álcool sesquiterpeno oleoso, que apresenta diversas atividades biológicas dentre elas, citotoxicidade. Apesar dos diversos estudos com essa molécula, pouco se sabe das atividades biológicas de seu análogo natural, o óxido de bisabolol A. Além disso, o sistema purinérgico tem sido relacionado com progressão e desenvolvimento tumoral. Assim, o objetivo deste trabalho foi avaliar os efeitos de dois promissores novos agentes quimioterápicos (alfa-bisabolol e óxido de bisabolol A) e correlacionar esses efeitos com possíveis modulações do sistema purinérgico, que é considerado um novo alvo terapêutico em linhagens de glioma e meduloblastoma. Nós observamos que a atividade da ecto-5'-nucleotidase, importante enzima do sistema purinérgico, é aumentada tanto em linhagens de glioma quanto de meduloblastoma quando estas são tratadas com alfa-bisabolol. Vimos também que esse aumento na atividade deu-se por um efeito direto do tratamento sob a enzima, com uma redução da expressão do mRNA da ecto-5'-nucleotidase em linhagem de glioma e sem alteração no imunoconteúdo dessa enzima em linhagens de meduloblastoma. Em linhagens de glioma, adicionalmente, a atividade citotóxica do alfa-bisabolol foi correlacionada com estímulo ao receptor de adenosina A_3. Ainda, as linhagens estudadas foram sensíveis aos tratamentos com alfa-bisabolol e óxido de bisabolol A. Em conclusão, os dados obtidos nesse trabalho demonstram que tanto o alfa-bisabolol quanto o óxido de bisabolol A são interesseantes novas possibilidades terapêuticas para os tumores cerebrais e que a atividade citotóxica do primeiro composto envolve modulação do sistema purinérgico. Mais estudos são necessários para entender a efetividade desses tratamentos in vivo e se o tratamento com o óxido de bisabolol A também modula o sistema purinérgico.

Palavras chave: Glioma, Meduloblastoma, alfa-bisabolol, óxido de bisabolol A, sistema purinérgico, ecto-5'-nucleotidase

Abstract

Among all the cancers that can affect the central nervous system, the brais tumors are the most prevalent. Two of this tumors deserve special attention: glioma and medulloblastoma once they are the most prevalent in adults and children respectively. Alpha-bisabolol is a small oily sesquiterphene alcohol that presents diverse biological activities, among them, citotoxicity. In despite of the diverse studies using this molecule, little is known about the biological activities of its natural analogue bisabolol oxide A. Besides, the purinergic system has been related to tumor development and progression. Therefore, the objective of this work was to evaluate the effect of two new promising chemotherapic agents (alpha-bisabolol and bisabolol oxide A) and correlate this effects with possible modulations of the purinergic system which is considered an interesting therapeutical target to glioma and medulloblastomas. We observed that the activity of ecto-5'-nucleotidase, an important enzyme of the purinergic system, is increased in both glioma and medulloblastoma cell lines when the cells are treated with alpha-bisabolol. It was also observed that this increase on activity was due to and direct effect of this treatment in the enzyme. Also, the treatment lead to a reduction on mRNA level of ecto-5'-nucleotidase on glioma cell line and no alterations on the imunocontent of the enzyme was observed on medulloblastoma cell lines. Additionally, the citotoxic activity of alpha-bisabolol on glioma cell line was correlated with modulation on A_3 adenosine receptor. Moreover, the cell lines used in this work were sensible to the treatments with alpha-bisabolol and bisabolol oxide A. In a conclusion, the data obtained in this work demonstrate that alpha-bisabolol and bisabolol oxide A are both interesting new therapeutical possibilities to brain tumors and the citotoxic activity of the first compound is related with modulations on the purinergic system. More studies are needed to better understand the effectiveness of these treatments in vivo and if the compound bisabolol oxide A also promotes a modulation on the purinergic system.

Key-words: Glioma, Medulloblastoma, apha-bisabolol, bisabolol oxide A, purinergic system, ecto-5'-nucleotidase.

Lista de abreviaturas

ADP- adenosina difosfato

AMP- adenosina monofosfato

ATP- adenosina trifosfato

CBTRUS – do inglês Central Brain Tumor Registry of the United States

DMSO - Dimetilsulfóxido

ecto-5'-NT/CD73 - ecto-5'-nucleotidase

ENTPDases - ectonucleosídeo trifosfato-difosfohidrolases

ENPPs - ectonucleotídeo pirofosfatase/fosfodiesterases

GPI - glicosil-fosfatidilinositol

GBM - glioblastoma multiforme

LCA- do inglês "large and anaplasic cells"

OMS - Organização Mundial da Saúde

SHH- sonic hedgehog

SNC - Sistema Nervoso Central

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Sumário

1. Introdução

1.1 Tumores Cerebrais

Diversas neoplasias podem atingir o sistema nervoso central e dentre elas, os tumores cerebrais são os mais prevalentes (Fisher et al. 2007). A incidência desses tumores vem crescendo e apenas nos Estados Unidos, estima-se que cerca de 69.720 novos casos de tumores cerebrais primários tenham sido diagnosticados em 2013 (Bohan 2013, Fisher et al. 2007).

Em adultos, os tumores cerebrais primários são bastante raros e constituem apenas 2% dos casos totais de cânceres diagnosticados. Em crianças, entretanto, eles são os tumores sólidos mais prevalentes, ficando atrás apenas das leucemias (Huttner 2012). Além da variação de incidência de acordo com a faixa etária, outros fatores como gênero, etnia e geografia influenciam a prevalência dos tumores cerebrais. Considerando o gênero, a taxa de incidência em homens vem crescendo mais do que no sexo oposto, sendo a taxa anual de incidência corrigida por idade cerca de 3,7 casos a cada 100.000 homens e 2,6 a cada 100.000 mulheres . Quanto à variação geográfica, no Japão são registrados menos da metade dos casos de tumores cerebrais que em países do nordeste da Europa. A incidência em países em desenvolvimento também é bastante inferior do que em países desenvolvidos, fato que é atribuído a um melhor acesso ao diagnóstico e existência de exames mais precisos (Bondy et al. 2008, Fisher et al. 2007).

A taxa de sobrevivência varia de acordo com a malignidade do tumor e diferem minimamente entre os diferentes sexos e etnias, sendo a idade o principal fator que influencia o prognóstico. A taxa de sobrevida após cinco anos em crianças chega a 65%, enquanto que em adultos na faixa etária de 55 a 64 anos não ultrapassa os 11% (Fisher et al. 2007, Gurney and Kadan-Lottick 2001).

Considerando todos os casos, apenas 37,7% dos pacientes sobrevivem por mais de dois anos após o diagnóstico, porcentagem que cai para 30% após cinco anos de diagnóstico (Fisher et al. 2007). Em um estudo retrospectivo dos anos 1980 a 1998 realizado no Brasil, foi observado que os óbitos por câncer cerebral cresceram 59% nesse período, com um crescimento médio de 2,8% ao ano (Monteiro and Koifman 2003).

Pouco se sabe sobre os fatores de risco para essas neoplasias. Diversos estudos epidemiológicos de causa-efeito foram realizados nos últimos anos, mas os achados são pouco consistentes. Em geral, esses estudos são conduzidos com um n amostral bastante reduzido em função das altas taxas de mortalidade da doença e da raridade desses tumores (Bondy et al. 2008, Gurney and Kadan-Lottick 2001). Além disso, há bastante discrepância quanto ao tipo tumoral e aos métodos de classificação usados nesses estudos (Bondy et al. 2008).

Os tumores cerebrais se mostram como um grupo bastante heterogêneo, possuindo grande variação quanto ao de sítio de origem, as características morfológicas, as alterações genéticas, ao potencial de crescimento, à extensão de invasão, à tendência à progressão e recorrência bem como a variada resposta ao tratamento (Gurney and Kadan-Lottick 2001). Quanto à origem, esses tumores podem ser subdivididos em tumores primários, que se originam diretamente no cérebro e secundários, provenientes de metástases de tumores primários em órgãos que não o cérebro. Os tumores primários podem ser ainda classificados como malignos ou benignos, sendo os primeiros fortemente associados à perda de qualidade de vida e a altas taxas de óbito (Lemke 2004) e segundo a Organização Mundial da Saúde (OMS), os tumores primários são classificados em uma escala que varia de I a IV de acordo o tipo celular, alterações nas taxas mitóticas e

presença de necrose e pleomorfismo. Quanto maior o grau, maior a agressividade e a malignidade do tumor (Lemke 2004).

Dentre todos os tumores cerebrais, dois merecem especial atenção: gliomas e meduloblastomas. Estes tumores são os mais recorrentes em adultos e crianças, respectivamente. (Swartling et al. 2013).

1.1.1 Gliomas

Gliomas são cerca de 36% dos tumores primários que afetam o Sistema Nervoso Central (SNC) (Adamson et al. 2011) e mais de 80% dos tumores malignos que afetam esse sistema (Schwartzbaum et al. 2006). A incidência anual atualmente é estimada em 5,26 casos a cada 100 000 habitantes ou 17 000 novos casos por ano apenas nos Estados Unidos. É uma doença mais comum entre a sexta e oitava década de vida e a estimativa é que o número de casos aumente com o aumento da expectativa de vida populacional (Omuro and DeAngelis 2013).

Os gliomas apresentam uma grande variação clínica dependendo do grau de malignidade (Sathornsumetee et al. 2007). A Organização Mundial da Saúde classifica os gliomas em quatro grupos (I a IV, onde o último é o mais maligno) definidos por graus crescentes de indifenciação celular, anaplasia e agressividade (Chintala et al. 1999, Kleihues et al. 1993, Omuro and DeAngelis 2013). Os tumores de grau I são os astrocitomas pilocíticos, que acometem principalmente crianças com idade entre 5 a 15 anos, apresentando baixa capacidade invasiva e sendo facilmente removidos por ressecção cirúrgica. Astrocitomas de baixo grau são classificados como grau II e ainda são considerados menos malignos por crescerem de forma relativamente lenta, podendo ou não invadir o tecido cerebral normal

adjacente. Os tumores de grau III e IV são considerados altamente malignos e em geral levam a óbito dentro de 15 meses. Os tumores de grau III são os astrocitomas anaplásicos, que possuem alta taxa de recorrência, crescimento e invasividade ao tecido normal. Por fim, o tumor de grau IV denominado glioblastoma multiforme (GBM), é o mais agressivo e infelizmente, o mais comum (Henriksson et al. 2011, Sathornsumetee et al. 2007). Os pacientes que desenvolvem GBM apresentam o pior prognóstico entre todos os grupos, com uma baixa sobrevida. A análise histopatológica desses tumores evidencia focos de necrose, neovascularização, núcleos mitóticos e grande infiltrado inflamatório (Dai and Holland 2001, Konopka and Bonni 2003, Wen P. Y. and Kesari 2008). Os glioblastomas por sua vez, podem ser divididos em primários, que surgem como um tumor agressivo e altamente invasivo, sem nenhum histórico prévio de tumores e que se desenvolvem rapidamente. E secundários, que são progressões de astrocitomas de baixo grau. Esses se desenvolvem lentamente e acometem pacientes mais jovens(Ohgaki and Kleihues 2009).

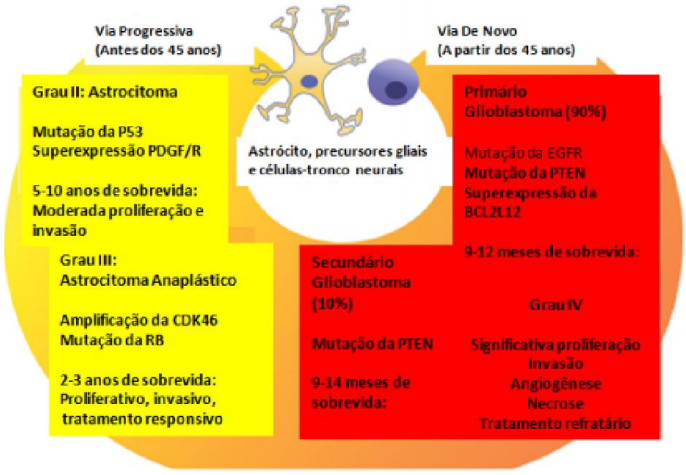

Figura 1: Esquema comparativo entre grau de malignidade tumoral, sobrevida e alterações moleculares que levam à formação de glioblastomas primários ou secundários. Adaptado de (Nieto-Sampedro et al. 2011).

Além da maior prevalência em idosos, é identificada também uma maior ocorrência em homens e em pessoas brancas (Fisher et al. 2007). Dentre os fatores de risco relacionados a estes tumores estão a exposição à radiação ionizante, mutações gênicas e histórico familiar (Schwartzbaum et al. 2006). Os sintomas relacionados com a doença são em geral dores de cabeça (matinal principalmente), náuseas, vômitos, confusão mental, mudança de personalidade, perda de memória, convulsões, incontinência urinária, diminuição da visão periférica e alteração de personalidade (Wen e Kesari, 2008, (Omuro and DeAngelis 2013).

Atualmente, o tratamento pode consistir em cirurgia, visando a ressecção tumoral, seguida de radioterapia e quimioterapia. Apesar das três opções de tratamento, a sobrevida dos pacientes permanece curta, entre 12 e 15 meses (Henriksson et al. 2011, Sathornsumetee et al. 2007), pois dentre os tratamentos utilizados, apenas a radioterapia se mostra capaz de aumentar significativamente a expectativa de vida (Henson 2006, Salazar et al. 1979). A dificuldade do tratamento se deve principalmente a dois fatores: a alta capacidade de infiltração no tecido normal desse tumor e a insensibilidade à quimioterapia. O GBM se infiltra no parênquima cerebral de tal forma que a retirada total do tumor sem afetar áreas importantes do SNC é praticamente impossível. O uso de quimioterápicos não é eficaz graças à presença da barreira hematoencefálica e devido à grande heterogeneidade celular desses tumores, que possuem em sua massa tumoral não só células neoplásicas altamente proliferativasmas também muitas células do sistema imune. Os quimioterápicos atuais não possuem lipofilia suficiente para atravessar eficazmente a barreira hematoencefálica e também são ineficazes em combater todos esses tipos celulares (Nieto-Sampedro et al. 2011, Wen P. Y. and Kesari 2008). Dos agentes quimioterápicos atuais, apenas a temozolamida se

mostrou eficiente no combate à doença e ainda assim, existe uma porcentagem bastante importante de pacientes que não se beneficiam desse tratamento graças a capacidade de as células tumorais se tornarem resistentes à esse medicamento (Bruyere et al. 2011). Por essas razões, o desenvolvimento de novos tratamentos que possam ser usados em associação com os quimioterápicos existentes e a descoberta de novos alvos terapêuticos se tornam necessários.

1.1.2 Meduloblastomas

Dentre os tumores cerebrais que podem acometer as crianças, os meduloblastomas são os mais prevalentes correspondendo a cerca de 20% de todos os tumores do SNC que acometem essa faixa etária. Apesar da alta prevalência na infância, com um pico de prevalência aos seis anos de idade, casos desse tipo de tumor são relatados em pacientes de todas as faixas etárias, desde bebês até adultos (Northcott et al. 2012). Essa patologia foi inicialmente descrita como um glioma cerebelar o que mudou em 1925 quando Bailey e Cushing nomearam meduloblastoma (Bailey and Cushing, 1925). Não só por sua elevada prevalência, mas também por ser o tumor que acomete o SNC com maior chance de evoluir para um quadro metastático, esse é um dos tumores pediátricos mais estudados (Mazloom et al. 2010).

Os meduloblastomas são tumores embrionários neuroepiteiais, altamente vascularizados, possuem alta taxa de proliferação e são classificados como tumores grau IV de acordo com a Organização Mundial da Saúde (Brandes and Paris 2004, Gilbertson and Ellison 2008, Martinez Leon 2011). Por definição, os meduloblastomas tem origem na quarta região ventricular (Packer 1999), sendo a

localização mais comum o cerebelo (94,4% dos casos), geralmente na linha média do vermis inferior (75% dos casos) (Sumer-Turanligil et al. 2013) e cresce em direção ao quarto ventrículo (Martinez Leon 2011). Pacientes com meduloblastoma comunmente apresentam sintomas relacionados à alta pressão intracraniana devido ao crescimento tumoral e à hidrocefalia secundária a obstrução tumoral. As manifestações clínicas dependem da idade do paciente, tamanho do tumor, se a doença é local ou disseminada. Os sintomas clássicos são dores de cabeça matinais, com vômitos, irritabilidade e letargia. Crianças com as fontes ainda abertas podem apresentar aumento na circunferência da cabeça (Crawford et al. 2007, Martinez Leon 2011).

Pouco se sabe sobre a etiologia dessa doença e os fatores de risco ainda não estão bem estabelecidos. Em função da maior incidência ser em crianças, acredita-se que existem alguns fatores na primeira infância que podem influenciar o desenvolvimento desses tumores. Peso grande ao nascimento tem sido associado positivamente com risco aumentado de meduloblastoma. Além disso, crianças cujas mães tiveram alguma infecção viral durante a gestação, possuem onze vezes mais chances de desenvolver malignidades do sistema nervoso central, entre eles, o meduloblastoma. Exposição parental a pesticidas também tem sido associada positivamente ao desenvolvimento de MB (Massimino et al. 2011).

Os meduloblastomas podem ser classificados de acordo com sua histologia e/ou perfil molecular. A Organização Mundial Da Saúde (OMS) leva em conta na sua classificação à histologia desses tumores, e assim os divide em clássico, desmoplásico/nodular, meduloblastoma com nodularidade extensiva, anaplásico e de células grandes. Destas variações, o anaplásico e o de células grandes mostram

certo grau de sobreposição e foram, portanto, agrupados em um único subgrupo, intitulado LCA (do inglês “large and anaplasic cells”) (Massimino et al. 2011).

Quanto ao perfil molecular, os meduloblastomas são divididos em dois grandes subgrupos: aqueles que possuem o mecanismo de tumorigênise conhecido (grupo WNT, que possuem mutações na via de sinalização da proteína homônima e SHH que possuem mutação na via Sonic Hedgehog) e os com mecanismo desconhecido (grupo 3 e 4) (Tabela 1). Cada subtipo é tão diferente que alguns autores defendem que podem ser consideradas doenças diferentes, com a necessidade de protocolos independentes de tratamento (Archer and Pomeroy 2012). O grupo WNT possui melhor prognóstico de todos os grupos, com mais de 95% dos pacientes sobrevivendo a doença. Os casos são relativamente bem distribuídos entre os gêneros. Esse tumor é o menos comum dos quatro subtipos, sendo apenas diagnosticado em um a cada dez pacientes com meduloblastoma. Tipicamente ocorrem em crianças com mais de três anos e geralmente não produzem metástase. Os tumores deste subtipo possuem taxas de sobrevida variando entre 60 a 80%. Quanto a sua prevalência, é bastante frequente em infantes e em adultos, sendo responsável por poucos casos em crianças. Os tumores enquadrados como grupo 3 são aos que se atribui o pior prognóstico. São tumores restritos a pacientes pediátricos e mais comuns em meninos do que em meninas. Por fim, os tumores do grupo 4 são os mais comuns, sendo diagnosticado em cerca de dois a cada cinco casos. Adultos acometidos com esse subtipo tem uma expectativa de vida a crianças afetadas pelo mesmo. É cerca de três vezes mais comum e homens do que em mulheres (Northcott et al. 2012).

