PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
FACULDADE DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

NATÁLIA FONTANA NICOLETTI

AVALIAÇÃO DO PAPEL DOS RECEPTORES B_1 E B_2 DE CININAS E DOS CANAIS DE CÁLCIO VOLTAGEM DEPENDENTES TIPO-P/Q E -N EM MODELO DE GLIOMA *IN VITRO* E *IN VIVO*

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Tese apresentada como requisito para a obtenção do grau de Doutor pelo Programa de Pós-Graduação em Biologia Celular e Molecular da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul.

Orientador:

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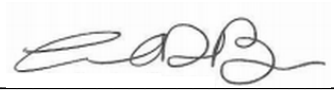

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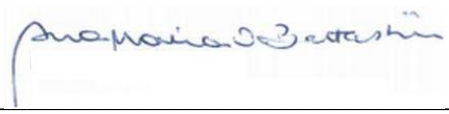

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Porto Alegre
2015

Aos meus sobrinhos,
Valentina e João Vítor,
para que sejam sempre criativos e curiosos.

Agradecimentos

Às queridas mestres Fernanda Bueno Morrone e Maria Martha Campos, meu sincero agradecimento, com carinho e amizade. Vocês, em seus diferentes modos de trabalho e vida, foram fontes de inspiração e aprendizado durante todos estes anos. Obrigada pela oportunidade e pela confiança.

Dr. Réjean Couture, merci de m'avoir accueilli dans votre laboratoire à l'université de Montreal. Je vous remercie pour la confiance dans mon projet de recherche. C'était un moment très heureux de ma vie scientifique et personnelle. Merci infiniment!

Cher Jacques, je vous remercie pour tout ce vous avez fait pour moi à l'université de Montreal. Merci beaucoup pour toute l'aide dans le laboratoire e pour votre amitié et confiance.

Chers amis, Joseph Haddah, Menakshi Bhat, Nejla Tidjane, Rose Mari Vianna, Somaya Hachana et Virginnie Rhéaume, vous étiez très gentil avec moi dans mon temps à Montréal. Merci pour l'amitié et pour les classes de français. Je vous garde dans mon coeur.

Ao Professor Marcus Vinicius Gomez, agradeço pela oportunidade de trabalhar com a encantadora Phoneutria nigriventer e pela concessão da bolsa de estudos, que possibilitou a dedicação exclusiva a este trabalho.

Thais Erig, minha querida aluna de iniciação científica, obrigada por dividir comigo as dúvidas e as frustrações da bancada. Tenho certeza que a amizade e o carinho são mútuos. Obrigada pelo teu excelente trabalho e total empenho.

Aos queridos amigos do Laboratório de Farmacologia Aplicada e Cultura Celular, agradeço pela amizade, carinho e pelos bons momentos com cafézinho e trocas de ideias. Vocês fizeram os meus dias mais felizes e divertidos. Foi muito bom dividir este tempo com vocês. Vocês são incríveis!

Aos amigos do Instituto de Toxicologia e Farmacologia agradeço pelo carinho, apoio, amizade e pelos diversos ensinamentos durantes estes anos de pós-graduação.

Aos queridos amigos Fabiana de Melo, Eduardo Zimmer e Rose Vianna, agradeço a acolhida e as dicas em Montréal, e por serem tão gentis e solícitos na minha adaptação. Vocês dividiram comigo um momento de grande crescimento pessoal e profissional. Vocês moram no meu coração!

Às amigas Anna Siebel, Caroline Calice, Daiana Renck, Elen Fagherazzi, Kelem Vedovelli e Laura Roesler agradeço a amizade sincera, troca de ideias, parceria e companheirismo durante toda a minha formação.

Pai e Mãe, obrigada por nunca negarem a realização de um sonho. Obrigada pelo incentivo e por me encorajarem a sempre seguir em frente, com a certeza e a segurança de ter para onde voltar. Obrigada pelas raízes e pelas asas!

Vô Rosalinda, obrigada pelo amor, torcida e orações. Eu ainda vou precisar muito de tudo isso.

Meus irmãos, Rodrigo, Flávia e Nanau, partes da minha criação e da minha vida. Obrigada por serem fonte de tantos exemplos. Vocês contribuíram muito para a minha formação pessoal. Sorte de quem nasce e já tem companhia.

Vavá e João, meus sobrinhos queridos, obrigada por tornarem minha vida mais alegre e por proporcionarem momentos sem agenda, sem relógio e sem pressa. Vocês são fonte de amor e inspiração.

Aos meus tios e primos, obrigada pela torcida e orgulho que sempre demonstram. É sempre muito bom receber o incentivo e o carinho de vocês.

Marcelo, meu noivo, meu parceiro, meu melhor amigo, meu amor. A tua cumplicidade, segurança e força me trouxeram até aqui. Obrigada por tantas vezes ser meu ponto de equilíbrio e por incentivar de forma incondicional meu sonho pelo doutorado sanduíche. Este título também é teu.

À minha querida Popô, que me leva para passear todas as manhãs e finais de tarde, agradeço pelos momentos de vento no rosto, serenidade e reflexão. A ela e a todos os cães que já cruzaram a minha vida, agradeço por despertar em mim o mais puro sentimento de solidariedade e cuidado.

À CAPES, CNPq, FINEP e PUCRS agradeço pelo financiamento ao longo destes quatro anos.

"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale."

Marie Curie

RESUMO

O glioblastoma apresenta a maior incidência entre todos os gliomas e se caracteriza como o mais agressivo e fatal (grau IV) dos tumores primários do SNC. Atualmente o glioblastoma é considerado uns dos grandes desafios da oncologia e da neurocirurgia, devido ao seu caráter altamente agressivo. A sobrevida média dos pacientes é bastante baixa e o prognóstico é desfavorável, já que a grande maioria destes tumores apresenta um padrão difuso e infiltrativo de crescimento, o que dificulta as abordagens atuais para a terapia tumoral. Neste estudo foram analisados os efeitos dos receptores da família dos GPCRs de cininas e dos canais de cálcio voltagem-dependentes (CCVD) como base para possíveis alvos no tratamento dos gliomas malignos, a fim de caracterizar novas abordagens terapêuticas. O efeito da sinalização desencadeada pela BK e da sinalização Ca^{2+}-dependente pode estar envolvida na regulação do crescimento e progressão dos gliomas e na migração das células tumorais. Neste sentido, este trabalho visou explorar o papel dos receptores B_1 (B_1R) e B_2 (B_2R) de cininas e da sinalização de Ca^{2+} via CCVD tipo-P/Q e -N em modelo de glioma *in vitro* e *in vivo*.

Ensaios em cultura celular utilizando as linhagens de glioma humano U-138MG e U-251MG demonstraram que a ativação dos B_1R e B_2R pelo uso dos agonistas des-arg^9-BK (1-100 nM) e BK (1-100 nM) aumentou a proliferação das linhagens celulares testadas, através da ativação das vias ERK1/2 e PI3K/Akt. Enquanto que a exposição aos antagonistas seletivos para estes receptores, SSR240612 (1-30 µM) e HOE-140 (1-100 µM), provocou intensa morte celular com características de necrose/apoptose. A parte *in vivo* compreendeu a técnica de implante das células GL261 de glioma (grau IV) em animais C57/BL6 e *knockout* para os B_1R e B_2R. A deleção apenas do B_1R provocou um importante crescimento tumoral nos animais *knockout* para este receptor, enquanto que os animais com deleção de B_2R não tiveram o desenvolvimento tumoral alterado. Notavelmente, tanto a deleção gênica como o antagonismo farmacológico combinado dos receptores B_1 e B_2 (SSR240612; 25 nmol/sítio + HOE-140; 50 pmol/sítio) diminuiu o crescimento tumoral e o índice mitótico dos gliomas implantados.