Tabela 1: Aspectos clínicos dos subgrupos de meduloblastoma. Adaptado de Northcott *et at.* 2012

	WNT	**SHH**	**Grupo 3**	**Grupo 4**
Prevalência (total de casos)	10%	30%	25%	35%
Prevalência de acordo com o sexo (M/F)	1/1	1,5/1	2/1	3/1
Histologia	Clássico; muito raramente LCA	Clássico > desmoplástico/ nodular> LCA > nodularidade extensiva	Clássico > LCA	Clássico; raramente LCA
Metástase ao diagnóstico	~5-10%	~15-20%	~40-45%	~35-40%
Sobrevida global (após 5 anos)	~95%	~75%	~50%	~75%

As taxas médias de sobrevida, considerando todos os subtipos de meduloblastomas, é de cerca de 75%, consequência de protocolos de tratamento

que envolvem cirurgia, radioterapia e por vezes quimioterapia (Gajjar et al. 2013). A terapia de escolha é a cirurgia, que deve ser o mais radical possível. Uma ressecção cirúrgica adequada é importante e pacientes com uma doença residual superior a 1.5 cm^2 pós cirurgia tem um pior prognóstico. A radioterapia tem sido aceita como o tratamento pós operatório mais eficaz para os meduloblastomas. Estudos mostram que cerca de 40 a 70% dos pacientes estão vivos e com sobrevida de 5 anos livre de progressão de doença após tratamento com essa ferramenta terapêutica. A utilização de quimioterapia, por sua vez, ainda é um tanto quanto controversa (Brandes and Paris 2004). Alguns estudos demonstraram eficácia da quimioterapia como adjuvante pós radiação em crianças com estágios avançados da doença (Jenkin et al. 2000, Merchant et al. 1996), mas esse beneficio não foi obsevado em pacientes com doença em estágios mais iniciais (Abacioglu et al. 2002, David et al. 1997). Apesar da ausência de dados que suportem fortemente o uso de quimioterapia nesses tumores, hoje se acredita que a quimioterapia é benéfica, ao menos em pacientes com grandes riscos de recorrência e em casos onde há desenvolvimento de metástase (Brandes and Paris 2004) e nesses casos, os pacientes são tratados com agentes quimioterápicos como vincristina, carboplatina, ciclofosfamida e cisplatina (Mazloom et al. 2010).

Apesar das promissoras taxas de sobrevivência, os tratamentos empregados atualmente são altamente danosos para os pacientes, não só pelos efeitos adversos em curto prazo, mas também, e principalmente por serem altamente prejudiciais para a cognição dos pacientes e por levarem a problemas de desenvolvimento, neurológicos, neuroendócrinos e psicossociais (Archer and Pomeroy 2012, Northcott et al. 2012). Além disso, cerca de 10% dos pacientes acabam desenvolvendo metástases o que limita bastante o prognóstico, reduzindo a expectativa de vida

para em média oito meses. As metástases podem ser tanto no próprio SNC como extraneurais, sendo os focos mais comuns os ossos, a medula óssea e os linfonodos. Sítios metastáticos também podem ser identificados no pulmão e fígado, e estão associados com prognóstico ainda mais limitado: cerca de dois meses (Mazloom et al. 2010). O cenário atual, onde os pacientes submetidos ao tratamento sofrem com grandes perdas de qualidade de vida, associado com a incapacidade desses mesmos tratamentos em prolongar a vida dos casos metastáticos, evidencia que novos estudos devem ser realizados objetivando encontrar soluções terapêuticas mais eficazes e seguros.

1.2 Camomila

A camomila (*Matricaria recutita L., Chamomilla recutita L., Matricaria chamomilla*), é uma das plantas medicinais mais estudadas e documentadas (McKay and Blumberg 2006, Srivastava et al. 2010). Essa planta é uma das mais importantes plantas medicinais nativas do sul europeu, e que foi amplamente difundida pelo mundo todo, podendo ser encontrada em diversos continentes e países, inclusive no Brasil e é considerada um dos chás mais consumidos do mundo (McKay and Blumberg 2006, Singh et al. 2011). Ela tem sido usada em preparações fitoterápicas e em forma de chá a centenas de anos, desde o antigo Egito, Grécia e Roma. A camomila está incluída na farmacopeia de 26 países e suas indicações tradicionais são flatulência, cólica, histeria e febre intermitente. O óleo essencial que se extrai das flores, possui mais uma série de efeitos terapêuticos, como antiinflamatório e antiséptico, tratamento de dores estomacais, diarreia, náusea. Além disso, como uso externo, tanto o óleo essencial, quanto o chá e a própria

planta macerada são indicadas para erupções cutâneas, como cicatrizante, e inflamações de boca e olhos (Singh et al. 2011).

Mais de 120 componentes já foram identificados nas flores de camomila. Aminoácidos, polissacarídeos e ácidos graxos, estão presentes em cerca de 10% das cabeças das flores. O rendimento da extração de óleo essencial a partir das flores gira entre 0,4-2%, sendo os maiores componentes desse o terpenóide alfa-bisabolol e seus óxidos (≤ 78%) e azulenos, incluindo o camazuleno (McKay and Blumberg 2006).

1.2.1 Alfa bisabolol e óxido de bisabolol A

O alfa-bisabolol (Figura 2) é um pequeno álcool sesquiterpeno oleoso com massa molecular de 222,37 Da. Principal componente do óleo essencial da Camomila, ele também é encontrado em outras plantas, apresentando atividade cicatrizante e sendo amplamente utilizado em cosméticos e fragrâncias. (Cavalieri et al. 2009, Cavalieri et al. 2004, Piochon et al. 2009). Recentemente, estudos têm demonstrado uma ampla atividade biológica desse composto incluindo efeitos despigmentantes, antimutagênicos, antiinflamatórios, antifúngico, antibacteriano e gastroprotetor (Bezerra et al. 2009, Gomes-Carneiro et al. 2005, Kim et al. 2008, Rocha et al. 2011). O alfa-bisabolol é um composto bastante lipofílico, que apresenta forte tendência à oxidação. Os produtos de oxidação são essencialmente dois: óxido de bisabolol A (Figura 2) e óxido de bisabolol B. Estudos mais atuais já identificam atividade biológica desses óxidos no combate a dor e ao edema (Tomic et al. 2013).

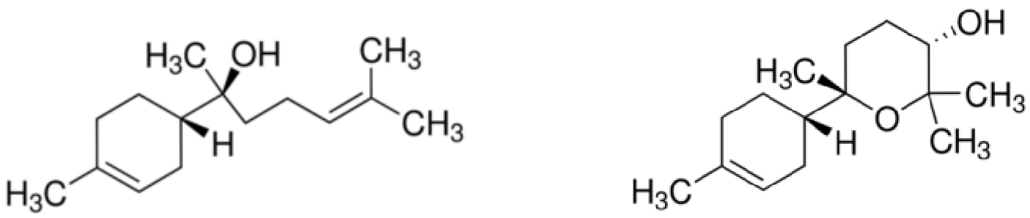

Figura 2: Estrutura molecular do alfa bisabolol (esquerda) e óxido de bisabolol A (direita).

Nos últimos anos, foi descoberta também uma importante atividade citotóxica do alfa-bisabolol frente a células tumorais. Os primeiros estudos foram realizados em linhagens celulares de glioma, onde foi verificada uma forte atividade dose-dependente do composto, levando as células tumorais a apoptose mesmo em baixas concentrações. Esse resultado é bastante interessante, uma vez que a apoptose é uma morte celular reconhecidamente mais desejável para tratamento tumoral uma vez que é uma morte sem extravasamento de conteúdo intracelular e não há recrutamento de sistema imune. Além disso, nesse mesmo estudo, quando células normais de glia de ratos foram tratadas com alfa-bisabolol nas mesmas concentrações das células tumorais, não foi observado morte celular das células sadias, evidenciando assim uma seletividade do composto e comprovando a sua baixa toxicidade (Cavalieri et al. 2009, Cavalieri et al. 2004).

A atividade citotóxica do alfa-bisabolol foi observada não só em gliomas, mas também em outros tipos de células tumorais, como melanoma, adenocarcinoma de mama, carcinoma de fígado e carcinoma de pulmão (Cavalieri et al. 2009, Chen et al. 2010, Piochon et al. 2009) e mais recentemente em câncer pancreático e leucemia (Cavalieri et al. 2011, Seki et al. 2011). Em todos esses estudos, o tipo de morte celular desencadeado foi a apoptose.

Devido as suas promissoras atividades biológicas, vem crescendo o interesse na produção e estudo de derivados do α-bisabolol, objetivando uma melhoria de suas propriedades físico-químicas, já que este é pouco solúvel em fluídos biológicos devido a sua alta lipofilia, bem como de um efeito farmacológico mais pronunciado (da Silva et al. 2010, Piochon et al. 2009). Apesar do interesse em avaliar a atividade de compostos sintéticos derivados do α-bisabolol, na literatura existem pouquíssimos estudos sobre as atividades biológicas do derivado natural óxido de bisabolol A, um derivado mais hidrofílico, o que poderia implicar em uma melhor solubilidade deste nos fluídos biológicos.

Os efeitos biológicos do óxido de bisabolol A ainda são muito pouco conhecidos. Até hoje, sabe-se apenas que esse composto possui atividade antiinflamatória (Jakovlev et al. 1979), antimicrobiana (Simionatto, et al, 2009) e mais recentemente, viu-se que esse composto é capaz de reduzir a capacidade proliferativa de células de leucemia, sem efeitos citotóxicos a células normais não proliferativas (Ogata-Ikeda et al. 2011). Assim, faz-se promissor não só o estudo dos efeitos biológicos desse composto *per se*, como a comparação dos efeitos destes com os efeitos desencadeados pelo análogo não oxidado, uma vez que a alteração de lipofilia do óxido de bisabolol A pode ser interessante em condições biológicas.

1.3 Sistema purinérgico

A sinalização purinérgica está envolvida em diversos processos biológicos (Figura 3). No meio extracelular, purinas e pirimidinas funcionam como importantes moléculas sinalizadoras. Ligando-se aos receptores purinérgicos, essas moléculas são capazes de mediar os mais diversos efeitos biológicos, entre eles o controle da

agregação plaquetária, controle do tônus vascular, respostas antiinflamatórias, diferenciação celular, apoptose e o desenvolvimento de diversos tipos de tumores, entre eles os gliomas e meduloblastomas (Cappellari et al. 2012b, Morrone et al. 2006, Ralevic and Burnstock 1998, White N. and Burnstock 2006, Yegutkin et al. 2011). Nas células gliais, nucleotídeos e nucleosídeos estão também correlacionados com proliferação celular. O catabolismo alterado destas moléculas no meio extracelular de gliomas pode indicar uma importante contribuição destas no crescimento e sobrevivência anormal das células cancerígenas (Braganhol et al. 2009, Morrone et al. 2003, Wink et al. 2003).

Ectonucleotidases são enzimas localizadas na membrana celular, possuindo seu sítio catalítico voltado para o meio extracelular. Essas enzimas são responsáveis pela degradação sucessiva de nucleotídeos de purinas e pirimidinas tri, di e monofosfatados aos seus respectivos nucleosídeos (Burnstock 2007, Ipata et al. 2011). A família das ectonucleotidases é constituída pelas ENTPDases (ectonucleosídeo trifosfato-difosfohidrolases), ENPPs (ectonucleotídeo pirofosfatase/fosfodiesterases), fosfatase alcalina e ecto-5`-nucleotidase/CD73 (Zimmermann, 2001).

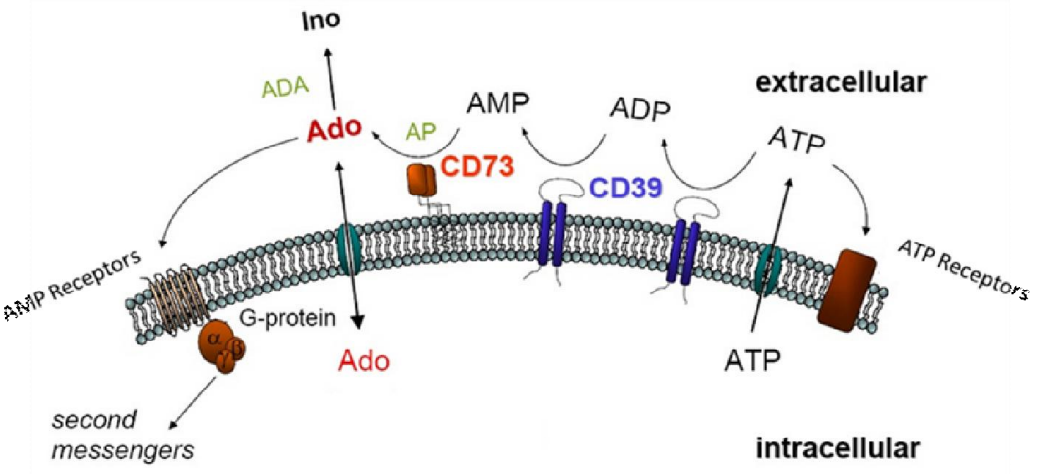

Figura 3: Overview do sistema purinérgico. Disponível em http://tu-dresden.de/die_tu_dresden/fakultaeten/medizinische_fakultaet/inst/phy/forschung/deussen/scienceinfo/ado/document_view?set_language=en (Adaptado).

Nessa família de enzimas, podemos destacar a ecto-5'-nucleotidase (ecto-5'-NT/CD73), que é responsável pela quebra de nucleotídeos monofosfatados, dando origem ao nucleosídeo respectivo, sendo o AMP o substrato preferencial. Essa enzima é constituída por 2 subunidades glicoprotéicas, apresentando o zinco e outros íons metálicos divalentes ligados ao seu domínio N-terminal e encontra-se ancorada na membrana plasmática através de uma âncora lipídica de glicosil-fosfatidilinositol (GPI) (Zimmermann 1992). Essa enzima é a principal responsável pela formação de adenosina a partir de AMP no meio extracelular, portanto o controle da sua atividade enzimática é essencial para a manutenção de níveis adequados desse nucleosídeo (Zimmermann, 2001).

Estudos têm correlacionado a ecto-5'NT/CD73 com modulação do crescimento, adesão, migração, diferenciação celular e interação entre células e entre célula e matriz celular. Além disso, também já foi proposto o envolvimento da ecto-5′-NT/CD73 na resistência à drogas (Braganhol et al. 2007, Cappellari et al. 2012a, Quezada et al. 2013). Em condições patológicas, como, por exemplo, nos glioblastomas e em diversos outros carcinomas humanos, a atividade e expressão dessa enzima se encontra aumentada, revelando uma importante correlação entre essa enzima e a progressão tumoral (Spychala 2000, Wang L. et al. 2008). Além disso, dados publicados por nosso grupo de pesquisa sugerem que alterações na via de sinalização purinérgica podem estar envolvidas na malignidade dos gliomas e meduloblastomas (Braganhol et al. 2009, Cappellari et al. 2012b, Morrone et al. 2003, Morrone et al. 2005, Morrone et al. 2006, Wink et al. 2003) e na proliferação celular de linhagens celulares de glioma (Bavaresco et al. 2008).

A adenosina, o produto final da hidrólise do ATP e que é gerada pela atividade da enzima ecto-5'-NT/CD73, tem múltiplas funções importantes como uma molécula sinalizadora extracelular, mediando efeitos biológicos em células normais e tumorais (Ralevic and Burnstock 1998, Spychala 2000). Os efeitos exercidos por essa molécula são regulados pela concentração de adenosina extracelular, a expressão diferencial dos receptores de adenosina e pelo mecanismo de transdução de sinal ativado pela ligação do agonista ao receptor. A ação da adenosina pode se dar pela ligação da molécula à quatro receptores distintos, são eles os receptores A_1, A_{2A}, A_{2B}, e A_3, todos ligados à proteínas G e atuam modulando a atividade da adenilciclase de forma inibitória (A1 e A3) ou estimulatória (A2A, A2B) (Palmer and Stiles 1995). Os receptores do tipo A_1 são distribuídos amplamente em todos os tecidos e medeiam diversos efeitos biológicos como depressão cardíaca, vasoconstrição e efeitos protetores durante isquemia. Os receptores A_2 são divididos em dois subtipos graças à sua afinidade diferencial de seus sítios de ligação à adenosina. O receptor A_3, que foi descoberto posteriormente aos outros, é descrito como modulador de morte celular em cardiomiócitos, em astrócitos e em células de glioma (Bernardi et al. 2007, Jacobson et al. 1999, Ralevic and Burnstock 1998).

Graças à estreita relação entre o sistema purinérgico e o desenvolvimento e progressão tumoral, vem crescendo o interesse na utilização dessa via de sinalização como alvo terapêutico no tratamento de diversas neoplasias (Burnstock 2012). Corroborando com essa teoria, trabalhos anteriores do nosso grupo revelaram que a modulação do sistema purinérgico se mostra valiosa na terapia de neoplasias do sistema nervoso central (Bernardi et al. 2007, Braganhol et al. 2007, Morrone et al. 2006).

1.4 Tumores do sistema nervoso central, derivados de bisabolol e sistema purinérgico

Os tumores do sistema nervoso central são um grande desafio terapêutico pela malignidade e pela nobreza do tecido em que ele se instala. Além disso, a barreira hematoencefálica atua como um importante protetor desses tumores já que ela impede a passagem de muitos dos quimioterápicos conhecidos para o tecido acometido (Van Meir et al. 2010). Como consequência, novas drogas, que sejam eficazes e capazes de atravessar essa barreira e novos alvos terapêuticos, para conseguir combater mais eficientemente essa malignidade, devem ser estudados. Nesse cenário, tanto o os derivados de bisabolol, nova terapia, como o sistema purinérgico, novo alvo terapêutico, surgem como possíveis alternativas no tratamento dessa malignidade.

O alfa-bisabolol surge nesse cenário como um interessante potencial quimioterápico graças a sua forte atividade antitumoral. Devido ao seu grande potencial, cresce também o interesse em estudar análogos dessa molécula, naturais ou sintéticos. Muito ainda do mecanismo de ação do alfa-bisabolol precisa ser estudado para que possamos entender por quais vias essa droga exerce sua atividade. Dentre os possíveis alvos moleculares desse composto, está a ecto-5'-nucleotidase (ecto-5'NT/CD73) isso porque hoje já se sabe que o alfa-bisabolol é capaz de se intercalar nos "rafts" de membrana (Darra et al. 2008) e essas regiões são ricas em glicoproteína P, que é o local de ancoragem da ecto-5'-nucleotidase (Zimmermann 1992).

2. Objetivo geral

Considerando a promissora atividade citotóxica do alfa-bisabolol em uma ampla gama de tumores, a importância do sistema purinérgico na progressão tumoral e a inexistência de estudos com o derivado natural do alfa-bisabolol, o óxido de bisabolol A, o objetivo dessa dissertação foi avaliar os efeitos antitumorais das duas moléculas em questão e correlacionar os efeitos observados com possíveis alterações no sistema purinérgico.

2.1 Objetivos específicos

a) Avaliar a atividade citotóxica do alfa-bisabolol sob linhagens de glioma e de meduloblastoma;

b) Estudar a correlação dos efeitos de citotoxicidade do alfa-bisabolol com os efeitos sobre o sistema purinérgico, especialmente sobre a enzima ecto-5'NT/CD73 e o receptor de adenosina A3.

c) Avaliar efeitos citotóxicos do óxido de bisabolol A em linhagens de glioma e meduloblastoma, comparando esses efeitos com o alfa-bisabolol.

3. Resultados

Os resultados serão apresentados em três capítulos, na forma de manuscritos a serem submetidos a periódicos científicos.

3.1 Capítulo 1

Involvement of the purinergic system in the death promoted by alpha-bisabolol in gliomablastoma cells

Manuscrito a ser submetido para o periódico European Journal of Pharmacology

Involvement of the purinergic system in the death promoted by alpha-bisabolol in gliomablastoma cells

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Abstract

Gliblastoma miltiforme is the most common primarily tumors on central nervous system. In despite of advances in standard treatment, the prognosis of patients with the disease remains poor. Alpha bisabolol (α-bisabolol), the main component of *Matricaria chamomilla,* essential oil have been recently reported as a potent cell death inductor on glioma cell line. The most well characterized enzymatic source of adenosine is the ecto-5'-nucleotidase (ecto-5'-NT/CD73), an enzyme present in the lipid rafts. Once alpha-bisabololis rapidly incorporated into lipid rafts, we decided to evaluate a possible effect of this compound on ecto-5'-NT/CD73 and the effect of adenosine on cell death induced by the compound. Exposure of C6 rat glioma cells and U138-MG human glioma cells lead to a decrease on cell viability, being the rat cell line more sensitive to the treatment. Ecto-5'-NT/CD73 activity was increased in cells treated with α-bisabolol. Pre treatment with MRS 1220, a selective A_3 antagonist increased nearly 60% cell number in the concentrations of 45 μM and 55 μM. Our data indicate a strong influence of alpha-bisabololon ecto-5'-NT/CD73 activity and suggest that the enzyme and the A_3 adenosine receptor are at least partially involved in the anti-proliferative effect of α-bisabolol.