Para compreender o envolvimento dos CCVD tipo-P/Q e -N na fisiopatologia dos gliomas foram utilizadas frações da toxina da aranha *Phoneutria nigriventer* (PhTx3-3 bloqueadora de canais do tipo-P/Q; Phα1β bloqueadora de canais do tipo-N) e ω-conotoxinas provenientes do *Conus magus* (MVIIC bloqueadora de canais do tipo-P/Q; MVIIA bloqueadora de canais do tipo-N). Os experimentos *in vitro* evidenciaram que o bloqueio dos canais de Ca^{2+} tipo-P/Q e -N pelas toxinas PhTx3-3 (0.3 - 100 pM), Phα1β (0.3 - 100 pM) e MVIIA (0.3 - 100 pM) inibiram a proliferação e a viabilidade das linhagens celulares M059J, U-138MG e U-251MG de glioma humano, com intensa característica de morte celular por apoptose. Os resultados utilizando o modelo de glioblastoma *in vivo*, demonstraram que ambas as toxinas bloqueadoras dos canais do tipo-N, Phα1β (50 pmol/sítio) e MVIIA (10 pmol/sítio), foram efetivas em diminuir o

crescimento e a progressão tumoral nos animais tratados, com intensa ativação de astrócitos e micróglia, destacando o possível envolvimento do sistema imune na inibição do crescimento tumoral.

Através do uso de ferramentas moleculares e farmacológicas, nossos resultados demonstraram o envolvimento importante tanto dos B_1R e B_2R de cininas, como dos CCVD tipo-P/Q e -N no desenvolvimento dos gliomas malignos. Desta maneira, podemos propor que o bloqueio farmacológico combinado de antagonistas seletivos para os receptores B_1 e B_2, assim como a inibição dos CCVD tipo-P/Q e -N surgem como potenciais alvos terapêuticos no manejo dos tumores cerebrais e podem representar alternativas promissoras no tratamento dos gliomas.

ABSTRACT

Glioblastoma (grade IV) is among the most prevalent primary intracranial tumors and is considered a challenge in oncology and neurosurgery due to the highly aggressive nature, and the elevated mortality rates. The location of the tumor and its invasive nature avoid the standard-of-care therapy, which includes surgical resection followed by radiotherapy and chemotherapy. Nevertheless, the current gold standard treatment has not been effective to prevent tumor evolution, as indicated by the poor survival rates. In this context, we analyzed the GPCRs for kinin and the high-voltage-gated calcium channels (VGCC) as feasible new therapeutic approaches in malignant gliomas. Thereby, the signaling triggered by bradykinin or the disruption of calcium signaling might contribute with pivotal mechanisms underlying glioma progression, such as cell proliferation. Therefore, the aim of this study was to further evaluate the relevance of B_1 (B_1R) and B_2 (B_2R) kinin receptors as well as the P/Q- and N-type VGCC in glioma development, by using in vitro and *in vivo* glioma model.

Cell culture assay showed that the treatment with the selective B_1R des-Arg^9-BK (1-100 nM) and B_2R BK (1-100 nM) agonists induced a marked enhancement of cell proliferation and viability through ERK1/2 and PI3K/Akt signaling, according to evaluation of U-138MG and U-251MG cell lines. Meanwhile, the incubation of either B_1R SSR240612 (1-30 µM) or B_2R HOE-140 (1-100 µM) antagonists induced a marked cell death with mixed apoptosis/necrosis characteristics. The *in vivo* mouse model of GL261-induced glioma of C57/BL6 or B_1R and B_2R *knockout* mice showed an uncontrolled tumor growing in KOB_1R mice. Conversely, there was no significant change of the tumor development in KOB_2R mice. Notably, the genetic ablation or the pharmacological combined antagonism of B_1R and B_2R (SSR240612; 25 nmol/site + HOE-140; 50 pmol/site) diminished the tumor progression as well as the mitotic index of the GL261-induced glioma.

To understand the potential anti-tumor effects of the blockade of P/Q- and N-type VGCC, we used animal-derived inhibitors namely PhTx3-3 (P/Q-type blocker) and Phα1β (N-type blocker) from *P. nigriventer*, or MVIIC (P/Q-type blocker) and MVIIA (N-type blocker) from *C. magus*. The PhTx3-3 (0.3 - 100 pM), Phα1β (0.3 - 100 pM) and MVIIA (0.3 - 100 pM) displayed a significant inhibitory effect on proliferation and viability of M059J, U-138MG and U-251MG glioma tested cell lines, and evoked cell death mainly with apoptosis characteristics. In the glioblastoma *in vivo* model, the N-type VGCC blockade by either Phα1β (50 pmol/site; i.c.v. and i.t.) or MVIIA (10 pmol/site; i.c.v.) caused significant reductions of glioma growth and progression. Of note, the N-type inhibition by Phα1β and MVIIA led to a marked increase of GFAP-activated astrocytes and Iba-1-positive microglia in the peritumoral area, which might be related to the inhibitory effects of immune system in tumor development.

Using molecular and pharmacological approaches, our data provide clear evidence on the beneficial effects of the simultaneous inhibition of both B_1R and B_2R as well as the P/Q- and N-type blockade on glioma development. Thus, we propose that the combined selective antagonism of B_1R and B_2R, such as the P/Q-, and especially N-

type high-VGCC inhibition could markedly modify the tumor progression, which might represent an attractive alternative for the treatment of malignant gliomas in the future.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS

BHE - Barreira hematoencefálica

BTB - Barreira hematoencefálica tumoral

BK - Bradicinina

CCVD - Canais de cálcio voltagem-dependentes

EGF - Fator de crescimento epidermal

ECA - Enzima conversora da angiotensina

EGFR - Receptor de fator de crescimento epidermal

VEGF - Fator de crescimento vascular endotelial

FGF - Fator de crescimento de fibroblasto

PTEN - Fosfatase homóloga e tensina deletado do cromosso 10

GBM - Glioblastoma

MAPK - Proteína cinase ativada por mitógenos

MTIC - Monometil-triazenoimidazol-carboxamida

NO - Óxido nítrico

PDGF - Fator de crescimento derivado de plaquetas

PhTx - Phoneutriatoxina

PKC - Proteína quina C

GPCR - Receptor acoplado à proteína G

SNC - Sistema nervoso central

TMZ - Temozolamida

SUMÁRIO

Capítulo I

1. INTRODUÇÃO

1.1 Os Gliomas

Os tumores primários do sistema nervoso central (SNC) constituem um dos grandes desafios da oncologia e da neurocirurgia por apresentarem um elevado índice de mortalidade devido ao seu caráter altamente agressivo. Neste contexto, os gliomas representam cerca de 40% de todas as neoplasias do SNC em adultos (1) e entre os adolescentes são a terceira causa mais comum de morte por câncer (2). A sobrevida média em cerca de um terço dos pacientes é bastante baixa e o prognóstico é desfavorável, já que a grande maioria destes tumores apresenta um padrão difuso e infiltrativo de crescimento, o que dificulta as abordagens atuais para a terapia tumoral, que incluem ressecção cirúrgica, radioterapia e quimioterapia (2, 3).

Pacientes com gliomas podem exibir sintomas como cefaleia, vômitos, papiledema (síndrome de hipertensão intracraniana), déficit cognitivo e convulsões, explicados pelo grande comprometimento do tecido nervoso circunjacente ao tumor (1-3). Os subtipos histológicos dos gliomas são classificados de acordo com a linhagem glial de que se originam – astrocitomas, oligodendrogliomas, oligoastrocitomas (mistos) e ependimomas. O grau de malignidade dos gliomas é determinado pela Organização Mundial de Saúde, sendo considerados quatro graus para classificar os astrocitomas, os quais são agrupados em baixo grau (I e II – fibrilar e pilocítico, respectivamente) ou alto grau (III e IV – anaplástico e glioblastoma, respectivamente), com base na presença ou ausência de características histopatológicas como malignidade celular, índice mitótico, proliferação microvascular e necrose (4, 5).