Keywords: Glioma**,** α-bisabolol; Ecto-5'-NT/CD73; adenosine.

1. Introduction

Glioblastoma Multiforme is the most aggressive brain tumor and median survival on these patients normally does not exceed 15 months. Despite of a standard treatment, which includes surgical resection, radiotherapy and chemotherapy, the patient's prognosis remains poor (Henriksson et al. 2011, Sathornsumetee et al. 2007). The main reason for this is that the tumor cannot be completely removed by surgery and the tumor cells are able to become resistant to temozolomide, the chemotherapy drug most commonly used (Bruyere et al. 2011). Hence, the development of alternative treatments and the discovery of new therapy targets are needed.

Chamomile (*Matricaria recutita L., Chamomilla recutita L., Matricaria chamomilla*) is one of the oldest and most well documented medicinal plant (McKay and Blumberg 2006, Srivastava et al. 2010). The main component of its essential oil is alpha-bisabolol (α-bisabolol) a small, nontoxic, sesquiterpene. Alpha-bisabolol is also found in the essential oil of other plants and presents wound-healing activities, being widely used in cosmetics (Cavalieri et al. 2009, Cavalieri et al. 2004, Piochon et al. 2009). Recently, some studies have explored other biological activities of this molecule which includes depigmenting, antimutagenic, anti-inflamatory, antifungal, and cytotoxic effects on some cancer cell line such as glioma, melanoma, breast adenocarcinoma, liver carcinoma and lung carcinoma (Cavalieri et al. 2009, Chen et al. 2010, Piochon et al. 2009).

Adenosine, the final product of ATP hydrolysis, represents an important extracellular signaling molecule that mediates diverse biological effects in both normal and tumor cells (Ralevic and Burnstock 1998, Spychala 2000). Among the various adenosine functions, cell growth, differentiation and death are regulated

according to its extracellular concentration and expression of the different adenosine receptors. Adenosine can act on adenosine or P1 receptor that are subdivided into 4 subtypes (A_1, A_{2A}, A_{2B}, and A_3) all of them coupled to G proteins. Among these receptors, the A_3 adenosine receptor has already been described as cell death modulator in cardiomyocites, brain astrocytes and in glioma cells (Jacobson et al. 1999, Ralevic and Burnstock 1998).

The most well characterized enzymatic source of extracellular adenosine is the ecto-5'-nucleotidase (ecto-5'-NT/CD73). This enzyme catalyzes the dephosphorylation of nucleotide monophosphates, leading to the respective nucleoside. Evidences relate ecto-5′-NT/CD73 to modulation of cell growth, differentiation and to influence on cell–cell and cell–matrix interactions (Braganhol et al. 2007, Zimmermann 1992). In some pathological conditions, such as glioblastoma multiforme, the enzyme activity and expression is increased (Spychala 2000). This data was also reported to some other human carcinoma types, revealing an important correlation between this enzyme and the tumor progression (Wang L. et al. 2008).

Considering that alpha-bisabololis are able to be incorporated into lipid rafts (Cavalieri et al. 2009), that lipid rafts are rich in glycosyl phosphatidylinositol-anchored proteins (Simons and Ikonen 1997) and that ecto-5'-NT/CD73 is anchored in the cell surface via glycosyl phosphatidylinositol (Zimmermann 1992), the aim of the present study is to evaluate the effect of alpha-bisabololin on the activity of ecto-5'-NT/CD73 and investigate a possible involvement of this enzyme in the cell death triggered by this compound.

2. Material and Methods

2.1. Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin 0.25%, trypsin/ EDTA solution and Fetal bovine serum (FBS) were all obtained from Gibco (Carlsbad, CA, USA). TRIzol, SuperScript III First Strand, PCR Super Mix, Platinum SYBR Green qPCR SuperMix-UDG and SYBR Safe DNA Gel Stain were obtained from Invitrogen (Carlsbad, CA, USA), M-MLV RT and dNTPs were obtained from Promega (Madison, Wisconsin, USA), MRS 1220 (9-Chloro-2-(2-furanyl)-5-((phenylacetyl) amino)-[1,2,4]triazolo[1,5-c]quinazoline) was obtained from Tocris Cookson (UK).

2.2. Maintenance of cell line

C6 rat glioma cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA). Cells were cultured in DMEM, containing penicillin/streptomycin 0.5 U/ml and supplemented with 5% fetal bovine serum (FBS). Cells were kept at 37°C, minimum relative humidity of 95% in an atmosphere of 5% CO_2.

2.3. Alpha-bisabolol treatment

Alpha-bisabolol was prepared in dimethylsulphoxide (DMSO, Sigma). The final concentration of DMSO in each well was 0,5%, the same concentration used for the DMSO vehicle control. The glioma cells were seed in 6, 24 or 48 well plates, according to the experiment to be realized. To the MTT assay, the cells were treated with 30 μM, 45 μM, 60 μM, 75 μM, 100 μM and 115 μM of α-bisabolol, for 48h. To the

others experiments, the concentrations of 35μM, 45μM and 55μM of α-bisabolol were used. To evaluate the direct effect of alpha-bisabolol on the ecto-5′-NT/CD73 catalytic activity, cells were exposed to the drug for 10min in incubation medium and during the enzymatic assay. In the experiments performed with MRS1220, a selective A_3 adenosine receptor antagonist, it was added in the concentration of 1μM, thirty minutes before the exposition to alpha-bisabolol treatments.

2.4. Assessment of cell viability assay

C6 glioma cells were seeded in a 48-well plate and after achieve semi confluence they were treated with alpha-bisabololfor for 48h. After treatment time, culture medium was removed and cells were washed twice with PBS. Then, 180 μL of culture medium and 20 μL of MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide]) were added to each well. The cells were incubated for 3 h, the medium containing MTT was removed, and the formazan crystal products were eluted in 200 μL of DMSO. The level of absorbance was read by an ELISA plate reader at 492 nm. The absorbance was linearly proportional to the number of live cells with active mitochondria.

2.5. Ecto-5'-NT/CD73 assay

After treatment with α-bisabolol, the 24 well plates containing C6 cells were washed three times with incubation medium (2 mM MgCl2, 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM Hepes, pH 7.4). The enzymatic reaction was started by the addition of 200 μL of incubation medium containing 2 mM AMP, at 37ºC. After 10

min, 150 uL of the incubation medium in contact with the cells was collected and transferred to eppendorf tubes containing trichloroacetic acid (5% final concentration, w/v) previously placed on ice. The green malachite method was used to measure the inorganic phosphate (Pi) release using KH_2PO_4 as a Pi standard (Chan et al., 1986), The non-enzymatic Pi released from nucleotide into the assay medium without cells was subtracted from the total Pi released during incubation, giving net values for enzymatic activity. After the enzymatic assay, cells were dried and solubilized with 100 µL NaOH 1.0 M. An aliquot was then removed and the protein was measured by the Coomassie Blue method (Bradford, 1976), using bovine serum albumin as standard. Specific activity was expressed as nmol Pi released/min/mg of protein.

2.6. Ecto-5'-NT /CD73 and A_3 mRNA expression analysis

Once the time of 48h of treatment was achieved, the total RNA was isolated with TRIzol Reagent according to the manufacturer's instructions. 0,5µg of total RNA were added to each cDNA synthesis reaction using the SuperScript-III RT pre-amplification system. Real-time PCRs were carried out in the Applied-Biosystem Step One Plus cycler using Platinum® SYBR® Green qPCR SuperMix-UDG following the manufacturer's instructions. The real time PCR reactions were performed using the following temperature protocols: 2 min at 50°C, 2 min at 95°C, followed by 40 rounds of 95°C for 15 s, annealing at primer specific temperature for 30s. The same program was used for the amplification of the gene of reference, which were β-actin and β2-microglobulin. All results were analyzed by the $2^{-\Delta/\Delta CT}$ method (Livak and Schmittgen, 2001)

The following sequences of primers were used: ecto- 5′-nucleotidase/CD73: forward 5' CAA ATC TGC CTC TGG AAA GC 3´ and reverse 5' ACC TTC CAG AAG GAC CCT GT 3'. For A_3 forward: 5′-CTG CGA GTC AAG CTG AC-3′ and reverse: 5′-GTC CCA CCA GAA AGG ACA-3′. β- actin: forward: 5′-GGT CAT CAC TAT CGG CAA T-3′ reverse: 5′-GAA TGT AGT TTC ATG GAT GC-3′. β2-microglobulin: forward 5´-TCC TGG CTC ACA CTG AAT TC-3´ reverse 5´- CTT TGT GGA TAA ATT GTA TAG CA -3´.

2.7. Assessment of cell number

After 48h of treatment with α-bisabolol, as described above, medium was removed, cells were washed twice with phosphate buffered saline (PBS), detached with 0,05% trypsine/EDTA and immediately counted in a haemocytometer.

2.8. Statistical analysis

Data are expressed as mean ± SD and were analyzed for statistic significance either by One Way analysis of Variance (ANOVA), followed by post-hoc comparisons (Tuket test) or Two Way ANOVA using GraphPad Prisma Software. Differences were considered significant in relation to control when $p<0,05$.

3. Results

3.1. Cell viability is decreased in cells treated with α-bisabolol

The MTT assay was used to investigate the effect of alpha-bisabolol on cell viability. C6 cells were treated with 30 μM, 45 μM, 60 μM, 75 μM, 100 μM and 115 μM of α-bisabolol. The increase on drug concentration lead to a bigger decrease on the percentage of viable cells (67,39% ± 7.09, 36,56% ± 9,48, 41,83% ± 9,81, 24,27% ± 3,21, 17,81% ± 0,28, 14,04% ± 2,37 of viable cells, respectively) when compared to non-treated cells (Figure 1). The IC_{50} calculated was approximately 45μM. Therefore, to the next experiments, we used a concentration below the IC_{50} (35 μM), the concentration of 45 μM and a concentration above the IC_{50} (55 μM) to the other experiments, No differences between the cell viability of control cells versus DMSO treated cells were observed (data not show).

3.2. Treatment with alpha-bisabolol increases ecto-5'-NT/CD73 activity

To evaluate the effect of alpha-bisabolol on the ecto-5'-NT/CD73 activity, glioma cells were exposed to the molecule for 10 minutes before enzyme evaluation, to assess the direct effect of the drug on ecto-5´-NT/CD73, or during 48h in culture, to verify a long-term effect of the drug on the enzyme. In both treatments, the cells were then incubated with adenosine-5'-monophosphate (AMP) as substrate and the inorganic phosphate released was measured as described in Material and Methods.

When the C6 glioma cells were exposed for 10 minutes to α-bisabolol, a significant increase in the enzyme activity was observed in all concentrations tested when compared to control (Figure 2A). Again, no differences between the enzymatic activity of control cells versus DMSO treated cells were observed. The intermediate concentration tested (45 μM) was able to induce the bigger increase on activity among the three concentrations tested with a peak on enzymatic activity of 188 ±

6,02 nmol Pi/min/mg. In the concentration of 55 μM the enzymatic activity (142,8 ± 6,03 nmol Pi/min/mg) was diminished by 25% when compared to the higher activity observed and returning to the same level detected at the 35 μM concentration (142,0 ± 2,59 nmol Pi/min/mg).

After 48h of treatment with alpha-bisabolol in cell culture the same pattern of enzymatic activity activation was observed, with a peak of activity in the intermediary concentration tested (Figure 2B), and a decrease in the ecto-5'-NT/CD73 activity was also observed in the higher concentration tested, which becomes similar to the activity in the lower concentration (119,3 ± 5,87 nmol Pi/min/mg, 147,5 ± 16,17 nmol Pi/min/mg, 119,5 ± 9,41 nmol Pi/min/mg for treatment concentrations of 35 μM, 45 μM and 55 μM, respectively).

Since alpha-bisabolol treatment promoted a significant enhancement on ecto-5´-NT/CD73 activity, it was evaluated if this increase was a result of an increase in the mRNA expression. Real time PCR was performed and surprisingly, it was observed a decrease in mRNA levels by 1,5 and 1,3 fold for the concentrations of 45 μM and 55 μM of alpha-bisabolol, respectively when compared to DMSO treated cells. The treatment with 35 μM was not able to alter the level of mRNA expression when compared to DMSO treated group (Figure 3). No differences between control and DMSO groups was also observed (data not shown)

3.3. The decrease on cell number is partly mediated by A_3 receptor

Considering that ecto-5'-NT/CD73 stimulation can increase the levels of adenosine in the extracellular medium around glioma cells, and the possible involvement of A_3 adenosine receptor in the cell death the participation of this

receptor was investigated. For this purpose cells where pre-treated with 1 μM of MRS1220 for 30 minutes before alpha-bisabolol exposure for 48h in culture. Figure 4 shows that 35 μM MRS1220 did not affect the alpha-bisabolol inhibitory effect. On the other hand the effect on cell number decrease caused by 45 μM and 55 μM of alpha-bisabolol was significantly reverted by the treatment with A_3 antagonist (from 14,81% ± 2,78 to 33,96± 2,83 and from 19,12± 5,33 to 38,13 ± 7,67 for 45 μM and 55 μM, respectively). It is possible to observe that the group of cells pre-treated with MRS1220 was less affected by α-bisabolol, and the number of cells counted for the groups MRS1220 plus alpha-bisabolol 45 μM and MRS1220 plus alpha-bisabolol 55μM came to be double of the treatment with alpha-bisabolol alone (Figure 4). No difference between control and DMSO treated cells were observed.

Once observed that MRS1220 reverts, at least partially, the effects of alpha-bisabolol treatment, it was evaluated if the treatment could modulate A_3 expression. It was observed that all concentrations tested were able to increase A_3 adenosine receptor mRNA levels in about 1.8 times (Figure 5).

4. Discussion

Glioblastoma multiforme is the most aggressive tumor in the CNS and the therapeutic options are limited (Henriksson et al., 2011). In despite of the recent advances, this brain tumor remains a challenging disease to treat and the prognosis for the patients is still poor (Clarke et al. 2010). This scene evidences the necessity of research and development of new drugs and new therapy targets.

Recent studies have shown the potent effect on inducing cell death of alpha-bisabolol in a large variety of cancer cell lines, including glioblastoma cells. (Cavalieri et al. 2004, Chen et al. 2010, Piochon et al. 2009). Since alpha-bisabolol is

rapidly incorporated into lipid rafts (Cavalieri et al. 2009) and ecto-5'-NT/CD73, an enzime that has already been described as a potencial new terapeutic target (Baqi et al. 2010, Zhang 2010), is residing in the lipid rafts (Zimmermann 1992), we decided to study a possible influence of alpha-bisabolol on ecto-5'-NT/CD73 activity and a possible correlation between changes in the enzymatic activity and cell death induced by α-bisabolol.

An important source of extracellular adenosine is the AMP hydrolysis catalyzed by ecto-5'-NT/CD73 and adenosine has been reported to stimulate cell proliferation and tumor growth (Morrone et al. 2003, Spychala 2000), being determinant in the cell progression pathway and as an angiogenesis promoter (Merighi et al. 2006). Although all the pro-tumor functions of adenosine, it has also been reported that this molecule acting via A_3 receptor induces cell death (Merighi et al. 2003). Also, drugs which are capable to induce cell death may promote an increase on this receptor expression (Bernardi et al. 2007).

In the present study, we first evaluated the effect of alpha-bisabolol on cell viability. A decrease of 30% on glioma cell viability is already observed with 30 uM of alpha-bisabolol. Moreover, in the higher concentration tested, it was observed a rate of only 14% of viable cells, reinforcing the strong cell death effect triggered by alpha-bisabolol in this cancer cell line (Cavalieri et al., 2004).

When the ecto-5'-NT/CD73 activity was assessed, it was observed a strong activation on this enzyme in the three concentrations tested both in the long-term (48h) and after 10 minutes of treatment. Furthermore, it was observed a peak of activity in the intermediated concentration tested. Although in previous studies from our laboratory it was observed a linear dose-response decrease or increase of ecto-5'-NT/CD73 activity after different treatments (Bernardi et al. 2007, Braganhol et al.

2007) there are at least three hypotheses to explain the reason why in the 55μM concentration promoted a decrease on enzymatic activity when compared to 45 μM concentration: (a) ATP may be released both in physiological and pathological conditions (Inoue et al. 2007). Damaged or dying cells may release ADP and mainly ATP in the extracellular medium (Bodin and Burnstock 2001) and these nucleotides are effective competitive inhibitors for ecto-5'-NT/CD73 (Zimmermann 1992). Considering that glioma cell lines have a poor ATPase and ADPase activity (Wink et al. 2003), it might be occurring an accumulation of these two nucleotides on extracellular medium, leading to ecto-5'NT/CD73 inhibition. (b) Previous results of our group showed that increases on cell confluence leads to an increase of ecto-5'-NT/CD73 activity (Bavaresco et al. 2008). The treatment with alpha-bisabolol leads to a decrease of confluence, once a large number of cells die when exposed to this compound. It is possible that this reduction on confluence or cell number influences the activity of ecto-5'-NT/CD73, resulting in a decrease in the enzyme activity in the higher concentration tested. Finally, (c) the observed effect might also be explained by a non specific chemical effect of alpha-bisabolol once the molecule is highly lipophilic and might be not completely soluble at the supposed concentration of 55 μM.

Once the ecto-5-NT/CD73 activity was increased, we decided to evaluate if it was a result of an increase on mRNA expression. Surprisingly, we observed a tendency to decrease on mRNA expression. This decrease might be a part of a feedback autoregulatory loop as previously described (Vuaden, et al, 2007). This feedback could be a response of the cell to the damage caused by the alterations on basal ecto-5´-NT/CD73 activity. Once the activation of this enzyme is leading to cell death trough activation of A_3 receptor by the product of the enzymatic reaction, the

transcriptional levels of the enzyme might be reduced. This results, together with the increase of enzyme activity with only 10 minutes of alpha-bisabolol exposure, make us to believe that the up-regulation on ecto-5´-NT/CD73 activity is related with the ability of the drug to insert in the lipid rafts. In hypoxia, where the lipid membrane content is affected, it has already been observed an enhancement of ecto-5´-NT/CD73 activity, with no alterations on the total protein (Ledoux, S., et al, 2003).

We then decided to go further on investigating the effect of ecto-5´-NT/CD73 activation on C6 glioma cells. In presence of high levels of adenosine, as occurs in high activity of ecto-5'-NT/CD73, activation of A_3 adenosine receptor could induce cell death (Jacobson et al. 1999) in a large variety of cancer cell types, including glioma cell lines (Bernardi, et al., 2007; Kim et al., 2012). To evaluate the functionality of this receptor we performed the assessment of cell number, using MRS1220, a selective A_3 adenosine receptor antagonist, which was added to the cells in culture thirty minutes before the treatment with alpha-bisabolol. In the presence of MRS 1220, an increase of around 50% on cell number was observed when compared to cells treated only with alpha-bisabolol, indicating that this receptor is, at least partially, involved in the cell death induced by the alpha-bisabolol. This effect was observed in the intermediate and in the higher (45 μM and 55 μM) concentrations tested. In the lower concentration (35 μM) the pretreatment with the antagonist did not reverted the observed decrease on cell number. The cell number in the group pretreated with MRS1220 however remained inferior to the control group. This suggests that other mechanisms besides induction of cell death via A_3 receptor are involved in the cytotoxic effect of α-bisabolol. We then wanted to verify if the treatment could induce an alteration on the receptor level. We then observed that the cells treated with alpha-bisabolol had the transcriptional levels of A_3 receptor enhanced.

In conclusion, the present work establishes by the first time the effect of alpha-bisabolol on an enzyme of the purinergic system and shows an interesting correlation between ecto-5'-NT/CD73 activity and the effect of cytotoxicity presented by this drug. The results presented herein are in accordance with works previously published, which described an important cytototoxic effect of alpha-bisabolol on glioma cell line that leads to cell death mainly by apoptosis. Previous studies also reported that this compound is promptly accumulated in the brain (Cavalieri et al. 2004) factor that contribute to believe that the use of this molecule is very promising for the adjuvant treatment of glioblastoma.

Acknowledgement

This study was supported by the Brazilian funding agencies: Fundação de Amparo a Pesquisa do Rio Grande do Sul (FAPERGS), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). F.B. Mendes was recipient of a CNPq fellowship.