Os gliomas podem ser derivados de astrócitos, oligodendrócitos e células ependimárias, apresentando morfologia e expressão gênica semelhantes às observadas para estas células (4, 6). Durante o desenvolvimeto, as células primitivas do neuroepitélio proliferam em neuroblastos e glioblastos e se diferenciam em neurônios e células gliais, que desempenham papel importante em condições fisiológicas e patológicas. As células gliais são capazes de proliferar em uma variedade de gliomas, sendo os tumores de linhagem glial aqueles que constituem o maior grupo de tumores primários intracranianos (2, 7). Embora a transformação neoplásica das células gliais diferenciadas seja descrita como um mecanismo de gliomagênese (8), estudos recentes

demonstraram que células-tronco neurais podem estar intimamente envolvidas neste processo (4, 9).

Apesar de as vias de sinalização envolvidas no desenvolvimento de gliomas malignos já estarem relativamente bem caracterizadas, a origem celular destes tumores é desconhecida. O sistema nervoso adulto abriga células-tronco neurais com capacidade de auto-renovação, proliferação e diferenciação em tipos celulares maduros. Existem evidências crescentes de que estas células-tronco ou células progenitoras relacionadas possam originar células-tronco neoplásicas que, ao escapar dos mecanismos de controle de proliferação e diferenciação, dariam origem a gliomas de alto grau (4) (Figura 1). Sabe-se ainda que as células-tronco neurais são reguladas pelas mesmas vias de sinalização que estão ativas em tumores cerebrais (6, 10) e que os gliomas são constituídos por populações de células diferenciadas e uma pequena população de células tumorigênicas indiferenciadas e multipotentes. Embora ambas as populações contenham mutações tumorigênicas que podem contribuir para a gênese tumoral, somente as células-tronco neurais apresentam a característica de auto-renovação, sendo apontadas como possíveis responsáveis pela sustentação e propagação do tumor (11).

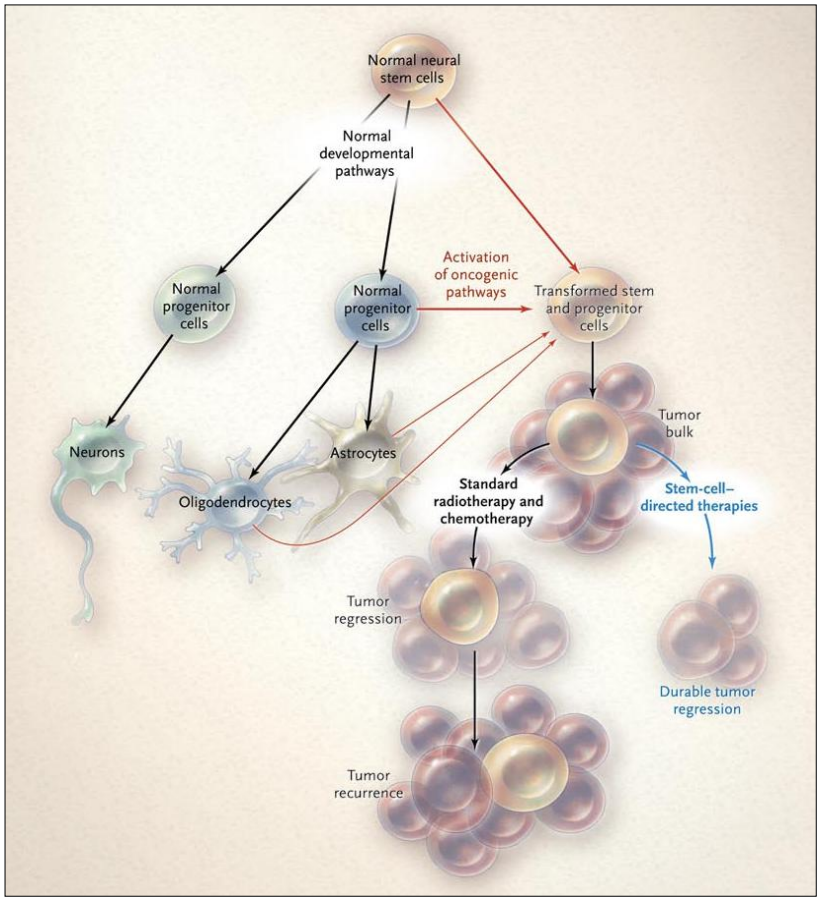

Figura 1. Mecanismos de resistência em células de glioma. Extraído de Wen & Kesari, 2008.

A incidência de tumores cerebrais tem aumentando ao longo dos últimos 30 anos e difere de acordo com idade, gênero e etnia (12, 13). Os gliomas são mais prevalentes em homens do que em mulheres e aproximadamente duas vezes mais comuns entre indivíduos caucasianos (14). A história familiar é raramente observada neste tipo de tumor, embora quando presente esteja associada a um risco duas vezes maior de surgimento de novos casos de glioma. Diferenças genéticas podem contribuir para elucidar a incidência relacionada às diferentes raças (13) e até então apenas algumas poucas variantes de susceptibilidade, tais como 20q13.33 (RTEL), 5p15.33, 9p21.3 (CDKN2BAS), 7p11.2 (EGFR), 8q24.21 (CCDC26), e 11q23.3 (PHLDB1), foram fracamente associadas ao desenvolvimento dos gliomas, refletindo uma possível interação entre subgrupos moleculares (15-17).

Tanto a predisposição genética quanto a exposição a fatores ambientais estão fortemente envolvidos na etiologia dos tumores primários do SNC e a exposição à radiação ionizante permanece como o fator de risco ambiental mais aceito (13, 18). Alguns estudos relacionaram uma maior incidência de tumores cerebrais em crianças submetidas à radioterapia na região da cabeça e pescoço e em indivíduos envolvidos em acidentes nucleares (14, 19). Por outro lado, estudos envolvendo o uso de telefones celulares e exposição a diferentes campos magnéticos não demonstraram uma relação consistente ao surgimento de tumores do SNC (20, 21), assim como a ocorrência de traumatismo crânio-encefálico, a exposição a pesticidas e a ingestão de alimentos contendo compostos N-nitrosos e aspartame também não foram relacionados a esta patologia (14, 19).

Diferente de outros tipos tumorais, hábitos alimentares e estilo de vida saudáveis não parecem estar relacionados a prevenção do surgimento dos gliomas, enquanto o diagnóstico precoce não melhora o prognóstico e a sobrevida dos pacientes que apresentam esta patologia (3). O tratamento padrão atual mais utilizado para o manejo dos gliomas consiste em ressecção cirúrgica total, seguida de quimioterapia e radioterapia. A ressecção cirúrgica é adotada como tratamento de escolha para os diferentes graus de malignidade dos gliomas (I-IV), embora a localização do tumor e sua natureza infiltrativa sejam fatores limitantes para a remoção completa da massa tumoral, o que permite a permanência de populações de células neoplásicas no tecido cerebral adjacente (7). Com relação à radioterapia e quimioterapia, o manejo do tratamento difere de acordo com o grau de malignidade. Para os tumores de baixo grau

(I e II), o tratamento de escolha é a radioterapia, usada de forma isolada ou associada a fármacos de segunda linha, como o bevacizumabe (Avastin®), anticorpo monoclonal recombinante humanizado que neutraliza seletivamente a atividade biológica do fator de crescimento vascular endotelial (VEGF). Para os gliomas de alto grau (III e IV) é comumente indicada à radioterapia, seguida por quimioterapia com temozolamida (TMZ) (Temodal®), agente alquilante que sofre uma rápida conversão química em pH fisiológico, formando o composto ativo monometil-triazenoimidazol-carboxamida (MTIC) devendo-se o efeito citotóxico principalmente à alquilação na posição O^6 da guanina (22), ou esquema de poliquimioterapia, que associa os fármacos procarbazina (Natulanar®, CEENU®), lomustina (Citostal®) e vincristina (Oncovin®) (7, 23). É importante ressaltar que fatores como a presença da barreira hematoencefálica (BHE) e a heterogeneidade histopatológica e molecular dos gliomas são fatores altamente limitantes para o sucesso da quimioterapia frente a estes tumores cerebrais.