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Figure Legends

Figure 1. Alpha-bisabolol decreases cell viability. Cells were treated with alpha-bisabolol for 48h. The DMSO control cells were treated with 0,5% of DMSO, same concentration of DMSO present in all the treatment concentrations. A mixture of 180 µL of medium and 20 µL of MTT was added to each well and incubated for 3h. The formazan product was eluted in 200 µL of DMSO and absorbance measured at 492 nm. No differences between control and DMSO cells were observed. Cell percentages were calculated in relation to control group. (A) C6 rat glioma cells. (B) U138-MG human glioma cells. The values represent means±S.D. Data is resultant of three independent experiments. * Significantly different from the DMSO group ($p<0,05$).

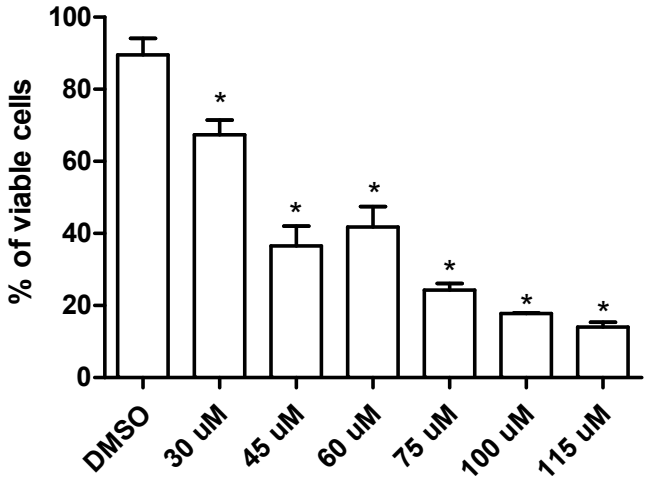
Figure 2. Treatment with alpha-bisabolol strongly stimulates ecto-5'-NT/CD73 activity. After alpha-bisabolol exposure, cells were incubated with 2 mM of AMP as described in Material and methods. The exposure to treatment was 10 minutes (A, C6 cells and C, U138-MG cells) or 48h in culture (B, C6 cells and D, U138-MG). Specific activity values are expressed as nmol Pi/min/mg protein and data as means±SD. Values are representative of at least three independent experiments. *Significantly different from the DMSO group ($p<0,05$).

Figure 3. Treatment with alpha-bisabolol changes ecto-5´-NT/CD73 mRNA expression. C6 Rat glioma cells were seeded and treated with α-bisabolol. After 48h of treatment, total RNA was isolated and cDNA was synthesized. Using specific primers, qPCR reaction was performed. The values represent means±S.D.. Data is resultant of two independent experiments.

Figure 4. Alpha-bisabolol effect on C6 cell number. After reached semi confluence, cells were treated with alpha-bisabololand with the indicated concentratios and kept in culture for 48h. DMSO group was treated with 0,5% of DMSO, which was the same concentration of DMSO present in all the treatments. In parallel, cells were pre-treated with 1μM of MRS1220, a selective A_3 antagonist, 30 minutes before the treatment with alpha-bisabolol and the cells were also kept in culture for 48h. Once the treatment time was achieved, the cells were immediately detached and counted in a hemocytometer. Data is resultant of three independent experiments and cell porcentage was calculated in relation to control group. * Significantly different from the DMSO group ($p<0,001$).*# Significantly different from the DMSO and from the treatment with alpha-bisabolol alone ($p<0,05$).No differences between DMSO and control group were observed.

Figure 5. A3 receptor mRNA expression is increased by alpha-bisabolol treatment. Rat glioma cells were seed and treated with α-bisabolol. After 48h of treatment, RNA was isolated and cDNA was synthesized. Using specific primers, qPCR reaction was performed. The values represent means±S.D. Data is resultant of four independent experiments.

Figure 1


% of viable cells
100
80
60
40
20
0
*
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*
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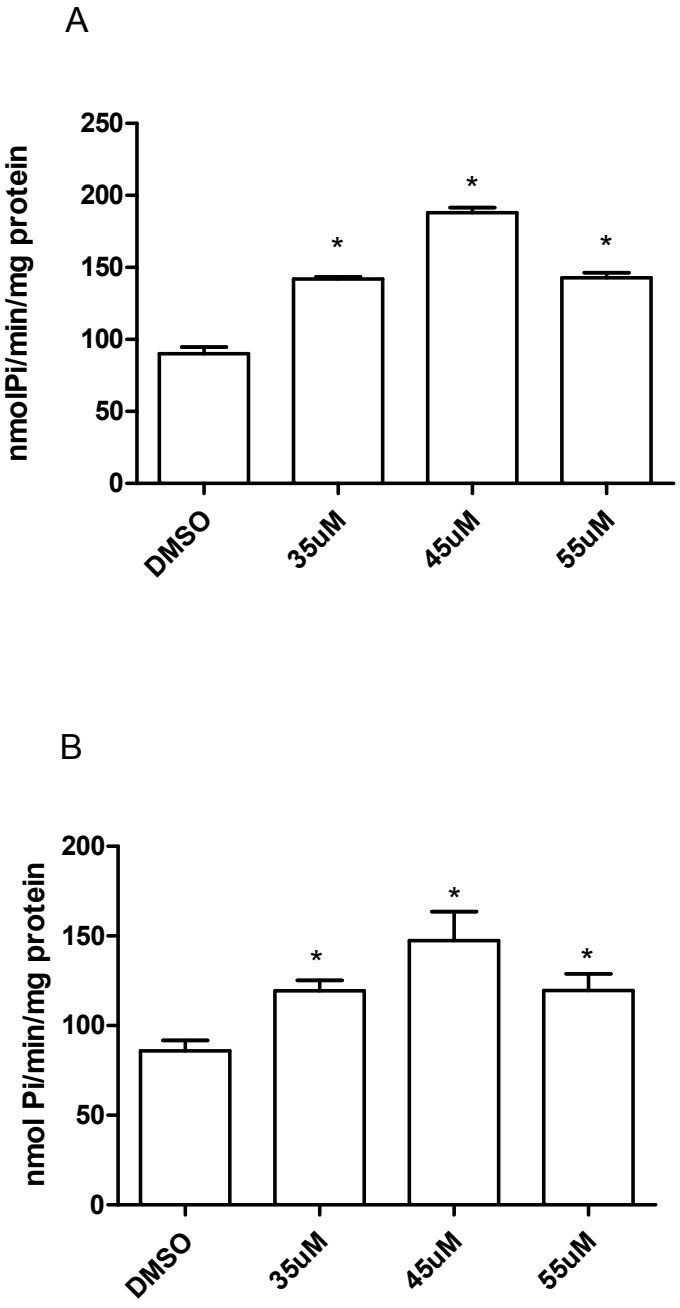
DMSO
30 uM
45 uM
60 uM
75 uM
100 uM
115 uM

Figure 2


A
nmolPi/min/mg protein
250
200
150
100
50
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35uM
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B
nmol Pi/min/mg protein
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35uM
45uM
55uM

Figure 3

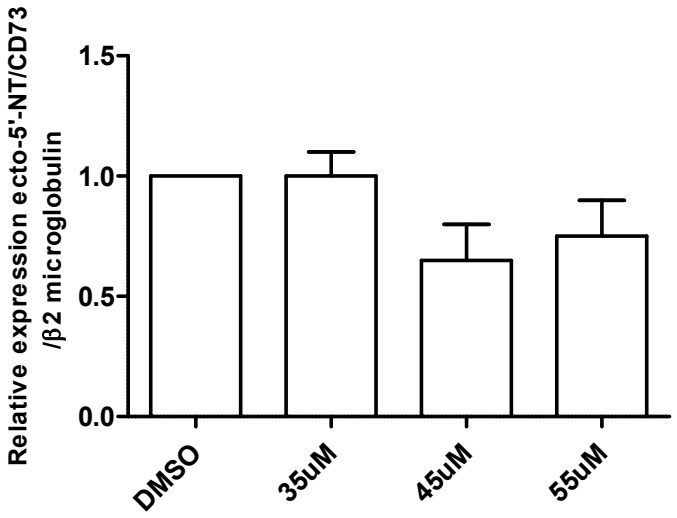


Figure 4

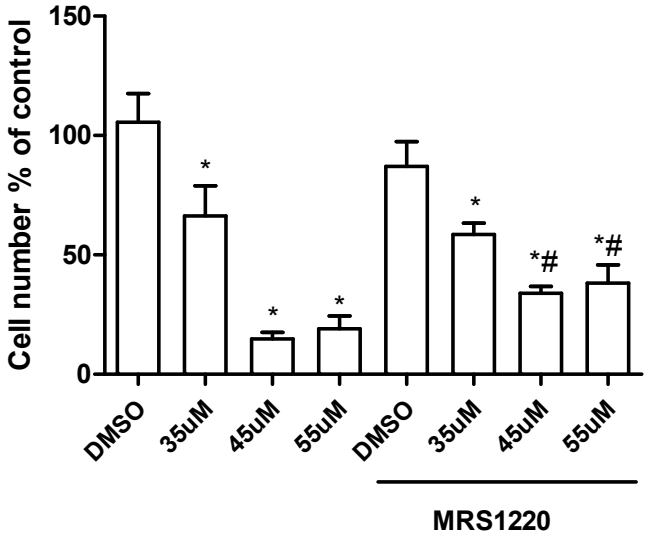
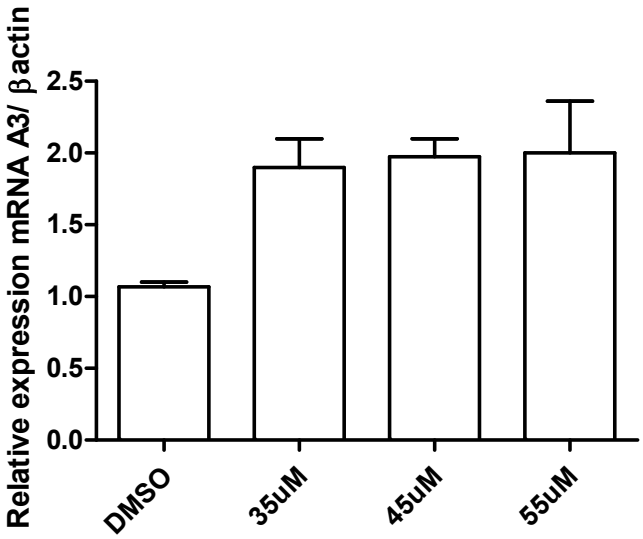


Figure 5


Relative expression mRNA A3/ βactin
2.5
2.0
1.5
1.0
0.5
0.0
DMSO
35uM
45uM
55uM

3.2 Capítulo 2

α-Bisabolol cytotoxic effect and ecto-5'-nucleotidase modulation on medulloblastoma cell lines

Manuscrito a ser submetido ao periódico British Journal of Pharmacology

α-Bisabolol cytotoxic effect and ecto-5'-nucleotidase modulation on medulloblastoma cell lines

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Summary

Background and purpose. Medulloblastoma (MB) is the most prevalent malignant brain tumor in children. The therapies currently cause considerable long-term side effects. Therefore, arises the need of new therapies and therapy targets to treat this disease. Alpha-bisabolol is a non-toxic molecule which exhibited interesting cytotoxic activity in a broad range of cancer cell lines. Ecto-5'-nucleotidase (Ecto-5'NT) is an enzyme strongly correlated with cancer progression. The aim of this work was to evaluate the effect of α-bisabolol on MB cell lines and a possible modulation of Ecto-5'NT.

Experimental approach. DaOY and D283 MB cell lines were treated with α-bisabolol for 48h. To evaluate cell cytotoxic effect, cell counting assay, Annexin/PI, acridine orange and cell cycle assay were performed. To evaluate the modulation of Ecto-5'NT activity, we performed an enzymatic assay and flow citometric for evaluation of protein expression.

Key results. Both cell lines tested have the cell number reduced when treated with α-bisabolol. The treatment triggers apoptosis on DaOY cell line, but not on D283 cell line. To both cell lines, the treatment lead to a direct increase on Ecto-5'NT activity, with no alteration on protein level.

Conclusions and Implications. We here demonstrate the potentiality of α-bisabolol to be used as a new or adjuvant therapy on MB. Also, we demonstrate that this treatment modulates Ecto-5'NT activity, an enzyme strongly correlated with cancer progression.

Keywords. Medulloblastoma, α-bisabolol, purinergic system, ecto-5'-nucleotidase.

Introduction

Medulloblastoma (MB) is the most prevalent malignant brain tumor in children, accounting for 20% of all central nervous system tumors in this age group. In spite of the high prevalence in childhood, cases of this tumor are related in patients of all ages (Northcott et al. 2012). MB occurs mainly in the cerebellum and posterior fossa, being able to migrate into other brain structures and also developing extraneural metastasis (Muoio et al. 2011). The World Health Organization (WHO) has classified MB as a grade IV tumor, the most malignant grade (Louis et al. 2007). The survival rates are considered promising due to treatment protocols that involves surgery, radio and chemotherapy (Gajjar et al. 2013). Although those therapies can cure a large number of patients, there are considerable long-term side effects, including developmental and neurological deficits (Northcott et al. 2012).

Among the factors that can influence tumor progression, one has received special attention in the last few years. The purinergic system has recently been found amended in many tumor types (Bavaresco et al. 2008, Cappellari et al. 2012b, Spychala 2000, Stagg and Smyth 2010, Stella et al. 2010). After the discovery of the new extracellular role of ATP (Burnstock 1972), a whole new way to see the ATP was developed. ATP can be released into the extracellular medium in response to physiological or pathological conditions. The extracellular hydrolysis of this molecule and the nucleosides di- and mono phosphatated ADP, and AMP are finely controlled by membrane bound ecto-enzymes (Burnstock 2012). The final step of the hydrolysis cascade is mediated by ecto-5'-nucleotidase/CD73 (ecto-5'NT), which hydrolyzes nucleoside monophosphates such as AMP to adenosine (Stagg and Smyth 2010). Ecto-5'NT, an ecto-enzyme bound to the plasma membrane by a glycosyl-phosphatidylinositol lipid anchor has been found to be overexpressed in several types of cancer (Stella et al. 2010, Zimmermann 2000). Several pro tumoral functions such as increased invasion, migration and adhesion are related to this enzyme and the interest of

studying it as new therapeutic target has increased in the past few years (Wang H. et al. 2012, Wang L. et al. 2008).

Alpha-bisabolol (α-bisabolol) is a small, oily, non-toxic sesquiterpene that is able to incorporate into lipid rafts (Darra et al. 2008). It has been used for years in cosmetic preparations and recent studies have demonstrated a broad range of biological activities such as antimutagenic, anti-inflammatory, cytotoxic antifungal, antibacterial and gastroprotective effects (Bezerra et al. 2009, Cavalieri et al. 2004, Gomes-Carneiro et al. 2005, Kim et al. 2008, Rocha et al. 2011). The cytotoxic activity was evaluated against several cancer cell lines, but no studies with medulloblastoma cells were performed yet (Cavalieri et al. 2004, Cavalieri et al. 2011, Chen et al. 2010, Seki et al. 2011).

Considering the promising activity of α-bisabolol against cancer cells and the absence of studies using this molecule on MB cell lines the objective of this study was to evaluate the citotoxic activity of α-bisabolol against a primary and a metastatic (D283) MB cell lines. Furthermore, considering the ability of this molecule to incorporate into the lipid rafts (Darra et al. 2008), that are rich in ecto-5'NT (Babiychuk and Draeger 2006), we also evaluated this enzyme activity of cells treated with this molecule. We could observe that α-bisabolol exhibits cytotoxic effect on the MB cell lines studied and that this treatment is able to stimulate Ecto-5'NT activity.

Material and Methods

Maintenance of cell lines

DaOY (representative of a human primary tumor) and D283 (representative of a human metastatic MB), originally obtained from ATCC (American Type Culture Collection), were kindly donated by Cancer Research Laboratory and Children's Cancer Institute (HCPA

and ICI-RS, Rio Grande do Sul, Brasil). Cells were grown and maintained in Dulbecco´s Modified Eagle Medium (DMEM) 1% containing 0.5 U/mL penicillin/streptomycin antibiotics and supplemented with 10% (v/v) fetal bovine serum (FBS). Cells were kept at 37^0C, in an incubator with minimum relative humidity of 95% and atmosphere of 5% of CO_2 in air.

Assessment of medulloblastoma cell number

Cells were seed at 24 well plates and allowed to grow until reach semi-confluence. After that, cells were treated with 5, 10, 20 and, 50 μM of α-bisabolol for 48 h. At the end of the treatments, 100 μl of 0.05% trypsin/EDTA (Gibco BRL) solution was added to detach the cells, 200 μl of culture medium was added to inactivate the trypsin and cells were counted immediately in a hemocytometer. In all experiments, statistical analysis was performed comparing data to that for vehicle control groups treated with 0,5% of DMSO.

Annexin V/PI assay

Apoptotic cells were quantified using na AnnexinV-fluorescein isothiocynate-PI (AnnexinV-FITC–PI) double staining kit, according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA). D283 and DaOY medulloblastoma cells were plated in 6-well plates and treated with 20 and 50 μM of alpha-bisabolol for 48 h. At the end of the treatments, cells were washed twice with cold PBS (pH 7.4) and counted. A volume correspondent of 10^5 cells were suspended in binding buffer containing FITC-conjugated AnnexinV and PI. Samples were then incubated for 15 min at room temperature in the dark. Apoptotic and necrotic cells were quantified using a FACS Calibur cytometer (FACS Calibur; BD Bioscience, Mountain View, CA, USA). Data obtained were analyzed by

FLOWJO®software (Tree Star, INC Ashland, OR, USA). Cells were classified as: live cells (Annexin$^-$/Pi$^-$ cells), apoptotic cells (Annexin$^+$ cells) and necrotic cells (Pi$^+$ cells).

Cell cycle analysis

Cells were plated in 6-well plates, and after reaching semi-confluence, treated with 20 and 50 μM of alpha-bisabolol for 48 h. At the end of treatments, the cell medium was removed and cells were washed twice with PBS (pH 7.4), harvest, centrifuged, and suspended with 400 μL staining solution [Tris-HCl 0.5 mM (pH 7.6); 3.5 mM trisodium citrate; 0.1% (v/v) NP40; 100 μg/mL RNAse; 50 μg/ml propidium iodide (PI)] at a density of 10^6 cells/ml. After 30 min, data were collected using a flow cytometer (FACS Calibur cytometric system; BD Bioscience, Mountain View, CA, USA) and analyzed by FLOWJO®software.

Acridine Orange assay

To evaluate acidic vacular organeles (AVOs), a marker of autophagic cells, the acridine orange assay was performed. D283 cells were plated in 6 wells plate and after reaching semi-confluence, were treated with 20 and 50 μM of alpha-bisabolol for 48 h. After this, cells were incubated with 1.0 μg/mL of acridine orange for 15 min at room temperature. To quantify the AVOs, treated cells were detached from the plate with 0.05% trypsin/EDTA and analyzed by flow cytometry, using FACS Calibur cytometric system.

Ecto-5'NT activity

Ecto-5'NT activity was assayed as described by Capellari et al (Cappellari et al. 2012b) . 24-Well plates containing medulloblastoma cells that were exposed to α-bisabolol for 48 h were washed three times with phosphate-free incubation medium (2 mM $MgCl_2$, 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM Hepes, pH 7.5). The enzyme reaction was

started by the addition of 200 µl of incubation medium containing 2 mM AMP at 37˚C. After 10 min of incubation for DaOY cell line or 30 min of incubation for D283 cell line, an aliquot of the incubation medium was transferred to a pre-chilled tubes containing trichloroacetic acid (TCA, final concentration 5%, v/v). To evaluate the direct effect, a pre-incubation of 10 minutes with α-bisabolol at 5, 10, 20 and 50 µM, which was continued during the incubation with the substrate AMP was performed. To verify the real participation of ecto-5'-NT activity in AMP hydrolysis, incubations with α,β-methyleneadenosine-5'-diphosphate (APCP), an ecto-5'-NT specific inhibitor, and Levamisole (LEV), an ecto-alkaline phophatases (ALPs) inhibitor, were performed, which were added together with AMPthe substrate. Inorganic phosphate released was measured by the malachite green method (Chan et al. 1986) and controls were performed to determine non-enzymatic inorganic phosphate release. Protein was quantified by the Comassie blue method (Bradford 1976) utilizing bovine serum albumin as standard. Specific activity was expressed as nanomol of inorganic phosphate released per min per mg of protein (nmol Pi/min/mg).

Ecto-5'NT protein immunodetection

The immunocontent of ecto-5´NT was evaluated by flow citometry using anti CD73-PE-conjugated antibody (BD Biosciences, San Diego, CA, USA). Briefly, the cells were detached with trypsin and washed twice with PFA (PBS, 1% FBS, 0.1% sodium azide). Next 5 x 10^5 were incubated for 30 min with the anti CD73 antibody, with gently vortex at the middle of incubation time, and two washes with PFA were performed at the end of incubation. Cell surface fluorescence was measured in FACSCalibur Flow Cytometer (BD Biosciences) and data were analyzed with FLOWJO®software.

Statistical analysis

Data were analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by a post-hoc test for multiple comparisons (Tukey test) using GraphPad Prism Software®(GraphPad Software, INC, La Jolla, CA, USA). Data are expressed as the mean±S.D. Differences were considered significant at $p<0.05$ in relation to the vehicle control (DMSO).

Results

Medulloblastoma cell number is reduced in cell treated with α-bisabolol

To evaluate the α-bisabolol citotoxicity, the cell counting assay was performed as described in Material and methods. As shown in figure 1, the number of both DaOY and D283 cell lines were decreased when cells are treated with α-bisabolol. For DaOY, the MB *in situ* representative cell line, the treatment at the concentrations of 20 and 50 μM caused a significant reduction on cell number compared to DMSO treated cells (64,46±16,25 and 51,91±10,39 % of remaining cells in relation to control, respectively). The D283 cells, a metastatic cell line, showed to be more sensitive to α-bisabolol treatment and the significant reduction of cell number was already observed in the concentration of 10 μM when a reduction of 40% on cell number was observed. At the concentrations of 20 and 50 μM, the reduction on cell number was slightly increased and only 50% of the initial cell number was counted.

Cell number reduction was related to apoptosis induction on DaOY cell line

Once it was observed that α-bisabolol was able to significantly reduce cell number in both cell lines studied, we questioned by which mechanism it may be occurring. First, the Annexin/Pi assay was performed (Figure 2). For DaOY cell line, an increase on apoptotic

cells (Annexin + cells) was observed in both concentrations tested (35% for 20μM and 45% for 50μM). To D283 cell line, no alterations on apoptotic/necrotic cell percentage was observed.

Next, the cell cycle progression was evaluated (Figure 3). This analysis revealed that neither DaOY nor D283 cell lines has their cell cycle progression affected when treated with α-bisabolol, indicating that this treatment does not affect cell proliferation.

Finally, once to D283 cell line neither increase of apoptotic/necrotic cells nor alterations on cell cycle progression were observed, we evaluated if α-bisabolol was able to increase autophagy. Over again, no alterations were observed when data do DMSO treated group and α-bisabolol treated cells were compared (Figure 4), indicating that autophagic cell death was not triggered by this compound.

α-bisabolol increased medulloblastoma Ecto-5'-NT activity with no increase on protein level

Considering that α-bisabolol is rapidly incorporated into the lipid rafts (Darra et al. 2008), which are rich in Ecto-5'NT enzyme (Babiychuk and Draeger 2006), and the important role of this enzyme on cancer and probably MB progression (Cappellari et al. 2012b, Wang L. et al. 2008), we decided to evaluate if the treatment with α-bisabolol could alter this enzyme activity. Both DaOY and D283 cells had their ecto-5'NT activity increased when treated with α-bisabolol in all concentrations tested (Figure 5). Although significant, in DaOY cell line, this increase was lesser and the enzyme activity was enhanced in 25% only for concentrations greater than 10 μM of α-bisabolol. To D283 cell line the enzyme activity was doubled for all the concentrations tested, indicating a higher tendency of this cell to have the ecto-5'NT activity modulated in the presence of α-bisabolol.

Since the activity was increased, we decided to evaluate if the ecto-5'NT protein expression was augmented (Figure 6). To our surprise, the immunocontent of ecto-5´-NT, evaluated by flow cytometry, was not altered to none of the cell lines after the treatment with α-bisabolol. Considering that besides ecto-5'NT, there are other enzymes, which are able to hydrolyze AMP into adenosine, such as ALPs (Picher et al. 2003), we decided to go further on the investigation of altered AMP hydrolysis observed when cells were treated with α-bisabolol. Therefore we performed enzyme incubations adding the specific inhibitors of ecto-5'NT and ALPs (APCP and LEV, respectively), during the experimental reaction (Figure 7). When AMP hydrolysis was accessed in DaOY cells, the addition of APCP at 1μM in the incubation medium of cells treated with 50μM of α-bisabolol, made the enzyme activity reach the levels of control while the addition of 1mM of LEV only lead to a slightly reduction, with no alterations when compared to the treatment with α-bisabolol alone. To D283 cells, although it was possible to observe a bigger reduction when cells were treated with APCP plus α-bisabolol, none of the inhibitors were able to significantly revert the stimulus triggerd by α-bisabolol on the ecto-5'NT activity.

Direct effect of α-bisabolol on ecto-5'NT activity

To better understand the ecto-5'NT increased activity, we investigate whether α-bisabolol was able to stimulate ecto-5'NT activity after a short period of treatment during the incubation of the cells with AMP. For this purpose, cells were treated with α-bisabolol during all long the pre- and incubation times with AMP and the nucleotide hydrolysis was evaluated. As shown in Figure 8, both cell lines has the Ecto-5'NT activity significantly increased in around 25% and 40% for DaOY and D283 cell lines, respectively, considering the higher concentration of α-bisabolol used in the assay (50 μM).

Discussion and Conclusions

Human brain tumors comprise different tumors that can be distinguished at both a histological and a molecular level (Swartling et al. 2013). Among this type of tumor which can affect children, MB is the most frequent one. In spite of presenting high rates of cure, the treatment used nowadays can cause diverse side effects at a long term period (Northcott et al. 2012). Besides, in thereabout 10% of patients, cases of metastasis are observed, which can occur either in the brain or in on extraneural sites (Mazloom et al. 2010). Metastatic disease is the most important negative prognostic clinical marker for MB patients and is highly associated with current treatment failure (Gessi et al. 2012). Thus more researches in new therapeutic options and target are needed.

Here, we describe the antitumor effect of α-bisabolol, a small, non toxic sesquiterpen on two MB cell lines, one representative of an *in sito* tumor (DaOY) and other representative of an extraneural metastatic site (D283). First, we performed the cell counting assay to verify if the treatment with α-bisabolol was able to reduce cell number, which could be indicative of a cytotoxic or antiproliferative effect. In DaOY cell line, it was needed a concentration of 20 μM to reduce the cell number while for D283 half of this concentration was enough to observe the same effect, evidencing a higher sensitivity of the metastatic cell line to this treatment. These results are in accordance of previous literature data, which showed that low concentrations of this molecule exhibited interesting antitumor effects (Cavalieri et al. 2004, Seki et al. 2011).

Next, we attempted to evaluate by which mechanism this decrease on cell number was occurring. Apoptosis is a programmed cell death which is a key regulator of physiological growth and tissue homeostasis (Fulda and Debatin 2006). The acquired resistance toward apoptosis is an important hallmark of most and perhaps all types of cancer, which leads not only to tumorigenesis but also to tumor progression. For this reason, it is largely accepted that

the restoration of apoptosis is a mechanism of cancer treatment with substantial therapeutic benefits (Hanahan and Weinberg 2000). Another cell death type, broadly known necrosis, can occur depending on the initial harmful stimulus or when apoptosis machinery fails or is actively inhibited (Voigt et al. 2014). Thus, we decided to evaluate if the treatment with α-bisabolol was able to induce one of this mechanisms of cell death. When DaOY cells are treated with α-bisabolol, is possible to observe an increase on Annexin V positive cells. Besides, when we compare the percentage of cell number reduction after treatment with α-bisabolol at 50 μM (50% approximately) and the percentage of positive Annexin V cells (45%) at the same concentration, it is possible to infer that in this cell line the mechanism of cell number reduction is trough apoptosis induction. This corroborates with the literature, where it was demonstrated that α-bisabolol induces apoptosis (Cavalieri et al. 2009, Chen et al. 2010). In the metastatic D283 cells, it was not possible to observe increase on Annexin V positive cells nor in PI positive cells, indicating that neither apoptosis nor necrosis were triggered by the treatment.

An important number of chemotherapy agents used nowadays exhibit antiproliferative effects by inhibiting cell cycle progression at a particular check points such as G0/G1, S, or G2/M (Drexler 1998, Fei et al. 2014). Hence, to further evaluate the mechanism involved in α-bisabolol growth repression, we carried out the flow cytometry analysis to verify alterations on cell cycle progression. Neither in DaOY, nor in D283 cell line, alterations on this parameter was observed, indicating that the reduction of cell number is really to an induction of cell death.

Our results so far demonstrated that the cell number reduction observed when DaOY cells are treated with α-bisabolol is due to apoptosis induction while for D283, we could not determine the causes of this reduction once no increase on apoptotic/necrotic cells or cell cycle arrest were observed. For this reason, we next evaluated if α-bisabolol could be

triggering authofagic cell death on D283 cells. Autophagy is the process by which cellular material is delivered to lysosomes for degradation and recycling. It has essential roles in survival, development, and homeostasis processes but can also promote cell death, depending on the cell context (Thorburn et al. 2014, Wen S. et al. 2014). There is an increasing interest in exploring autophagy as a mechanism of action in cancer therapy once it has demonstrated to be an alternative mechanism of cell death when cells are resistant to apoptosis (Lefranc et al. 2007, Thorburn et al. 2014). To evaluate autophagy on D283 cells, we used the acridine orange staining followed by flow citometric analysis. No differences between vehicle control cells or treated cells were observed, suggesting that α-bisabolol does not induces autophagic cell death on this cell line. More studies have to be performed to evaluate which other mechanism of cell death (anoikis, entosis or mitotic catastrophe, for example) is activated when D283 cells are treated with α-bisabolol.

In the past few years, the importance of the purinergic system on the development and cancer progression has been reported in diverse studies (Bavaresco et al. 2008, Spychala 2000, Stagg and Smyth 2010, Stella et al. 2010). The ATP released in the extracellular medium can be hydrolyzed by ecto-nucleotidase triphosphate diphosphohydrolases into ADP and AMP. The final step of this cascade, the conversion of AMP to the nucleoside adenosine, is catalyzed by Ecto-5'NT. Not only the enzymes involved is this process but ATP and the products of its hydrolysis, acting trough specific receptors has broadly been related to cancer progression (Burnstock 2002, Feng et al. 2011, Jackson et al. 2007). A special spotlight has to be made on Ecto-5'NT, which is highly expressed in a variety of solid malignant tumors, and was found to be involved in cancer cell growth, differentiation, adhesion, migration, metastasis, immune escape and drug resistance (Bavaresco et al. 2008, Quezada et al. 2013, Spychala 2000, Stagg et al. 2010, Zhang 2012, Zhi et al. 2007). Once this enzyme is broadly located on the lipid rafts (Babiychuk and Draeger 2006), and it was already demonstrated that

α-bisabolol is able to incorporate on theses lipid microdomains (Darra et al. 2008), we next evaluated if the treatment with α-bisabolol could somehow alter its catalytic activity. Both cell lines studied had the AMP hydrolysis activity increased under treatment for 48h with α-bisabolol with DaOY cells having an increase of about 25% on enzyme activity and D283 cells having it duplicated. With the use of levamisole, a specific ALPs inhibitor, we had a strong evidence that the increasing in the AMP hydrolysis was a result of Ecto-5'NT stimulus. Surprisingly, when the ecto-5´-NT protein level was accessed, no alterations between controls and treated cells were observed. To better investigate this apparent discrepancy, we exposed the cells to a short-time treatment with α-bisabolol and kept the treatment during the incubation time with the substrate AMP. We then verified the same pattern of increased enzyme activity, indicating that α-bisabolol increased Ecto-5'NT by acting directly on the enzyme activity. Other studies have also observed alterations on enzyme activity with no implication on protein expression. Ledoux *et al*., also demonstrated an enhancement of Ecto-5´NT activity, with no alterations on protein level (Ledoux et al. 2003), and we previously showed an inhibition on Ecto-5'NT activity in a direct effect way and also with no alterations on protein level in a bladder cancer cell line (Rockenbach et al. 2013).

In summary, the results present herein presents for the first time, the cytotoxic effect of α-bisabolol on two MB cell lines. We also demonstrated that this treatment leads to modulation of Ecto-5'NT, an enzyme of the purinergic system. Although the antitumor results seems promising, more studies has to be done to better understand how the cytotoxic effect is triggered on D283 cell line and if this results are reproductive in an *in vivo* animal model. Besides, more studies are needed to better comprise what are the implications of the Ecto-5'NT enhanced activity and the consequences of it.

Acknowledgement

This study was supported by the Brazilian funding agencies: Fundação de Amparo a Pesquisa do Rio Grande do Sul (FAPERGS), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). F.B. Mendes was recipient of a CNPq fellowship.

Statement of conflicts of interest

None

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Figures

Figure 1

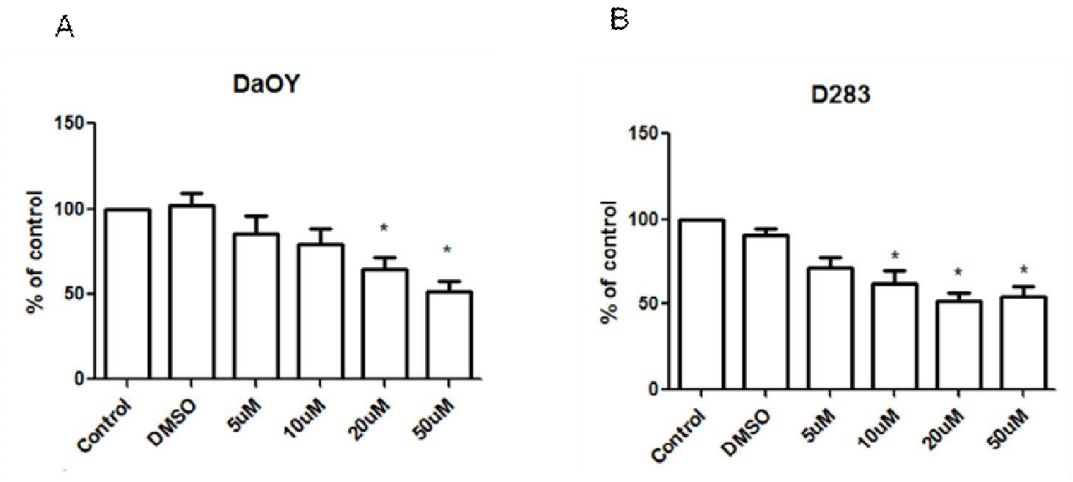

Figure 1 Cell number reduction is observed in cells treated with α-bisabolol. Cells were seed and let grown until reach semi-confluence. Treatment with 5, 10, 20 or 50 μM of α-bisabolol was performed for 48h. Cells were then detached and imediatelly conted in a haemocytometer. Data was obtained from at least four indepentent experiments and represented as mean±S.D. Statistical analysis was performed by one way ANOVA followed by Tuckey's test. *Significantly different from the DMSO-treated group ($p<0.05$).

Figure 2

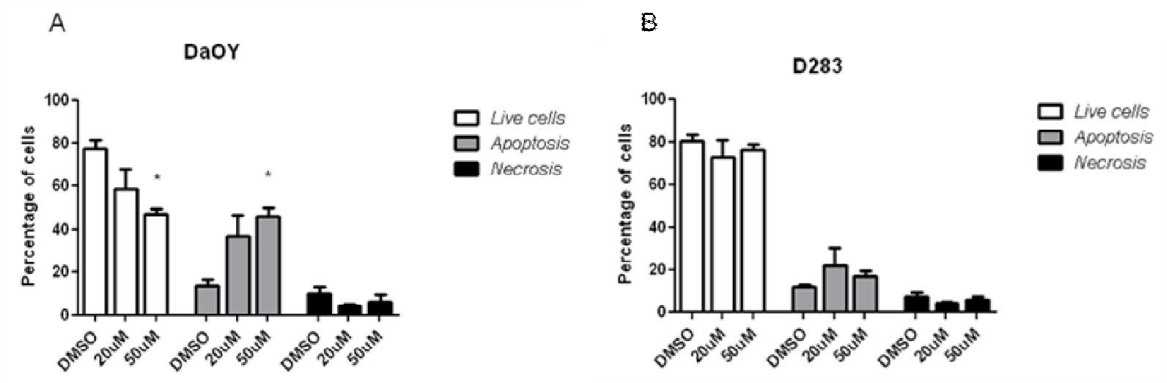

Figure 2 Treatment with α-bisabolol increased apoptotic cells in DaOY cell line. Cells were seed and let grown until reach semi-confluence. Treatment with 20 or 50 μM of α-bisabolol as performed for 48h. Cells were incubated with Annexin/PI for 15 minutes at room temperature and analysed by flow citometry. Data was calculated using the FLOWJO®software, Data was obtained from at least four indepentent experiments and are represented as mean±S.. One way ANOVA followed by post-hoc test (Tukey) was performed. *Significantly different from the DMSO-treated group ($p<0.05$).

Figure 3

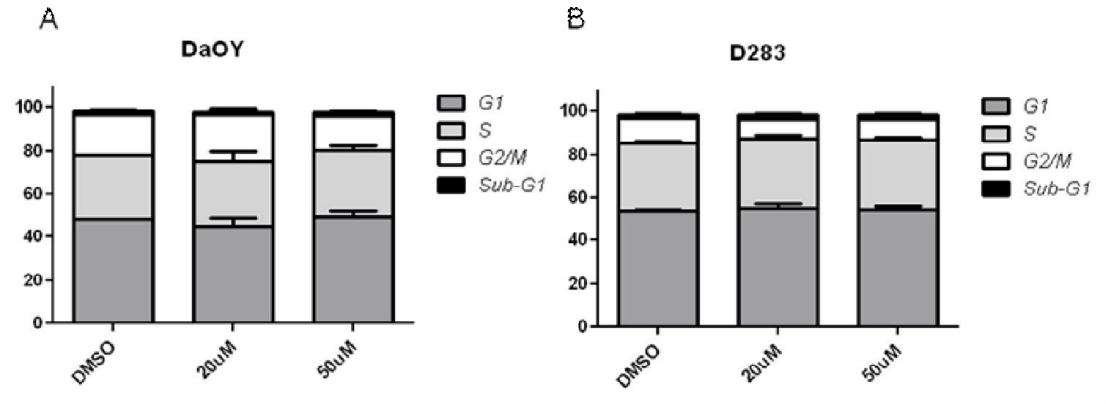

Figure 3. α-bisabolol does not alter the cell cycle progression. DaOY and D283 cell lines were treated with DMSO, 20 or a 50 μM of α-bisabolol for 48h. After treatment, cells were permeabilized and stained with propidium iodide for determination of cell cycle distribution (sub-G1, G1, S and G2/M) by flow cytometry. Peak areas in the histogram were calculated using FLOWJO®software. Data are representative of at least three experiments and are presented as the means±S.D and were analyzed by one-way ANOVA followed by post-hoc comparisons (Tukey). *Significantly different from the DMSO-treated group ($p<0.05$).

Figure 4

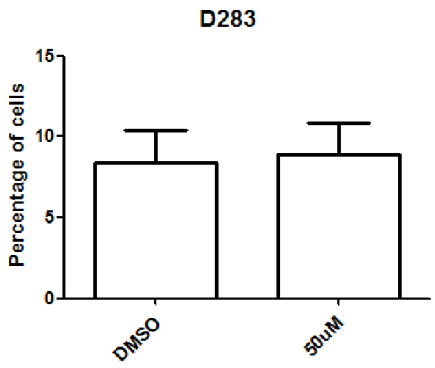

Figure 4. α-bisabolol treatment does not increased the presence of AVOs. Cells were treated with DMSO and α-bisabolol at 50μM for 48h. Acridine orange staining was performed in PBS solution at 1 μg/mL of the dye and after incubation cells were analyzed by flow cytometry. Data was obtaining from at least four independent experiments and are represented as mean±S.D. Statistical analysis was performed by one way ANOVA followed by Tuckey's test. *Significantly different from the DMSO-treated group ($p<0.05$).