1.1.2 O Glioblastoma

O glioblastoma (GBM) apresenta a maior incidência entre todos os gliomas e se caracteriza como o mais agressivo e fatal (grau IV) dos tumores primários do SNC, apresentando comumente aspectos infiltrativos e multifocais (4). O GMB pode ser dividido em dois subtipos, de acordo com as características clínicas apresentadas: GBM "primário" e "secundário" ou "*de novo*". O GBM primário surge na ausência de uma lesão pré-existente, enquanto o GBM secundário origina-se de um astrocitoma de baixo grau (8, 24) (Figura 2). A história natural dos gliomas de baixo grau não é totalmente compreendida e, desta forma, não se sabe qual o tempo de evolução necessário para uma neoplasia glial de baixo grau sofrer transformação maligna (25).

O GBM primário ocorre tipicamente em pacientes com mais de 50 anos de idade e é caracterizado por mutações em receptores de fator de crescimento epidermal (EGFR) ou perda de heterozigosidade do cromossomo 10q. Uma característica molecular marcante deste subtipo de GBM é a perda da região 10q22-25, porção cromossômica que abriga diversos genes supressores tumorais, entre eles o gene fosfatase homóloga e tensina (PTEN), além da deleção de p16. O GBM secundário ou *de novo* é mais prevalente em pacientes jovens que apresentam recidiva de gliomas de baixo grau ou astrocitoma anaplástico, e são menos frequentes que os GBM primários. Neste caso é comum os tumores surgirem como resultado do acúmulo gradual de

anormalidades genéticas, que incluem perda ou inativação de genes supressores tumorais p53, p16 e retinoblastoma (Rb) ou superexpressão de alguns receptores de proteases e fatores de crescimento derivados de plaquetas (PDGFR), além da perda de heterozigosidade do cromossomo 10q (4, 26, 27). Frente ao caráter altamente heterogêneo do GBM, recentemente sugeriu-se associar uma subclassificação molecular a fim de aprimorar a caracterização do GBM e direcionar o tratamento. Neste sentido, de acordo com a expressão gênica de um conjunto de mutações relacionadas ao desenvolvimento deste tumor, o GBM também pode ser caracterizado como proneural, neural, clássico e mesenquimal (28).

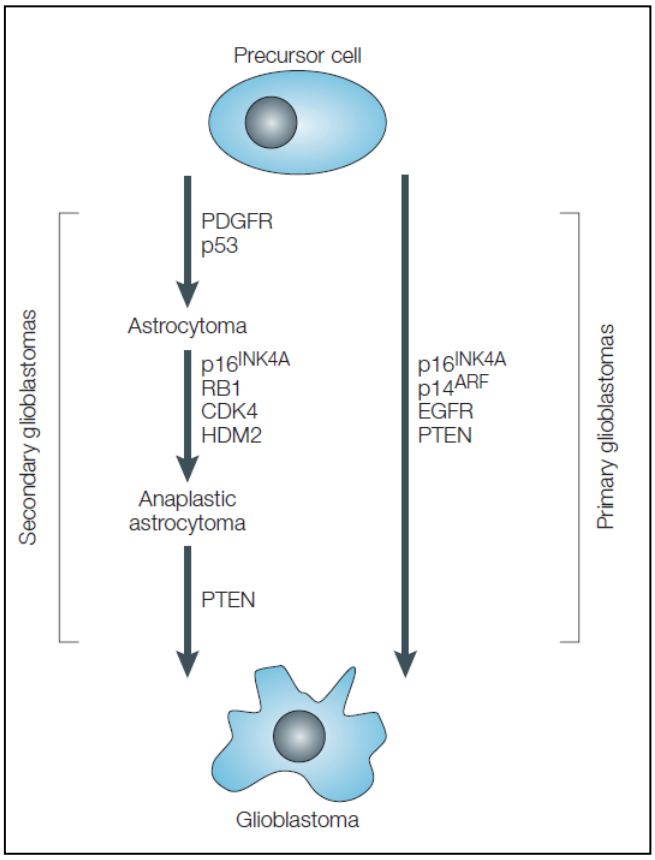

Figura 2. Vias moleculares envolvidas na formação do glioblastoma. Extraído de Rich & Bigner, 2004.

Apesar dos recentes avanços acerca da patogênese molecular do GBM, as opções atuais para a terapia tumoral ainda são muito limitadas e incluem ressecção cirúrgica, seguida de radioterapia e quimioterapia, que contribuem pouco para prolongar a sobrevida média dos pacientes, que varia de nove a doze meses (26, 29). Além disto, a eficiência da cirurgia para a remoção do GBM é dificultada pela natureza invasiva do tumor, sendo a infiltração do parênquima cerebral um fator limitante para o sucesso da ressecção cirúrgica. Desta forma, a recorrência do tumor é praticamente inevitável, já

que a ressecção invariavelmente permite a permanência de populações de células neoplásicas na topografia tumoral. Frente às limitações apresentadas pela ressecção cirúrgica, o tratamento com radioterapia e quimioterapia são de extrema relevância, a fim de eliminar as populações celulares de caráter neoplásico responsáveis pela rápida recidiva tumoral e baixa sobrevida. Atualmente o esquema de tratamento para o GBM primário mais utilizado emprega a TMZ, concomitante à radioterapia. Para pacientes idosos recomenda-se um esquema diferenciado de radioterapia isolada. Já para pacientes em recidiva, recomenda-se terapia de resgate, com tratamento associado, que compreende TMZ, associada à radioterapia e ao bevacizumabe (23, 30, 31).

Embora os esquemas de tratamento descritos sejam largamente utilizados e a TMZ seja um agente alquilante de considerável penetração na BHE, pouco se obteve de sucesso na sobrevida dos pacientes com GBM desde que estes agentes foram inseridos na prática clínica. Estudos clínicos demonstraram uma sobrevida média de 14,6 meses para pacientes com GBM que fizeram o uso combinado de radioterapia e TMZ, enquanto os pacientes submetidos apenas a radioterapia apresentaram uma sobrevida média de 12,1 meses (22, 23). De forma similar, um estudo recentemente analisou a associação do uso de bevacizumabe ao tratamento combinado com radioterapia e TMZ, sem sucesso no aumento da sobrevida e qualidade de vida dos pacientes com GBM (32). Além disso, a resistência do GBM à TMZ vem sendo amplamente descrita e comentada nos últimos anos (33, 34).

Assim, o GMB constitui-se em um desafio à neurocirurgia e à oncologia, devido ao seu caráter altamente agressivo e difícil abordagem terapêutica. Tal complexidade, combinada à presença de uma subpopulação de células-tronco tumorais e à variabilidade das alterações genéticas envolvidas na patogênse do GBM, contribuem para dificultar o entendimento e o tratamento deste tipo de tumor (26).

Mutações e a superexpressão em receptores intracelulares foram identificados em gliomas malignos, que apresentam uma heterogeneidade significativa (6). Sabe-se que tais eventos levam à ativação constitutiva de vias de sinalização e resultam em proliferação celular descontrolada, invasão e secreção de fatores angiogênicos. Um elemento importante no crescimento tumoral é o VEGF, que é o fator de crescimento angiogênico mais relevante, produzido pela maioria dos tumores sólidos. Seus receptores são altamente expressos em células endoteliais vasculares e predominam em vasos próximos ao tumor (6, 27, 34-36). Estudos recentes demonstraram que o aumento

da expressão de VEGF, está intimamente relacionado ao grau de vascularização, progressão e agressividade tumoral e ainda ao prognóstico clínico (6, 37, 38). Apesar do GBM ser caracterizado como altamente angiogênico e invasivo, este tipo de tumor raramente metastatisa, provavelmente pela ausência da circulação linfática no tecido cerebral e ainda pela existência da BHE (39). Além disto, mutações e super-expressão de receptores EGF, FGF-2 e PDGF podem resultar em ativação anormal de vias de transdução de sinal *downstream* dos receptores tirosina quinase ou interrupção das vias de parada do ciclo celular (40). Neste sentido, a inativação nas vias de p53 e Rb também são frequentemente identificadas e associadas a gliomas malignos (27, 36).

No GBM, a atividade de diversas vias de transdução de sinal relacionadas ao crescimento tumoral está significativamente elevada em relação ao parênquima cerebral normal e aos gliomas de baixo grau. Estudos demonstraram que a atividade de Ras e Akt encontra-se aumentada na maioria dos GBMs analisados (6, 41, 42), enquanto o baixo nível de expressão de PTEN, supressor tumoral que antagoniza as ações de PI3K, está diretamente relacionado com a malignidade dos gliomas (8). A redução ou perda do PTEN parece de grande importância para a progressão dos gliomas de baixo grau a GBM. Estudos em modelos animais confirmaram a importância da anormalidade destas vias na biologia dos gliomas (43, 44). A ligação entre o desenvolvimento tumoral e a perda de PTEN funcional revela que um aumento desproporcional na atividade da via PI3K/Akt e o aumento dos níveis de PI3´-fosforilados pode levar a modificações através da combinação de eventos como o aumento da proliferação celular, diminuição da apoptose e modificações na motilidade e adesão celular (27, 36, 45) (Figura 3).

Avanços recentes acerca dos mecanismos celulares e moleculares relacionados à patogênese do GBM têm encorajado pesquisadores a elucidar novos processos celulares e alvos moleculares a serem utilizados em promissoras abordagens terapêuticas. Além disto, estratégias para incluir agentes que promovam o aumento da permeabilidade da BHE com consequente aumento da eficácia do tratamento também são alvos das pesquisas. Frente aos achados, há necessidade de novos tratamentos que visem a modulação de vias de sinalização, a fim de aumentar a especificidade e a eficácia e de minimizar a toxicidade do tratamento.

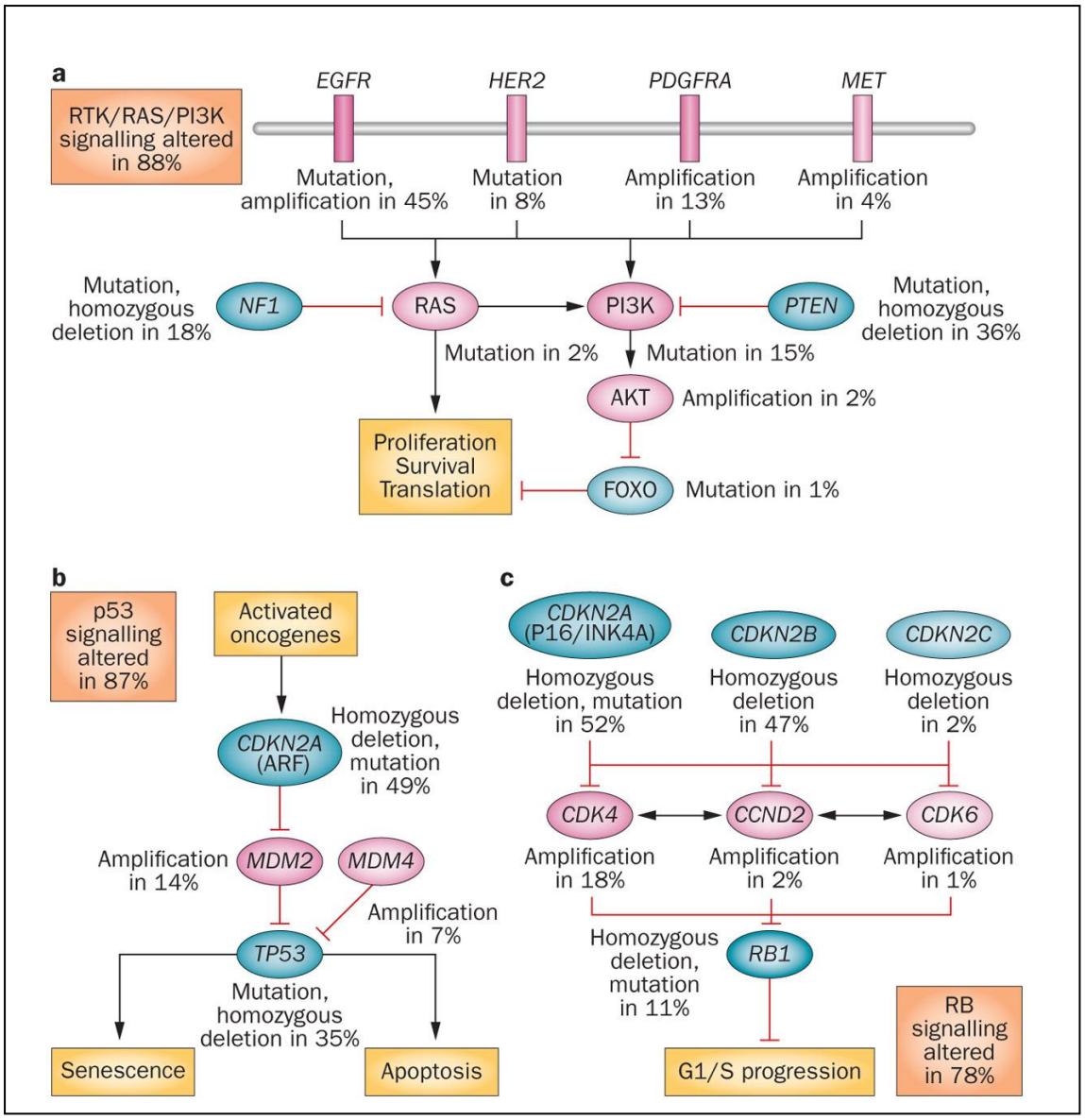

Figura 3. Principais vias de transdução de sinal alteradas na patogênese dos gliomas malignos. Extraído de Tanaka, S. *et al*., 2012.

1.2 A Bradicinina

A Bradicinina (BK) pertence à família das cininas, peptídeos biologicamente ativos que modulam eventos inflamatórios e estão presentes em condições como sepse, dano pós-isquêmico, dor, asma, pancreatite, diabetes, artrite reumatóide, alergia, cistite e gastrite, além do câncer (46-54). Componentes do sistema calicreína-cininas são alvos de estudo desde 1909, quando um princípio hipotensor foi encontrado na urina, mais tarde identificado como a calicreína. Em 1949, Maurício Rocha e Silva demonstrou que a incubação do veneno da cobra *Bothrops jararaca* ou de tripsina, com a fração

pseudoglobulina do plasma, resultava na liberação de um potente agente vasodilatador, que causava contração em preparações isoladas de íleo de cobaia. Baseados nesses resultados, os autores sugeriram o nome *bradicinina* para definir a nova molécula (do grego: *bradys* para lento; *kinesia* para movimento). As cininas são formadas a partir de α-globulinas denominadas cininogênios, os quais sofrem ação de enzimas plasmáticas ou teciduais denominadas calicreínas (55) e são degradadas pela ação de enzimas conhecidas como cininases (56). A BK e a Lys-BK (calidina) são metabolizadas pela ação da cininase II, também chamada enzima conversora da angiotensina (ECA) (57), que se encontra distribuída, especialmente, na membrana das células endoteliais e atua removendo o dipeptídeo da porção C-terminal da BK e da Lys-BK, resultando na inativação destes dois peptídeos. A cininase I, por sua vez, leva à produção dos metabólitos ativos des-Arg^9-BK e Lys-des-Arg^9-BK, a partir da BK e da Lys-BK, respectivamente (58, 59) (Figura 4).