Figure 5

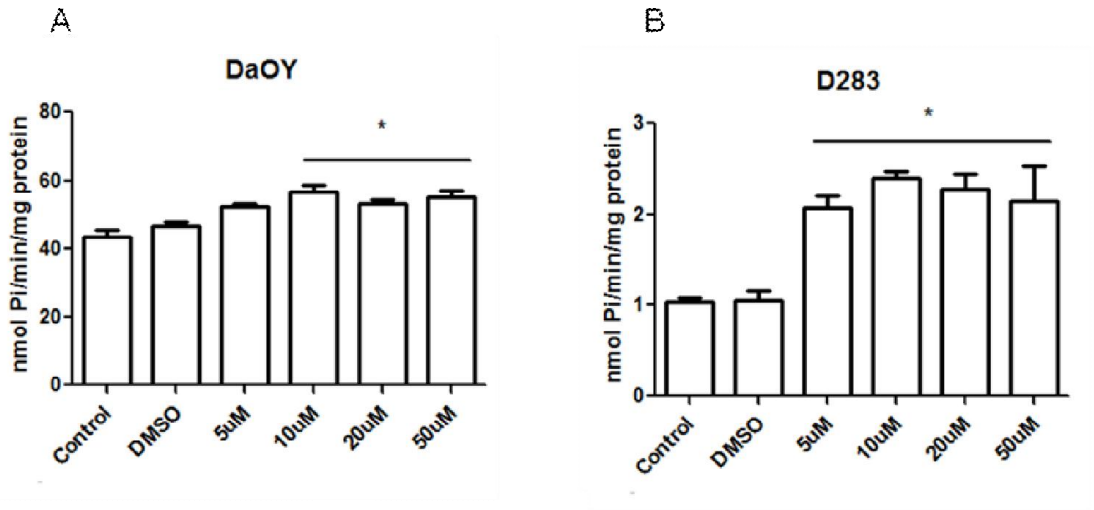

Figure 5. Medulloblastoma cell lines has the ecto-5'NT activity enhanced when treated with α-bisabolol. DaOY and D283 cell lines were treated with 5, 10, 20 or 50 μM of α-bisabolol for 48h. After treatment, cells were submitted to enzymatic assay for AMP hydrolysis by malachite green method. Specific activity of ecto-5'NT is expressed as nmol Pi/min/mg protein. Data represent the mean±S.D of at least four independent experiments. Data were analyzed by one-way ANOVA followed by post-hoc comparisons (Tukey). *Significantly different from the DMSO-treated group ($p<0.05$).

Figure 6

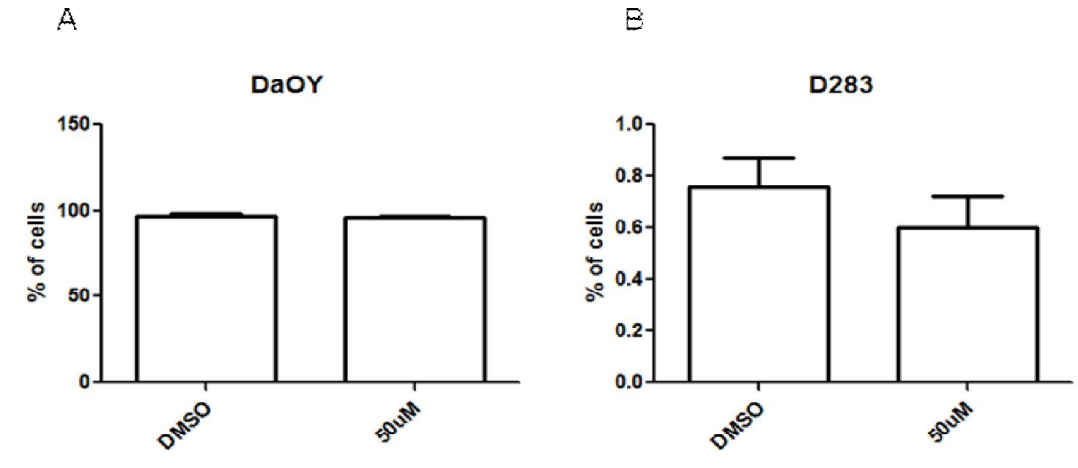

Figure 6. Ecto-5'NT expression is not altered in cells treated with α-bisabolol. Cells were treated with α-bisabolol 50μM for 48h, then, were detached and incubated with anti CD73 labeled antibody for 30min. At the end of incubation, cells were washed and ecto-5'NT immunocontent was analysed by flow citometry. Results are presented as mean±S.D of at least four experiments. Data were analysed by one-way ANOVA followed by post-hoc comparisons (Tukey) and no significative differences were observed.

Figure 7

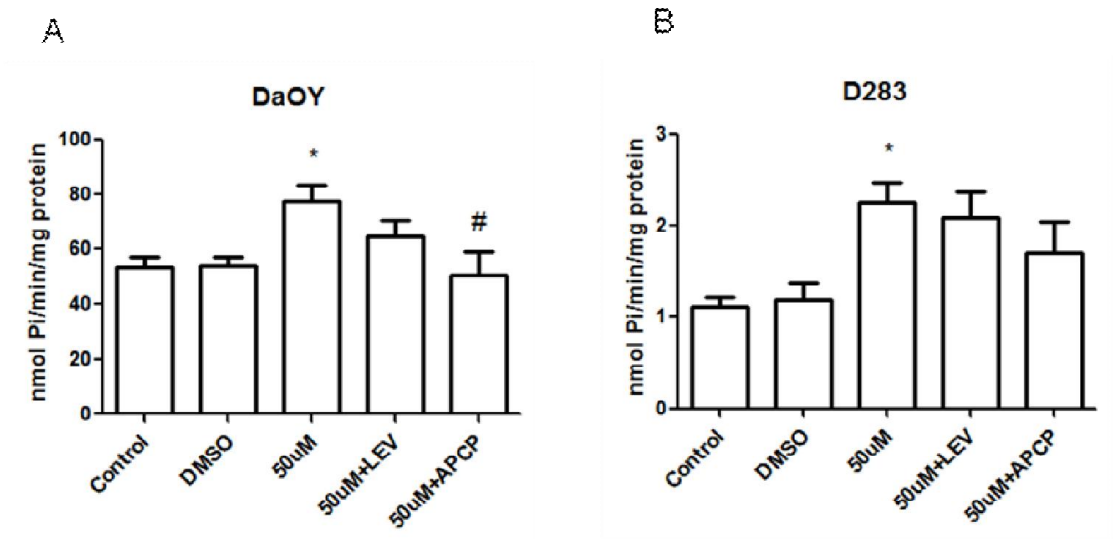

Figure 7 Incubation with the specific inhibitors levamisole (LEV) and α,β-methyleneadenosine-5'-diphosphate (**APCP) confirmed that the increased AMP hydrolysis is due to ecto-5'NT activity.** DaOY and D283 cell lines were treated with 50 μM of α-bisabolol for 48h. After treatment, cells were submitted to enzymatic assay for AMP hydrolysis by malachite green method. The specific inhibitors APCP and LEV were added together with AMP in the incubation medium of the enzymatic reaction. Specific activity of ecto-5'NT is expressed as nmol Pi/min/mg protein. Data represent the mean±S.D of at least four independent experiments. Data were analyzed by one-way ANOVA followed by post-hoc comparisons (Tukey). *Significantly different from the DMSO-treated group ($p<0.05$).

Figure 8

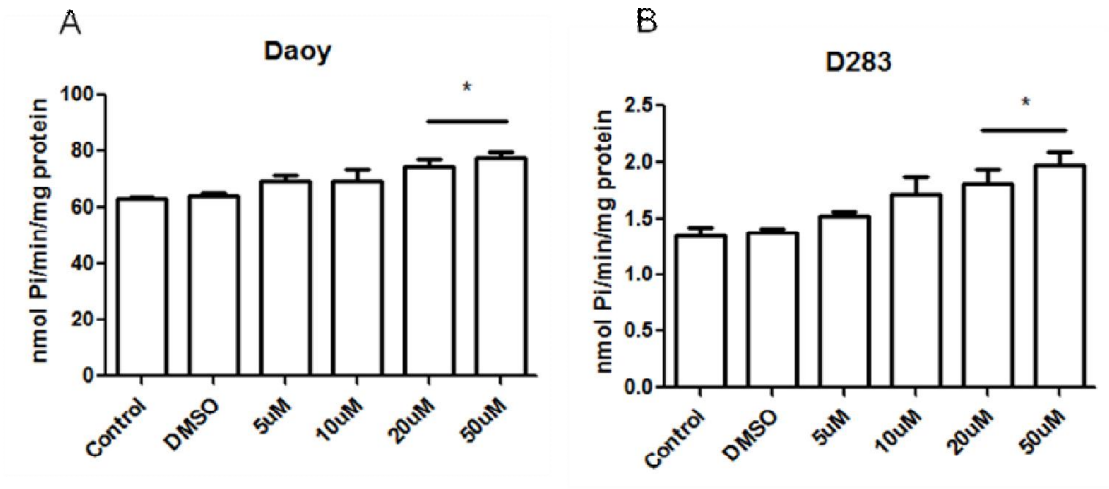

Figure 8. α-bisabolol treatment acts directly on Ecto-5'-NT increasing AMP hydrolysis activity. DaOY and D283 cell lines were seed and let grown until reach confluence. Cells were pre-incubated with α-bisabolol at the concentrations of 5, 10, 20 or 50 μM of α-bisabolol for 10 min. Cells were submitted to enzymatic assay for AMP hydrolysis by malachite green method, using AMP as substrate and in presence of α-bisabolol in the indicated concentrations. Specific activity of ecto-5'NT is expressed as nmol Pi/min/mg protein. Data represent the mean±S.D of at least four independent experiments. Data were analyzed by one-way ANOVA followed by post-hoc comparisons (Tukey). *Significantly different from the DMSO-treated group ($p<0.05$).

3.3 Capítulo 3

BISABOLOL OXIDE A CYTOTOXIC EFFECT ON GLIOMA AND MEDULLOBLASTOMA CELL LINES

Manuscrito a ser submetido ao periódico Anticancer Research

Bisabolol oxide A cytotoxic effect on glioma and medulloblastoma cell lines

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Key Words: Glioma, medulloblastoma, bisabolol oxide A.

Short Title: Citotoxic effect of bisabolol oxide A.

Date of Submission: 10/06/2014 (Experimental Work)

Abstract. Background/Aim: Brain cancer are a challenge disease to treat. Among them, the most prevalent tumors are medulloblastoma and gliomas in children and adults respectively. Alpha-bisabolol is a natural sesquiterpene that possesses an interesting cytotoxic activity and few studies were performed with the natural analogue bisabolol oxide A. Here we describe and compare the citotoxic effect of bisabolol oxide A with the effect triggered by alpha-bisabolol. **Materials and Methods:** Cells were treated with alpha-bisabolol or bisabolol oxide A for 48h. Sulphorhodamin B, to evaluate cytotoxicity and flow cytometry, to evaluate cell cycle, were performed. **Results and Discussion:** Treatment with alpha-bisabolol reduced cell viability more efficiently than bisabolol oxide A in the cell lines tested, except for human U251 cell line, were bisabolol oxide A was more efficient. The cell cycle assay performed in U251 treated cells revealed that in this cell line, bisabolol oxide A leads to cell cycle arrest while no effects on cell cycle were observed in cells treated with alpha-bisabolol. **Conclusion:** The results presented herein suggest that both alpha-bisabolol and bisabolol oxide A are promising chemotropic agents. The efficiency of treatments may vary depending on the molecular signature of each tumor.

Brain tumors are rare, but their incidence and mortality have increased in different countries. Although less is known about these tumors etiology, it is believed that genetic, hormonal and ambiental factors are involved (Monteiro and Koifman 2003). Those tumors are a heterogeneous group of neoplasms that vary widely on morphologic features, malignancy, site of origin and treatment response (Gurney and Kadan-Lottick 2001). Among all the possible tumors that can attain this noble tissue, two deserve special attention: medulloblastomas and gliomas. The first is the most common brain tumor in children and the second is the most common and lethal in adults (Swartling et al. 2013).

Medulloblastomas are the most common solid tumor in children and the second more prevalent tumor in this age group, being only leukemia more prevalent (Huttner 2012). This tumor usually arises from the cerebellum and is the major cause of morbity and mortality in pediatric oncology (Taylor et al. 2012). Although it is curable in approximately 70% of patients, the progress in improving survival using conventional therapies has stalled (Gottardo et al. 2014). Besides, the therapies currently are consider aggressive and the patients suffer from long-term side effects, including developmental, neurological, neuroendocrine and psychosocial deficits (Northcott et al. 2012).

Gliomas are divided into four subtypes, being the glioblastoma multiforme (GBM, referred to as grade IV by the World Health Organization) the deadliest one (Van Meir et al. 2010). The standard treatment nowadays consists in surgery, which can be followed by radiotherapy. This tumor, however, is highly infiltrative throughout the brain what makes difficult the complete resection. Moreover, remaining cancer cells are able to self-renew, creating a new tumor (Nieto-Sampedro et al. 2011). Also, this tumor exhibits robust cell proliferation, imumunosupression and intrinsic resistance to radio and chemotherapy and as a result, survival rates have not increased over the past years (Beier et al. 2011). In spite of the great efforts made over the past decades, few GBM patients survive more than five years and new efforts have to be made in order to develop new therapy approaches (Robins et al. 2009).

Once treating brain cancers is still a challenge in therapy, arises the necessity of developing new and more efficient drugs. In this scenery, a small, oily, non-toxic natural molecule known as alpha-bisabolol shows as an interesting new possibility of treatment. Being the main component of *Matricaria chamomilla* essential oil, alpha bisabolol exhibited an interesting cytotoxic effect in a large variety of cancer cell types, including glioma and medulloblastoma (Cavalieri et al. 2004, Cavalieri et al. 2011, Darra et al. 2008). Also, some works reported that synthetic analogues derived from this molecule had interesting antitumor effects (Piochon et al. 2009, Spitzner and Zepf 2006). Although synthetic molecule had been developed and tested, only one study to evaluate the cytotoxic effect of the natural analogue bisabolol oxide A has been performed (Ogata-Ikeda et al. 2011).

Considering the importance to study news therapies to treat brain cancer and that few studies of the cytotoxic effect of bisabolol oxide A had been performed, the objective of the present work was to evaluate the antitumor effect of this molecule in medulloblastoma and glioma cell lines and compare its effect with the one triggered by alpha-bisabolol.

Materials and Methods

Maintenance of cell lines. The rat C6, human U138 and U251 glioma cell lines were obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA). The medulloblastoma Daoy (representative of a human primary tumor) and D283 (representative of a metastatic site) cell lines were kindly donated by Laboratório de pesquisas em Câncer e Instituto de Câncer Infantil (HCPA and ICI-RS, Rio Grande do Sul, Brasil). Both cell lines were originally obtained from the ATCC (American Type Culture Collection). Cells were

grown and maintained in 1% Dulbecco's modified Eagle's medium (DMEM) containing antibiotics (0.5 U/ml penicillin/streptomycin) and supplemented with 5% (for C6 cell line only) and 10% (v/v) fetal bovine serum (FBS), all from Gibco BRL, Carlsbad, CA, USA. Cells were kept at a temperature of 37°C, minimum relative humidity of 95%, and atmosphere of 5% CO_2 in air.

Assessment of citotoxic effect. For sulphorhodamin B (SBR) (Sigma, St. Louis, MO, USA) assay, cells were seeded in 96-well plates and allowed to grow until reaching semi-confluence. Cells were treated with 10, 20, 50, 75, 100, 150 and 200 µM of alpha-bisabolol or bisabolol oxide A for 48 h. At the end of the treatments, cells were washed twice with PBS, fixed with TCA 10% (w/v), for 1h at 4^0C and next washed three times with distilled water. After complete drying the plates were stained with SBR 0,4% in acetic acid 1% for 15 minutes. Next, the stain excess was removed by washing the plates 5 times with acetic acid 1% and 100 µl of Tris-base pH 10 was added to each well. The absorbance was read at 492 nm. Cell percentages were calculated considering the control (cells without any treatment) as 100%.

Cell cycle analysis. Cells were plated in 6-well plates, and after reaching semi-confluence they were treated with 100 and 200 µM of alpha-bisabolol or bisabolol oxide A for 48 h. At the end of treatment, the cell medium was removed and cells were washed twice with PBS (pH 7.4), harvest, centrifuged, and suspended with 400 µL staining solution [Tris-HCl 0.5 mM (pH 7.6); 3.5 mM trisodium citrate; 0.1% (v/v) NP40; 100 µg/mL RNAse; 50 µg/ml propidium iodide (PI)] at a density of 10^6 cells/ml. After 30 min, data were collected using a flow cytometer (FACS Calibur cytometric system; BD Bioscience, Mountain View, CA, USA) and analyzed by FLOWJO® software.

Statistical analysis. Data were analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by a post-hoc test for multiple comparisons (Tukey test) using *GraphPad Prism Software*®. Data are expressed as the mean±S.E.M. Differences were considered significant at $p<0.05$.

Results

Alpha-bisabolol was able to reduce cell proliferation of glioma and medulloblastoma cell lines. The cytotoxicity of alpha-bisabolol and bisabolol oxide A was evaluated by the SBR assay. For this, D283 and DaOY medulloblatoma cells and C6, U138, U251 glioma cell lines were seed in 96 well plates and treated as described in Material and methods. A significant reduction of D283 cell proliferation was observed cells from the concentrations of 75 μM (69 ± 2,48 %, 64,99 ± 11,69 %, 42,67 ± 10,43 %, 42,73 ± 7,13 % of viable cells, to 75 μM, 100 μM, 150 μM and 200 μM of alpha-bisabolol respectively) (Figure 1A). The same concentrations were also efficient in reduce cell proliferation of DaOY cells in around 20% for 75 μM and 100 μM and 50% for 150 μM and 200 μM (Figure 1 C).

C6 was the most sensitive glioma cell line to alpha-bisabolol treatment (Figure 2A). Although treatment with 100 μM was needed to observe a reduction of 30% on cell proliferation was needed, in the higher concentration tested, only 25% of cells remained. In U138 cells (Figure 2C), only 150 μM and 200 μM of treatment were able to reduce cell proliferation and both in 30%. The U251 cell line reveald to be the less responsible to alpha-bisabolol treatment (Figure 2E), and 200 μM of treatment was needed to observe a significative cell proliferation reduction. Alpha-bisabolol was more efficient to reduce cell proliferation in the medulloblastoma cell lines than in glioma cell lines.

Bisabolol oxide A is not as potent as alpha-bisabolol. Cells were seed and treated for 48h with bisabolol oxide A at the same concentrations used to alpha-bisabolol. In DaOY cell line (Figure 1D), 200 μM of treatment was needed to induce 50% of cell reduction. D283 (Figure 1 B) cells needed at least a bisabolol oxide A concentration of 100 μM so the cell proliferation reduction could be observed and at the concentration of 200 μM only 34,66 ± 15,80 % of cells were observed.

In U138 cells, no effect on cell proliferation reduction was observed, in none of the concentrations tested (Figure 2D). In C6 cells (Figure 2B), cell reduction was observed in the same concentrations as for alpha-bisabolol with a less pronounced cytotoxic effect though (75,11 ± 18,93 %, 56,89 ± 18,03 and 48, 84 ± 19,35 % of viable cells in the concentration of 100 μM, 150 μM and 200 μM respenctively). Interestingly, from all cell lines tested, only U251 was more sensitive to bisabolol oxide A in comparison to the treatment of alpha-bisabolol (Figure 2F). Not only a tendency to reduce cell proliferation was observed in lower concentrations but the significative difference was reached in a lower concentration (150 μM

for bisabolol oxide A versus 200 μM for alpha-bisabolol). In the higher concentration tested, only 50% of cells remained alive after bisabolol oxide A treatment.

Bisabolol oxide A induces cell cycle arrest on U251 cell line. To evaluate alterations on cell cyle, U251 cells were seed in 6 well plates let grown until reach semi-confluence and treated for 48h with 100 and 200 μM of alpha-bisabolol or bisabolol oxide A (Figure 3). Cells treated with alpha-bisabolol exhibited a normal cell cycle profile, with no differences when the cell percentages are compared to the one observed to the control. When cells are treated with bisabolol oxide A, on the other hand, a cell cycle arrest was observed on S phase (25,91 ± 1,531 % of cells on this phase for 200 μM treatment versus 15, 07 ± 2,817 % on control group). Also, when compared to control, the treatment with bisabolol oxide A reduced the percentage of cells on G1 phase from 68,99 ± 7,135 % to control cells to 56,24 ± 9,021 % to 200 μM treatment.

Discussion

Brain tumors are a challenge in oncology. Independently of the tumor type, the most common therapy protocol is based in surgery, which can be followed by radiotherapy and/or chemotherapy (Bohan 2013, Fisher et al. 2007). The main issues of the current protocol is that the brain is a noble tissue and a complete and efficient resection usually is not possible without highly affecting the patient's quality of life (Bohan 2013). Besides, although radiotherapy have increased the life expectancy of the patients, the chemotherapic agents currently known are not really efficient to this disease treatment (Sengupta et al. 2012).

In the past years, alpha-bisabolol, a small, non-toxic, sesquiterpene has arrised as a new promising drug. Numerous studies has reported it has promissing biological activities, including depigmenting, antimutagenic, anti-inflamatory, antifungal, gastroprotector and antitumoral effects (Bezerra et al. 2009, Gomes-Carneiro et al. 2005, Kim et al. 2008, Rocha et al. 2011). The antitumor effect was reported to a large variety of tumor cell lines, including glioma, medulloblastoma, liver carcinoma, leukemia, pancreatic, for example (Cavalieri et al. 2004, Cavalieri et al. 2011, Chen et al. 2010, Seki et al. 2011). Its efficiency against cancer cell line reported prompted some authors to develop and test some synthetic analogues of this

compound, looking for a bisabolol-based but more effective molecule (Piochon et al. 2009, Spitzner and Zepf 2006). However, few studies were performed with the natural bisabolol anologue though, and so, this was the objective of this study.

We first treated a panel of two medulloblastoma cell lines and three glioma cell lines with alpha-bisabolol. To medulloblastomas, the concentration of 75 μM was sufficient to significantly reduce cell proliferation by 20% and 30% for DaOY and D283 respectively, while for glioma cells, the lower concentration needed to reduce cell proliferation was 100 μM and this concentration exert it effect only in the C6 cell line. For U138 cell line, a dose response tendency was observed but only at 150 μM and 200 μM it was possible to observe a significant reduction on cell proliferation. Finally, for U251 cells, a reduction of 30% was observed and only at the higher concentration tested. Next, we treated the same cell lines with bisabolol oxide A. DaOY cell line revealed to be very resistant to bisabolol oxide A, with decreasing on cell viability being observed only at the concentration of 200 μM. The metastatic medulloblastoma cell line D283 was sensible to bisabolol oxide A at the concentrations of 100, 150 and 200 μM, and although alfa-bisabolol was able to reduce the cell proliferation to this three concentrations plus at 75 μM the magnitude on cell proliferation reduction was similar to both treatment. In C6 glioma cells, the cell reduction was observed in the same concentrations as for alpha-bisabolol, with a different in magnitude, being alpha-bisabolol more potent that the natural analogue. U138 cell proliferation was not altered when cells were treated with bisabolol oxide A. From all the cell lines tested, only U251 glioma cell line revealed to be less sensitive to alpha-bisabolol than to bisabolol oxide A. At the higher concentration tested, 30% of cell death was observed when cells were treated with alpha-bisabolol and 50% when treated with bisabolol oxide A. The difference on cell sensitivity to the treatments is probably due to the differential protein expression in each cell line. Although both U251 and U138 cell lines were established from human glioblastoma tumors, for example, the protein identity expression is completely different for some key proteins that are related to cancer progression and development such as PTEN and p53 (Filippi-Chiela et al. 2013).

Once only the U251 cell line revealed to be more sensitive to bisabolol oxide A treatment, we decided to go deeper in the cell death mechanism triggered by those compounds in this cell line. Neither alpha-bisabolol nor bisabolol oxide A were able to induce apoptosis or necrosis on U251 cell line (data not shown). When cell cycle analysis was performed, it

was possible to observe an antiproliferative effect in cells treated with bisabolol oxide A, with a cell cycle arrest on S phase, accompanied with a reduction on G1 phase. Cell cycle deregulation, along with apopotosis inhibition, are two mechanisms involved in the tumor formation, and many chemotherapic agents act through the induction of apoptosis and cell cycle arrest to block the tumor formation process (Zamin et al. 2009). More studies have to be performed to understand which checkpoints of cell cycle are affected by this treatment and also to understand the implication of this arrest. Resveratrol, a natural compound that had been hardly studied to its antitumor activity, also promotes cell cycle arrest in some cell lines acting trough ATM/ATR–Chk1/2–Cdc25C–Cdc2 pathway, for example (Tyagi et al. 2005). It is also possible that this arrest be in fact a senescence induction, or an irreversible arrest. In this case, other pathways such as p38 MAPK cascade, and Rb and p53 tumor could be involved in the mechanism induced by bisabolol oxide A.

In summary, herein, we demonstrate that both alpha-bisabolol and bisabolol oxide A are effective against brain tumor cell lines. With in general, a stronger effect of alpha-bisabolol. More studies have to be performed to better understand the exact mechanism by which those drugs acts and to elucidate if in *in vivo* this more pronounced activity of alpha-bisabolol persists.

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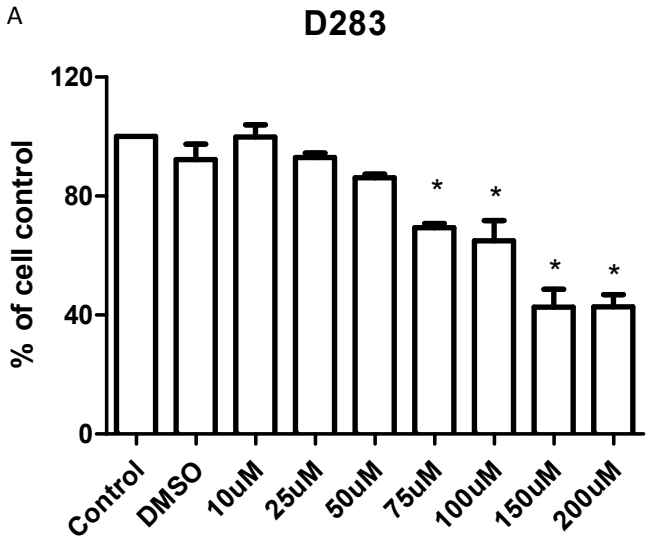
Figures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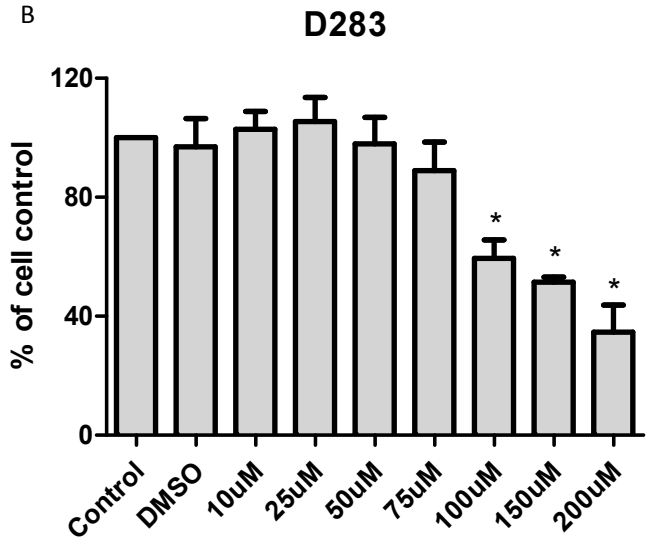
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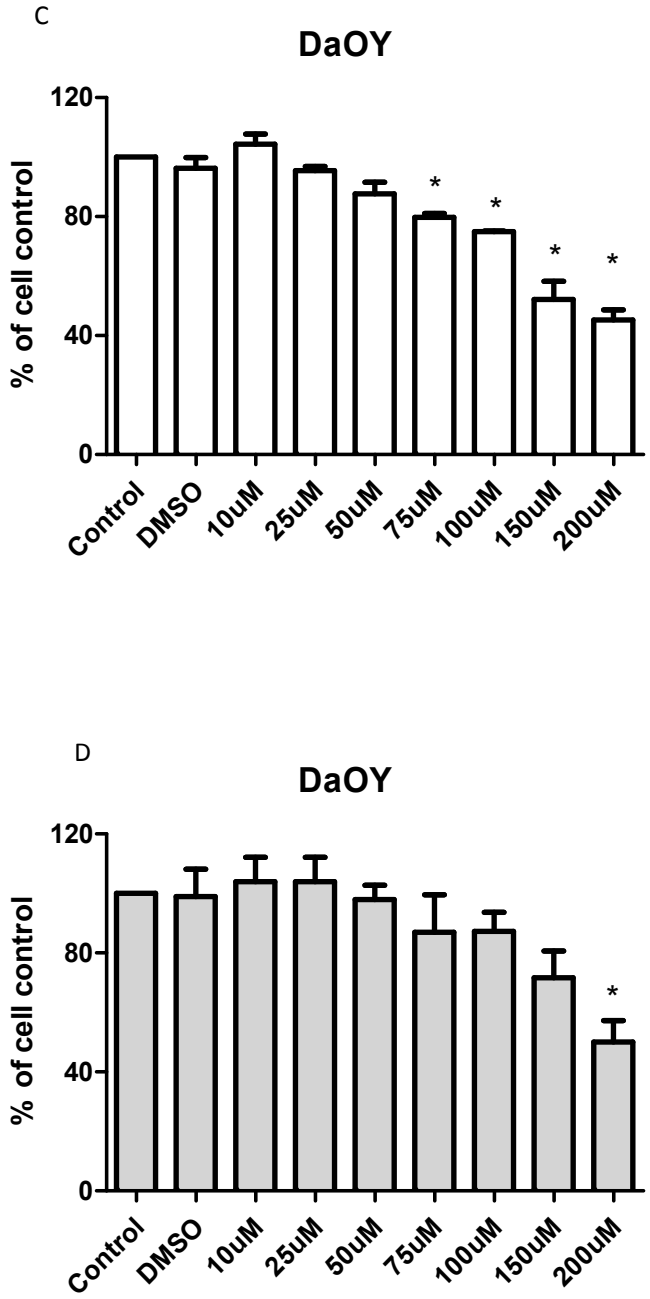

Figure 1: Comparative effect of alpha-bisabolol and bisabolol oxide A on medulloblastoma cell growth Cells were seed in 96 well plates and treated with alpha-bisabolol (A and C) or bisabolol oxide A (B and D) for 48h at the concentrations of 10 µM, 25 µM, 50 µM, 75 µM, 100 µM, 150 µM or 200 µM. After treatment, SBR assay was performed as described in Material and methods. Data was calculated in relation to control cells and the values represent

the mean ± S.D of four independent experiments. Data were analyzed by one-way ANOVA followed by post-hoc comparisons (Tukey test). *Significantly different from the DMSO-treated group ($p<0.05$).

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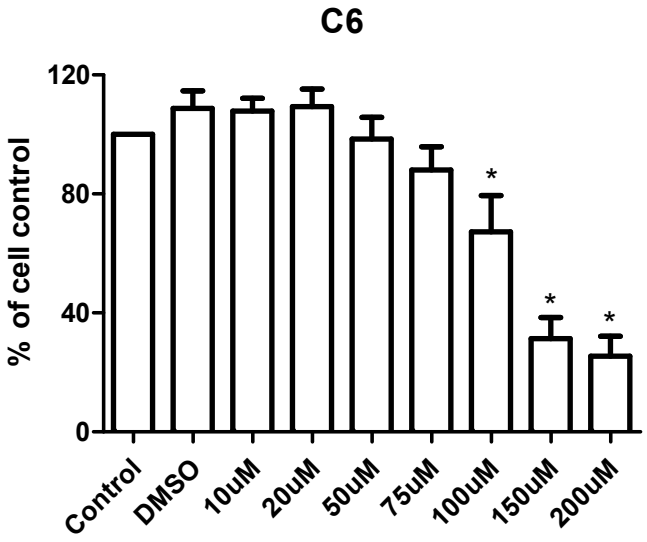

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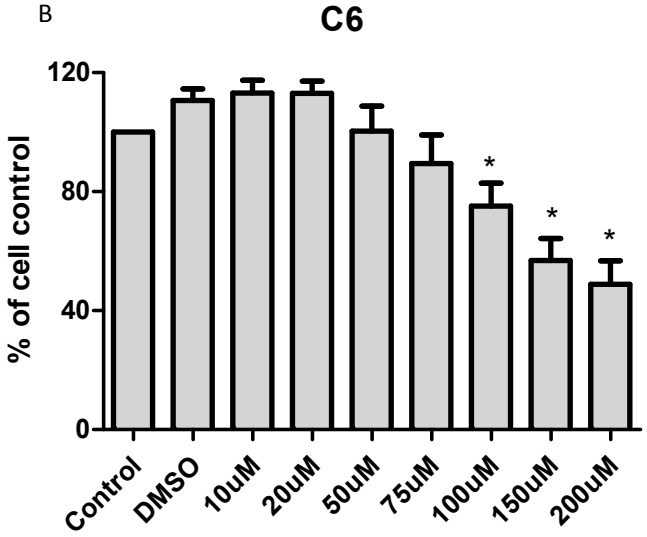

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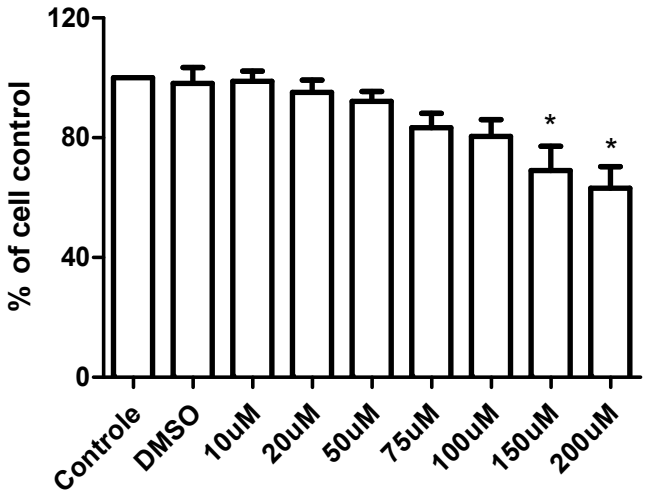
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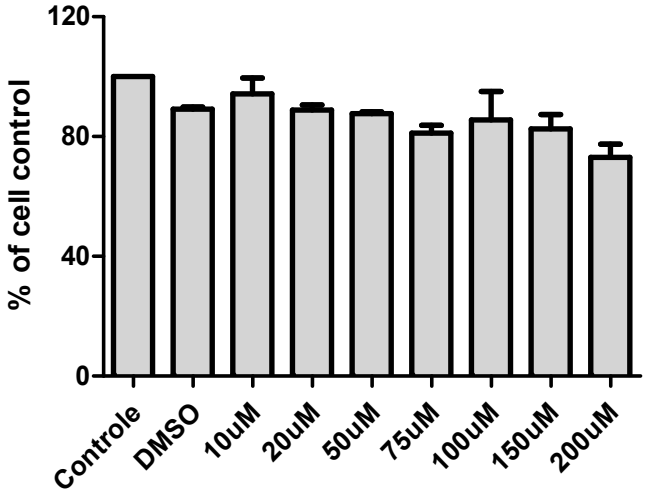
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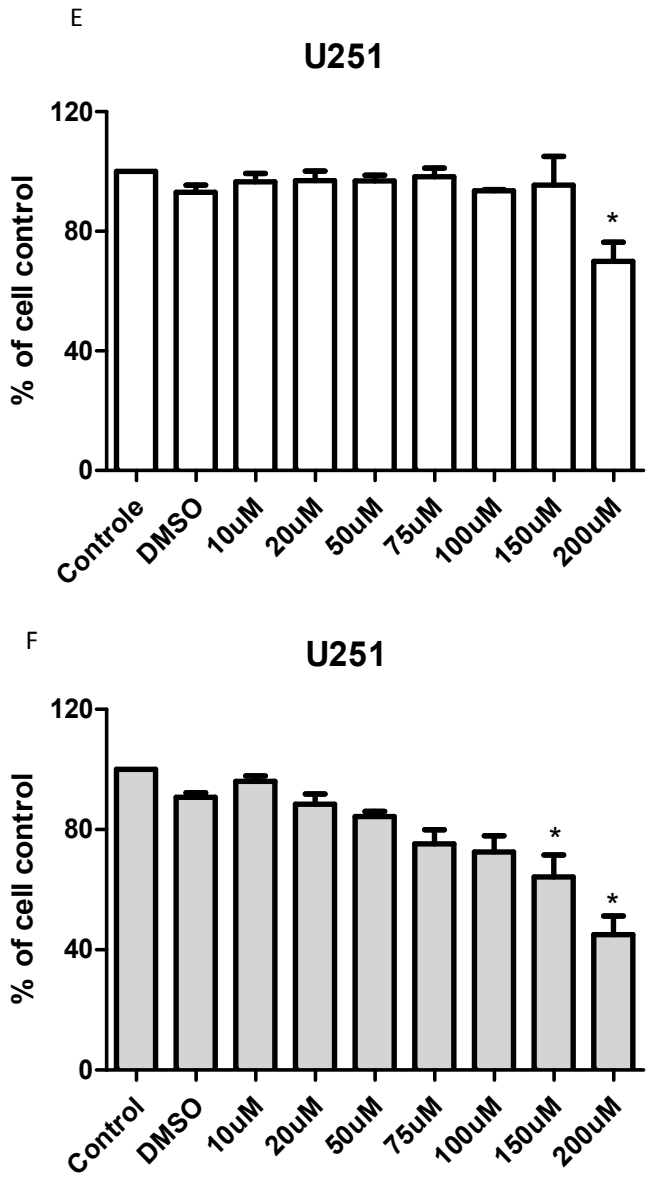

Figure 2: C6 and U138 cell lines are more sensitive to alpha-bisabolol and U251 cells were more sentitive to bisabolol oxide A treatment. After treatment with alpha-bisabolol (A, C, E) and bisabolol oxide A (B, D and F) cells were washed, fixed and SBR assay was performed. Data was calculated in relation to control cells. Data represent the mean ± S.D of four independent experiment and was analyzed by one-way ANOVA followed by post-hoc comparisons (Tukey). *Significantly different from the DMSO-treated group ($p<0.05$).

Graph in white are representative of alpha-bisabolol treated cells and graphs in gray are representative of bisabolol oxide A treated cells.

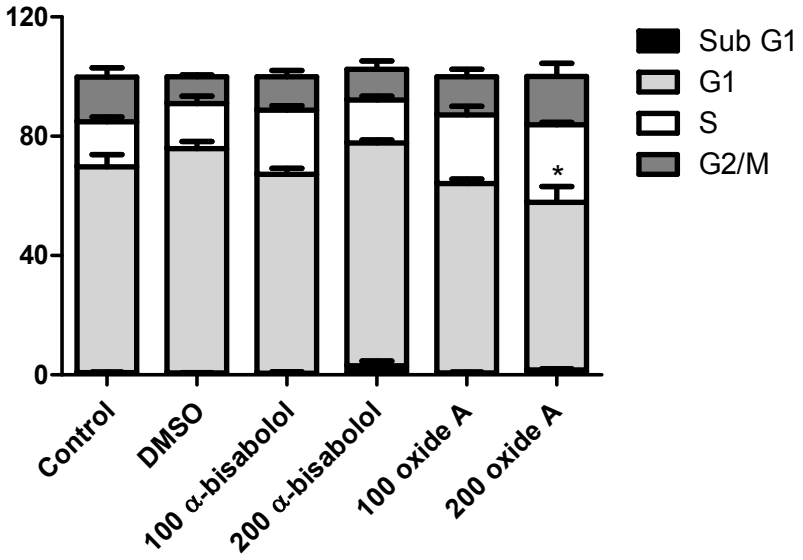

Figure 3: Cell cicle arrest on S phase is observed when U251 cells are treated with bisabolol oxide A. Cells were seed on 6 well plates and after reaching semi confluence, treated with alpha-bisabolol or bisabolol oxide A at the concentrations of 100 μM or 200 μM. Data are representative of three independent experiments. Statistic analysis was performed by ANOVA followed by Tuckey post-hoc test.). *Significantly different from the DMSO-treated group ($p<0.05$).