A BK possui uma meia-vida plasmática curta, que varia de 10 a 15 segundos (56), enquanto os peptídeos ativos des-Arg^9-BK e Lys-des-Arg^9-BK apresentam meia-vida plasmática de quatro a doze vezes maior, embora também sejam inativados pela ECA, mas de forma muito mais lenta (57, 60). A cininase II possui maior afinidade pela BK e pela Lys-BK do que a cininase I, o que pode mudar durante alterações inflamatórias. Este fato sugere que a formação dos metabólitos ativos *des-Arg* não ocorre *in vivo* sob condições fisiológicas (61).

A BK é um importante mediador envolvido em processos fisiológicos e patológicos, como a inflamação e vasodilatação (62, 63) e promove uma série de efeitos biológicos, como a liberação de NO e prostaciclinas, além de ser um importante componente na mobilização do Ca^{2+} intracelular (52, 64). As ações da BK e de outras cininas são mediadas por dois subtipos de receptores com sete domínios transmembrana acoplados à proteína G (GPCR, 7TM), denominados B_1 (B_1R) e B_2 (B_2R) – responsáveis por mediar a maioria dos efeitos das cininas em diferentes tipos celulares (37, 46, 55, 65).

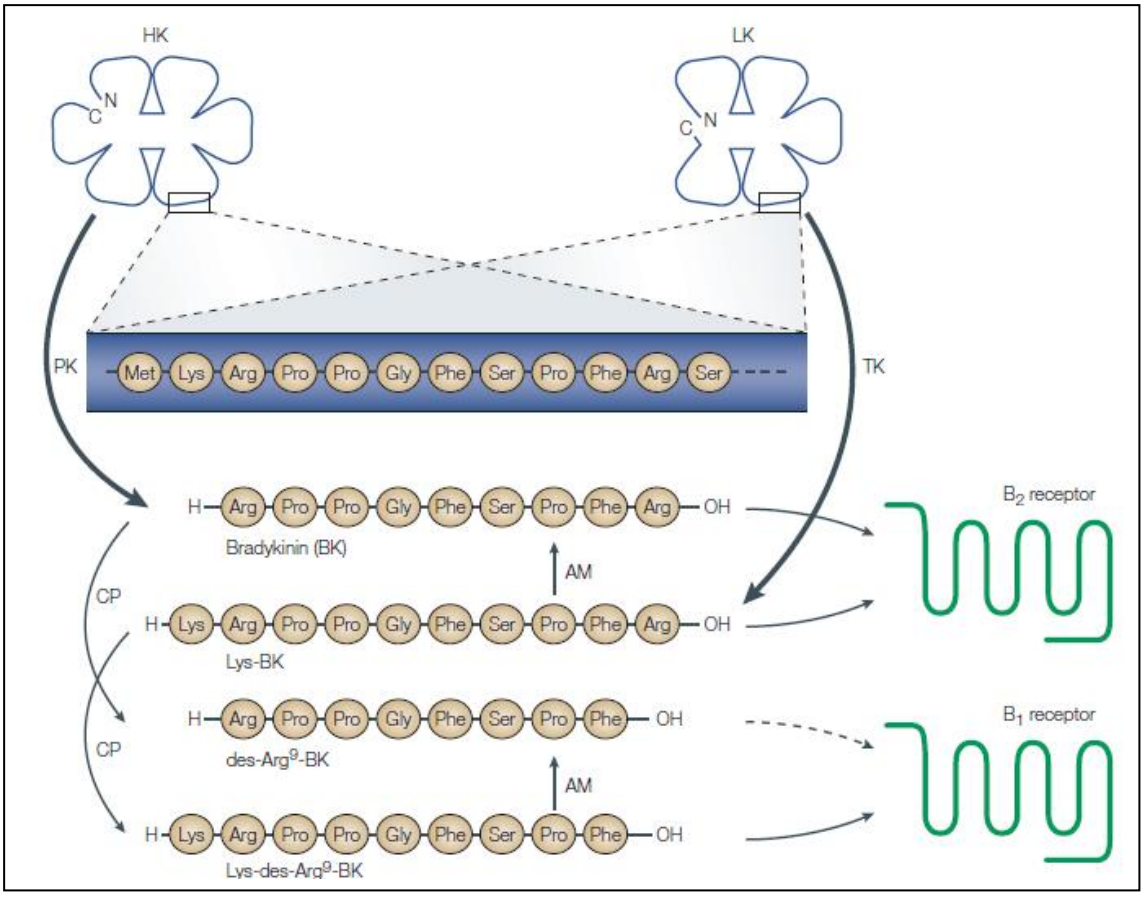

Figura 4. Representação esquemática do sistema calicreína-cininas. Extraído de Marceau & Regoli, 2004.

Os B_2R apresentam alta afinidade pela BK e pela Lys-BK; são constitutivamente expressos em diversos tecidos e considerados os principais mediadores dos efeitos fisiológicos da BK. Diferentemente dos B_2R, os B_1R se ligam aos metabólitos des-Arg^9-BK e Lys-des-Arg^9-BK e, não são comumente expressos em condições normais, mas podem ser induzidos após condições particulares como infecção ou trauma (46, 48, 53) (Figura 5). De forma interessante, ambos os receptores B_1 e B_2 são altamente expressos em células de glioma (66-69).

Com relação às vias de transdução de sinal, estas são semelhantes para ambos receptores, e requerem Ca^{2+} para a sinalização. Entretanto, os B_1R não sofrem dessensibilização e internalização, evento observado nos B_2R (55). Esta característica deve-se ao fato de os B_1R não apresentarem sítios de serina e tronina na extremidade c-terminal, os quais são necessários para o mecanismo de internalização via quinases, comum aos GPCRs (52). Além disso, o aumento de Ca^{2+} induzido pela ativação dos B_2R é transitório, ao passo que para os B_1R, este representa um evento sustentado.

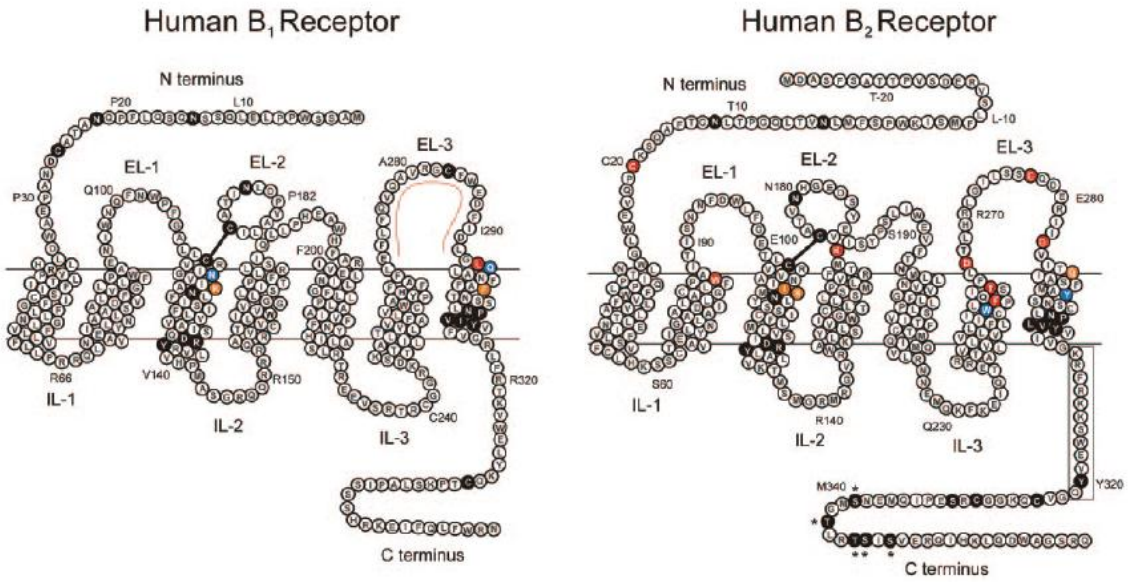

Figura 5. Representação esquemática dos receptores B_1R e B_2R. Extraído de Leeb-Lundberg, 2005.