4. Discussão

Segundo a Sociedade Americana de Câncer, os tumores cerebrais encontram-se entre as 10 maiores causas de mortes relacionadas ao câncer (American Cancer Society, 1998). Os tumores cerebrais primários são responsáveis por aproximadamente treze mil mortes por ano apenas nos Estados Unidos, de acordo com a Central de Registro de Tumores Cerebrais dos Estados Unidos (ou CBTRUS, do inglês Central Brain Tumor Registry of the United States), uma espécie de ONG, que compila e divulga os dados epidemiológicos dos tumores cerebrais naquele país (CBTRUS, 2000). Além disso, a incidência ajustada desses tumores entre os anos de 2006-2010 quando comparado a taxas de 1998 praticamente dobraram (CBTRUS, 1998, Ostrom et. al, 2013). Mais recentemente, acredita-se que a taxa de incidência, somente nos Estados Unidos, chega a 27,38 novos casos a cada 100.000 habitantes entre os adultos contra 5,26 novos casos em crianças e jovens entre 0 a 19 anos (Ostrom et al. 2013). Apesar de os dados epidemiológicos não parecerem alarmantes quanto ao número total de casos, é válido lembrar que por serem tumores que acometem um órgão do vital do corpo humano, a consequente morbi-mortalidade desses tumores é imensa. Dentre os diversos tumores cerebrais os gliomas e meduloblastomas merecem especial atenção por serem os mais prevalentes em adultos e crianças, respectivamente (Swartling et al. 2013).

O tratamento do câncer como um todo já configura um grande desafio, pois essa patologia possui seis ”hallmarks” (dentre eles sinalização sustentada de proliferação, insensibilidade a supressores de crecimento e resistência à morte celular), ou capacidades distintas e complementares, que possibilitam o crescimento e progressão tumoral, sendo que cada um desses “Hallmarks” é composto por

diversas alterações em diferentes vias de sinalização (Hanahan and Weinberg 2011). Assim, apesar de muitos serem os possíveis alvos farmacológicos para o tratamento, as células tumorais em resposta à terapia são capazes de diminuir sua dependência da Hallmark alvo do quimioterápico de escolha e se tornar mais dependente de outra, sem prejuízo a sua progressão (Hanahan and Weinberg 2011). Quando se considera os tumores cerebrais, o desafio é ainda maior, pois o cérebro é protegido contra substâncias potencialmente tóxicas pela barreira hematoencefálica, que também restringe a passagem de grande parte das moléculas antitumorais conhecidas (Patel et al. 2009). Assim, novas alternativas terapêuticas se fazem necessárias para o tratamento dessas neoplasias.

Moléculas provenientes de fontes vegetais são há muito tempo utilizadas no tratamento de diversas patologias. Em estudo realizado analisando medicamentos disponíveis no mercado entre o período de 1981 e 2002, foi constatado que cerca de 40% do total destes são derivados direto de vegetais (isolados ou obtidos por meio de semisíntese) ou foram sintetizados com base em grupos farmacofóricos de produtos naturais (Newman et al. 2000, 2003). Quando pensamos em fármacos antineoplásicos especificamente, esse índice sobe para cerca de 60% (Newman et al. 2003). Exemplos de quimioterápicos obtidos de plantas e que hoje são amplamente usados são o paclitaxel e a vincristina. O primeiro é um inibidor mitótico, que foi isolado a partir de uma árvore conhecida como teixo (*Taxus brevifolia)* e utilizado no tratamento de neoplasias de pulmão, ovário e mama, enquanto que o segundo é um alcalóide isolado da planta *Catharanthus roseus*, usada no tratamento de leucemias, linfomas e neuroblastoma. Ainda hoje a pesquisa de novas drogas se baseia em compostos presentes em diferentes espécies vegetais, devido às promissoras atividades biológicas destes.

Nesse cenário, onde as moléculas provenientes de plantas se mostram promissoras no tratamento contra o câncer e novas alternativas terapêuticas se fazem necessárias, o alfa-bisabolol surge como um novo promissor agente quimioterápico. A atividade citotóxica desse composto foi descrita pela primeira vez em 2004. Cavalieri e colaboradores utilizaram três linhagens de glioma para demonstrar a eficácia desse composto. Observaram que a concentração de 2,5 μM foi suficiente para reduzir em 50% a viabilidade de duas das linhagens testadas e que a linhagem mais resistente ao tratamento teve uma redução desta mesma magnitude com 5 μM de tratamento (Cavalieri et al. 2004). No presente trabalho, começamos a observar reduções significativas de viabilidade celular com concentrações maiores de tratamento (75 μM para as linhagens de meduloblastoma, 100 μM para a linhagem C6 de glioma de rato, 150 μM e 200 μM para as linhagens de glioma humano U138 e 200 μM para a linhagem U251, de acordo com os resultados obtidos pelo experimento utilizando sulfarodamina B). Essa aparente disparidade de resultados pode ser explicada porque Cavalieri e colaboradores realizaram uma estimativa da real fração solúvel de alfa-bisabolol nos meios de cultivo, uma vez que essa molécula é bastante hidrofóbica. A análise por HPLC realizada no estudo de Claviere e cols. mostrou que apenas 2,5% da quantidade inicial adicionada de alfa-bisabolol se encontrava realmente solúvel no meio, o que quer dizer que quando se tinha uma concentração teórica de 250 μM, por exemplo, apenas uma concentração de 6,25 μM estaria agindo efetivamente sobre as células ao passo que o restante estaria no meio de forma não solúvel e assim, todos os dados desse trabalho são expressos como concentração real de alfa-bisabolol no meio. Em nosso estudo, essa análise não foi realizada e nossos dados são sempre correspondentes à concentração total teórica da molécula. Uma vez que o solvente

usado em nosso estudo (DMSO) foi diferente do utilizado por Cavalieri e colaboradores (etanol), a extrapolação da estimativa da fração solúvel realizada por eles não é possível para os nossos dados. Apesar dessa alta lipofilia do composto parecer pouco apropriada para a utilização no tratamento uma vez que sua solubilidade em fluidos biológicos, que são hidrofílicos, ser baixa, vale lembrar que, por suas propriedades, a barreira hematoencefálica permite a passagem apenas de moléculas pequenas e lipofílicas (Loscher and Potschka 2005). Assim, por ser pequeno e bastante lipofílico, o alfa-bisabolol pode apresentar interessantes atividades *in vivo* no tratamento de cânceres cerebrais.

Diversos são os mecanismos de ação pelo qual um agente quimioterápico pode exercer seus efeitos. Ao mesmo tempo, a evasão da morte celular é considerada um “hallmark” do câncer. A apoptose, por exemplo, é um mecanismo normal de homeostasia tecidual que serve como uma barreira natural para o não desenvolvimento de tumores (Hanahan and Weinberg 2011). Assim, uma das estratégias da terapia antitumoral é justamente re-estabelecer esta capacidade das células tumorais de entrarem em apoptose. Essa alternativa é bastante interessante e desejável uma vez que quando ativada, não há extravasamento de conteúdo intracelular e como consequência, o sistema imune não é recrutado. Alguns agentes quimoterápicos são capazes de induzir apoptose em células tumorais, mas pode acontecer de as mesmas se manterem resistentes à esse mecanismo e então, diferentes abordagens devem ser tomadas (Roninson et al. 2001). Nesse sentido, tem aumentado a atenção dada à outros tipos de morte celular como catástrofe mitótica, autofagia e necrose (de Bruin and Medema 2008). Além dessas mortes celulares propriamente ditas, os fármacos antitumorais podem ainda possuir efeito

antiproliferativo, freando o crescimento tumoral por induzir a parada em alguma fase do ciclo celular.

Estudos realizados em linhagens de glioma, câncer pancreático e câncer hepático mostraram que o alfa-bisabolol foi capaz de induzir apoptose nas linhagens estudadas (Chen et al. 2010, Seki et al. 2011). Dentro dos racionais de nosso trabalho, realizamos estudos de mecanismo de morte celular nas linhagens de meduloblastoma humano D283 e DaOY, no capítulo 2 e na linhagem de glioma humano U251 no capítulo 3. Linhagem DaOY respondeu de acordo com os demais resultados da literatura e a diminuição do número de células verificado foi associado com a indução de apoptose. Na linhagem U251 não foi verificado nem parada no ciclo celular nem apoptose ou necrose em células tratadas com alfa-bisabolol, entretanto, não foram feitos estudos para avaliar a autofagia. Por fim, para a linhagem de meduloblastoma D283, o mecanismo pelo qual há redução no número de células não pode ser determinado. Apenas conseguimos sugerir que essa redução se dá por algum mecanismo diferente de apoptose, necrose, parada no ciclo celular e/ou autofagia. Considerando que os tipos de morte celular mais comuns observados em resposta a tratamentos com quimioterápicos são apoptose, necrose, autofagia e catástrofe mitótica, é possível supor que o tratamento com alfa-bisabolol leva à morte celular por catástrofe mitótica na linhagem D283. Para a linhagem de glioma U251, pode-se propor tanto catástrofe mitótica quanto autofagia. Entretanto, mais estudos se fazem necessários para confirmar essas hipóteses, para descartar outros tipos de morte de menor importância como “anoiks” (Frisch and Francis 1994) e “entose” (White E. 2007), por exemplo, bem como para descartar definitivamente os mecanismos mais comuns.

Por mais que esperássemos que nossos resultados concordassem com a literatura quanto ao tipo de morte induzida pelo tratamento, as particularidades dos tumores que se instalam em diferentes tecidos, bem como as mutações pontuais que deram origem a cada linhagem, mesmo que provenientes de tumores em um mesmo órgão tornam plausível essa diferença na resposta ao tratamento. O resveratrol, por exemplo, outro composto natural com atividade antitumoral bastante difundida, age por diversos mecanismos de ação, dependendo do tipo tumoral. Em linhagens de glioma, por exemplo, esse tratamento leva à apoptose precedida por parada no ciclo celular nas fases G1 e S (Figueiro et al. 2013). Em linhagem de tumores pancreáticos (Yang L. et al. 2014) e de bexiga (Zhou et al. 2014), por exemplo, apenas a apoptose é observada. Em tumores gástricos, foi descrito a indução de parada no ciclo celular e senescência em células tratadas com esse composto (Yang Q. et al. 2013) e em câncer colorretal foi observado morte por autofagia (Andreadi et al. 2014). Esse mesmo mecanismo de indução de autofagia em resposta ao tratamento foi considerado como sendo benéfica na progressão tumoral de células de melanoma tratadas com resveratrol (Wang M. et al. 2014). Além desses exemplos, onde um mesmo tratamento pode desencadear diferentes tipos de morte dependendo do tecido original do tumor estudado, também existem casos na literatura em que um mesmo tratamento, quando utilizado em linhagens diferentes de um mesmo tipo de câncer leva a respostas totalmente antagônicas: proliferação em uma das linhagens e morte na outra (Sakowicz-Burkiewicz et al. 2013). Assim, não é de surpreender que o alfa-bisabolol cause morte por apoptose em uma das linhagens de meduloblastoma estudada (DaOY), enquanto que na outra (D283), apesar de termos observado redução no número total de células, a indução de apoptose não tenha sido verificada. Seguindo este mesmo racional, não é

estranho que apesar de constar na literatura que o alfa-bisabolol causa apoptose em linhagens de glioma (Cavalieri et al. 2004), nós não a tenhamos observado na linhagem U251 de glioma humano estudada.

Considerando todas as atividades promissoras do alfa-bisabolol descritas até agora, nós nos questionamos se moléculas análogas e este também apresentariam atividade antitumoral. Dois estudos prévios na literatura realizaram síntese de moléculas derivadas do alfa-bisabolol. Da Silva e colaboradores sintetizaram uma série de oito thiosemicarbazonas a partir do alfa-bisabolol e testaram essas moléculas frente a uma série de oito linhagens tumorais, incluindo tumores de mama, pulmão e próstata. Algumas dessas moléculas se mostraram mais eficazes que o composto de partida, principalmente frente à linhagem de leucemia utilizada nesse estudo (da Silva et al. 2010). No outro estudo, foram sintetizadas uma série de glicosídeos de alfa-bisabolol. Essas moléculas foram testadas em células de glioma, câncer de pulmão, pâncreas, próstata, ovário, mama e colón, com interessantes atividades antitumorais. Apesar destes estudos promissores, não havia até então estudos avaliando a atividade antitumoral do derivado natural óxido de bisabolol A.

Assim, utilizamos o ensaio de Sulfarodamina B, e uma curva de dose bastante ampla (de 10μM a 200 μM) para avaliar os efeitos do óxido de bisabolol A em linhagens de glioma e meduloblastoma (Capítulo 3). Tratamentos pareados com alfa-bisabolol também foram realizados para possibilitar uma comparação de efeito entre ambas as moléculas. Para as linhagens de meduloblastoma, o alfa-bisabolol se mostrou mais eficiente. Dentre as linhagens de glioma estudadas, a linhagem C6 de ratos mostrou praticamente a mesma sensibilidade frente aos dois tratamentos. O tratamento com óxido de bisabolol A não surtiu nenhum efeito na linhagem U138 e a

linhagem U251 se mostrou mais sensível ao óxido que ao alfa-bisabolol. Por ter sido a única linhagem mais sensível ao óxido de bisabolol A, seguimos investigando os mecanismos da molécula apenas na linhagem U251. De acordo com nossos dados, essa maior sensibilidade pode ser explicada pelo fato que o óxido de bisabolol A foi capaz de induzir as células a uma parada no ciclo celular, enquanto que o alfa-bisabolol não. Esses dados são bastante preliminares e mais estudos precisam ser feitos para compreendermos porque apenas a U251 é mais sensível ao óxido de bisabolol A.

Em nossos estudos com o alfa-bisabolol, avaliamos também a influência deste sobre a ecto-5'-nucleotidase, importante enzima do sistema purinérgico. O câncer é considerado uma doença multifatorial, o que significa que para seu estabelecimento e progressão, diversos fatores devem estar envolvidos, sendo eles ligados ao ambiente, estilo de vida e características genéticas de determinada pessoa, que atuando de forma combinada, dão origem à doença (Bartsch and Hietanen 1996, Wogan et al. 2004). Do ponto de vista molecular, diversas são as proteínas e vias de sinalização alteradas e dentre esses, podemos destacar o sistema purinérgico. Alterações neste sistema já foram relatadas em diversos tipos de cânceres como bexiga (Rockenbach et al. 2014), melanoma (Dzhandzhugazyan et al. 1998) e também em gliomas (Wink et al. 2003) e meduloblastomas (Cappellari et al. 2012b).

Em gliomas, estudos anteriores de nosso grupo mostraram que linhagens celulares desses tumores possuem uma diminuição na capacidade de hidrolisar os nucleotídeos ATP e ADP e por outro lado, uma atividade aumentada de hidrólise de AMP (Wink et al. 2003), quando comparada a células normais, os astrócitos. Essa combinação de modulação enzimática parece favorecer o acúmulo de ATP, que

possui efeito citotóxico e pode levar a morte do tecido normal adjacente ao tumor e induzir, por outro lado, a proliferação de linhagens de glioma, e de adenosina, uma molécula pró-tumoral por estimular o crescimento tumoral e promover angiogênese e imunossupressão (Bavaresco et al. 2008, Morrone et al. 2005, Spychala 2000) . Assim, pode-se hipotetizar que tratamentos que modulem as atividades das enzimas responsáveis por essas catálises, fazendo com que elas voltem a apresentar o mesmo perfil das células normais, podem ser úteis no tratamento do câncer. Pensando na ecto-5'-NT/CD73, enzima alvo de nosso estudo, o objetivo seria desenvolver tratamentos que fossem capazes de inibir essa enzima, reduzindo assim, a hidrólise de AMP e conseqüentemente a redução de adenosina, nas células tumorais. Em um estudo anterior do nosso grupo, demonstramos que essa hipótese é válida. Quando células de glioma foram tratadas com quercetina, um flavonoide derivado de plantas,foi observada uma inibição da ecto-5'-NT/CD73 juntamente com uma diminuição no número de células (Braganhol et al. 2007). Entretanto, em estudo também de nosso grupo, demonstramos que a indometacina, um anti-inflamatório não esteroidal, induz a morte celular e um estímulo da atividade enzimática da ecto-5'-NT/CD73 (Bernardi et al. 2007), o mesmo efeito desencadeado pelo alfa-bisabolol, em linhagens de glioma como mostrado nos capítulos 2 e 3 desta dissertação. Tanto nesse estudo utilizando indometacina, quanto no tratamento com alfa-bisabolol, a morte celular pode ser correlacionada com o estímulo ao receptor de adenosina A_3, que é descrito como modulador de morte celular (Jacobson et al. 1999, Ralevic and Burnstock 1998), e que teve sua expressão aumentada em resposta aos tratamentos. Assim, parece que tratamentos que modulem a atividade da ecto-5'-NT/CD73, seja estimulando, seja inibindo, são interessantes para o tratamento de gliomas.

Em meduloblastomas, um estudo de nosso grupo demonstrou que tanto linhagens de meduloblastoma primário, DaOY, quanto células metastáticas, D283, possuem baixa atividade de hidrólise de ATP e ADP. Quanto ao AMP, a linhagem DaOY apresentou capacidade hidrolítica pronunciada desse nucleotídeo, enquanto que a linhagem D283 apresentou uma atividade hidrolítica bastante baixa, acompanhada também de uma expressão baixíssima dessa enzima. Esses resultados foram bastante surpreendentes, pois, como ao exemplo dos gliomas, o que se observa normalmente em células de câncer é uma atividade aumentada da ecto-5´-NT(Bavaresco et al. 2008, Stella et al. 2010). No estudo publicado por Cappellari e cols., sugerimos que para meduloblastomas a baixa expressão/atividade da ecto-5'-NT/CD73 pode estar relacionado com um fenótipo metastático, mais maligno desta patologia, e seria indicativo de um pior prognóstico. Assim, pode-se sugerir que tratamentos que aumentem a atividade da enzima ecto-5'-NT/CD73 podem reduzir a capacidade das células tumorais em gerar metástase, o que já seria um grande ganho para o tratamento destes tumores. Seguindo esse racional, o alfa-bisabolol seria uma alternativa bastante interessante de tratamento. Nós observamos que quando as linhagens estudadas foram tratadas com alfa-bisabol, a atividade da ecto-5'-NT/CD73 aumentou. Além disso, determinamos também que esse aumento é devido a um efeito direto do tratamento sobre a enzima, sendo o aumento da atividade independente de alterações na expressão da proteína. Mais estudos são necessários para que possamos entender as implicações desta atividade aumentada da ecto-5'-NT/CD73. A exemplo dos gliomas, a hidrólise aumentada do AMP pode estar gerando quantidades de adenosina suficientes para ativar algum receptor de adenosina, e desencadeando a morte celular por meio deste, por exemplo.

5. Perspectivas

Considerando os promissores resultados obtidos até agora, temos como perspectivas deste trabalho:

- Aprofundar o entendimento da morte induzida pelo alfa-bisabolol e óxido de bisabolol A, com foco nas linhagens D283 e U251 para o primeiro tratamento e nas linhagens C6, U251, DaOY e D283 para o segundo, estudando alterações em vias de sinalização celular e outros tipos de morte celular (como catástrofe mitótica, necroptose e entose, por exemplo) que podem estar ocorrendo nessas linhagens celulares

- Investigar as influências do aumento da atividade da ecto-5'-NT/CD73 nas linhagens DaOY e D283, principalmente sobre os receptores de adenosina.

- Avaliar possíveis alterações no sistema purinérgico induzidos pelo tratamento com óxido de bisabolol A, como alteração na expressão de receptores P1, bem como alterações na atividade e expressão de outras enzimas desse sistema.

- Conduzir estudos em modelo *in vivo* de implante de células de glioma em ratos saudáveis, com ambos os tratamentos, para entender a eficácia real destes.

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