Embora a BK esteja relacionada a diferentes efeitos fisiopatológicos, seus efeitos no SNC ainda são bastante discutidos. A BHE efetivamente separa a circulação sistêmica do SNC, criando uma espécie de proteção, de onde as pequenas e grandes moléculas hidrofílicas são excluídas (35, 70, 71). A BHE intacta impede a passagem de substâncias solúveis com peso molecular maior que 180 Daltons (Da), sendo que os quimioterápicos mais eficazes disponíveis atualmente apresentam peso molecular que varia de 200 a 1200 Da (72). Deste modo, a BHE pode atuar como uma camada protetora da massa tumoral, por limitar a chegada dos agentes antitumorais ao tecido-alvo. Além disto, os capilares existentes no tumor cerebral apresentam uma barreira hematoencefálica tumoral (BTB), que envolve o tumor e possui características particulares que a diferenciam da BHE. A integridade da BTB é heterogênea no tumor maligno e, se apresenta mais permeável no centro do tumor e menos permeável nas bordas que são ativamente proliferativas (73, 74). Deste modo, a quantidade de quimioterápico que atinge as bordas é mínima, o que favorece o comportamento infiltrativo do GBM e torna a ressecção cirúrgica completa praticamente impossível (73). Assim, mecanismos moleculares e bioquímicos alternativos têm sido utilizados para aumentar a permeabilidade da BHE e da BTB, como uma estratégia para facilitar a penetração dos fármacos utilizados no tratamento dos tumores do SNC (35, 71, 75).

Atualmente, o composto vasoativo mais eficiente no aumento da permeabilidade das BHE e BTB é a BK, ou seu análogo, o receptor mediador permeabilizador-7 (RMP-

7). O mecanismo envolvido no aumento da permeabilidade da BTB parece ativar uma cascata de transdução de sinal que inicialmente promove um aumento transitório nas concentrações de Ca^{2+} intracelular (76, 77), através da ativação da fosfolipase C e também do influxo de Ca^{2+} extracelular (65). O aumento de Ca^{2+} citosólico estimula a ativação do complexo Ca^{2+}/calmodulina quinase dependente e da NOS constitutiva, onde a produção de GMPc é facilitada pela ativação do NO, que medeia a ativação de guanilato ciclase (78). O aumento do Ca^{2+} intracelular e de GMPc ocasiona a contração das células endoteliais dos capilares, o que resulta na modificação das junções aderentes e aumento da permeabilidade vascular (79). Alguns estudos sugerem que a via do NO pode desempenhar um papel importante no aumento da permeabilidade da BTB após a infusão de BK (72, 80, 81).

Em modelos animais de tumor cerebral, foi demonstrado que a BK pode aumentar a permeabilidade da BHE, estimulando receptores presentes no endotélio e iniciando uma resposta via segundos mensageiros, capaz de promover a abertura das junções aderentes (82). Além disto, modelos de glioma em ratos demonstraram que a abertura da BTB induzida pela BK é seletiva, o que aumenta a permeabilidade desta barreira às drogas direcionadas aos tumores cerebrais, mas não aumenta a exposição do tecido cerebral normal aos efeitos tóxicos destes agentes antitumorais (65, 83). Alguns estudos sugeriram o envolvimento exclusivo do B_2R no aumento da permeabilidade das barreiras mediado pela administração de BK, já que tal permeabilidade é significativamente inibida pelo uso de antagonistas seletivos deste subtipo, o que não é observado utilizando antagonistas de B_1R (76, 84, 85). Por outro lado, um estudo recente descreveu o possível potencial de permeabilização local da BTB através do uso do agonista bioestável sintético de B_1R em modelo de glioma em ratos (67), fornecendo um novo mecanismo de modulação desta barreira através dos GPCRs.

Recentemente a BK vem sendo associada de forma importante a diferentes tipos de tumores, entre o quais os tumores cerebrais. Como descrito anteriormente, os gliomas apresentam uma rede vascular abundante, rica e dinâmica, de extrema importância para a sustentação e sobrevivência do tumor. Neste contexto, os receptores de BK tem-se destacado como alvo potencial na relação das células de glioma com a vasculatura tumoral e a angiogênese (34). Alguns estudos clínicos vêm sendo realizados com ligantes peptídicos e não peptídicos de B_1R e B_2R, focando no perfil favorável dos antagonistas de cininas em diversos modelos de câncer, particularmente em câncer de

mama, pulmão e próstata (86). Com relação aos tumores primários do SNC, um estudo de fase II utilizando o agonista B_2R RMP-7 associado a carboplatina não apresentou o sucesso clínico esperado em crianças com glioma (87, 88). Além disso, estudos realizados no SNC têm associado fortemente os receptores de cininas às funções cerebrais. Alguns autores sugerem o envolvimento pró-epileptogênico de B_1R e B_2R em modelo de epilepsia em camundongos (89). Já a ativação de B_1R parece promover um efeito deletério em modelo *in vivo* de Alzheimer, enquanto o antagonismo de B_1R melhora as funções cerebrovasculares, de aprendizado e memória (90).

Em um estudo desenvolvido por Zhao *et al*. (2005) foi possível observar que o grau de malignidade dos tumores cerebrais está diretamente relacionado aos níveis de expressão de B_2R nas células do tecido tumoral humano, onde os pacientes com tumores de alto grau (grau III) apresentaram os maiores níveis de expressão dos B_2R, enquanto as células do tecido edematoso presentes em torno do tumor dificilmente expressam este receptor (66). Um perfil semelhante foi encontrado em amostras de GBM humano com relação ao B_1R, onde pacientes com tumor grau IV apresentaram um nível aumentado de expressão deste receptor quando comparados à pacientes com tumores de baixo graus I e II (67).

1.3 Os Canais de Cálcio Voltagem-Dependentes

Os canais de cálcio voltagem-dependentes (CCVD) estão envolvidos em uma série de processos fisiológicos e patológicos que envolvem células nervosas e musculares, e sua presença define uma célula excitável (91). Os CCVD foram identificados por Paul Fatt e Bernard Katz em 1953, que observaram um evento interessante em músculo de crustáceos, onde após a inibição das correntes de Na^+, o músculo ainda apresentava potencial de ação (91, 92). Após esta descoberta, outros pesquisadores identificaram diferentes subtipos de canais em células excitáveis (93) e os canais voltagem dependentes foram classificados de diferentes modos (94).

Os CCVD pertencem a super família de canais iônicos protéicos transmembrana, na qual também estão incluídos os canais de sódio e potássio dependentes de voltagem. Estes canais medeiam o influxo de Ca^{2+} em resposta a despolarização da membrana e regulam diferentes processos intracelulares. O Ca^{2+} que entra nas células através dos CCVD, serve de segundo mensageiro na sinalização elétrica e na iniciação de eventos intracelulares como contração, secreção, transmissão sináptica e expressão gênica (94).

Os CCVD são formados pela subunidade principal α1 e pelas subunidades auxiliares α2, β, δ e γ. A subunidade α1 é a maior delas, onde se localizam os sensores de voltagem, o poro de condução iônica e o componente de portão do canal, além dos locais de regulação do canal por drogas e toxinas. As subunidades auxiliares modulam as propriedades do complexo canal, gerando a diversidade eletrofisiológica e farmacológica, em associação com a subunidade α1. Os CCVDs são comumente classificados em canais de baixo limiar de ativação (subtipo T) e de alto limiar de ativação (subtipos L, N, P/Q e R) (95). Até o momento, foram identificados nove subtipos de canais de Ca^{2+} dependentes de voltagem (figura 6) e essa diversidade deve-se a natureza da subunidade α1 formadora do poro principal. São descritas três diferentes famílias de subunidades α1: *Cav1* que codificam canais tipo-L, *Cav3* que codificam canais tipo-T e *Cav2* que codificam canais tipo-N, P/Q e R (96). Os canais de Ca^{2+} predominantes nos terminais sinápticos nervosos são aqueles do tipo-P/Q e -N, com os seus níveis de expressão variando ao longo do SNC. A diferente distribuição destes dois subtipos de canal reflete os papéis fisiológicos e patológicos distintos, ainda que sejam ativados e regulados por mecanismos comuns (97).

Os CCVD do tipo-P/Q (também descritos como *Cav2.1*) são canais pré-sinápticos de alta voltagem, os quais estão intimamente envolvidos em processos de excitação neuronal e liberação de neurotransmissores (98). Os canais do tipo-P foram inicialmente identificados em células de Purkinje do cerebelo (99), diferente dos canais do tipo-Q, encontrados nos neurônios cerebelares (100). Entretanto, ambos são caracterizados por sua sensibilidade a ω-agatoxina IVA – proveniente da fração do veneno da aranha *Agelenopsis aperta* – e são codificados pelo gene *CACNA1A*, onde o predomínio de *splicing* alternativos pode explicar as diferenças fenotípicas entre P e Q. Desta forma, os mesmos aparecem combinados como o tipo-P/Q e sua alta expressão no SNC faz deste subtipo de canal um alvo promissor ao que se refere a desordens neurológicas. Os canais do tipo-N (também conhecidos como *Cav2.2*) pertencem igualmente ao grupo dos canais ativados por alta voltagem e estão localizados preferencialmente no SNC, atuando na transmissão sináptica. Canais deste subtipo são codificados pelo gene *CACNA1B* e bloqueados por ω-conotoxinas, como a MVIIA, GVIA e CVID, isoladas do veneno de moluscos marinhos, entre eles o *Conus magus* e *Conus geographus* (97, 101).

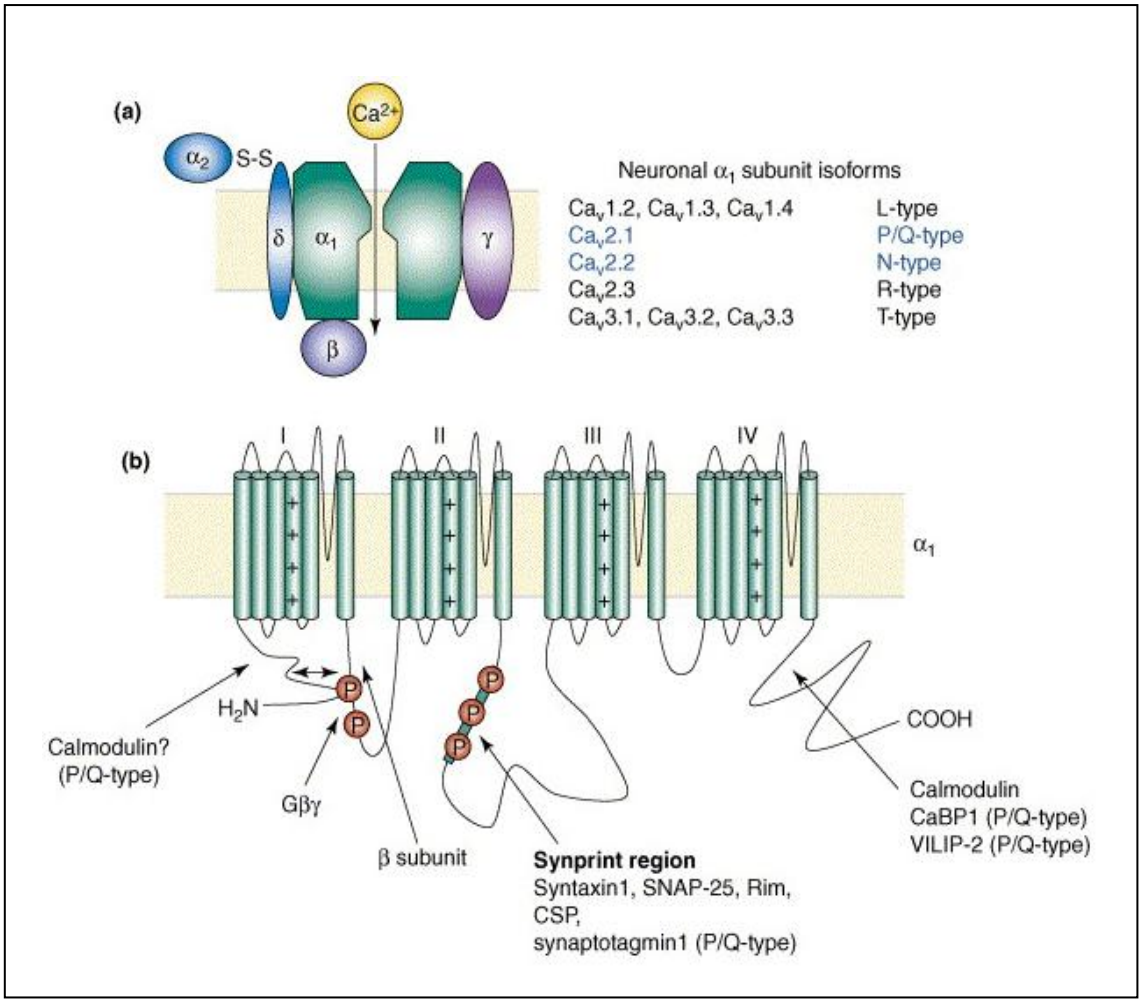

Figura 6. Aspectos estruturais dos canais de cálcio voltagem dependentes (CCVD). Extraído de Evans & Zamponi, 2006.

Com relação a patologias relacionadas ao SNC, estudos tem associado o bloqueio dos CCVD tipo-P/Q e -N com uma melhora na frequência das crises em pacientes com epilepsia (102), além de retardar o declínio cognitivo observado em pacientes com doença de Alzheimer (103). Neste sentido, o papel neuroprotetor destes canais tem sido extensivamente descrito nos últimos anos (104, 105). Além disso, os CCVD parecem estar envolvidos na sensibilização da dor central, proveniente da lesão nervosa ou em estados inflamatórios (106, 107). Em modelos animais de lesão nervosa, demonstrou-se que o bloqueio dos CCVD do subtipo N e P/Q foi capaz de reduzir os sinais comportamentais de nocicepção de origem neuropática (106).

De modo geral, o Ca^{2+} participa da regulação da homeostase e de inúmeros processos fisiológicos, como proliferação, apoptose, migração e expressão gênica, sendo a sua ação espacial e temporal de grande importância para desencadear uma resposta celular específica. Algumas patologias vêm sendo relacionadas com a regulação anormal da homeostase de Ca^{2+}, como a hipertensão, doenças

cardiovasculares, diabetes, doença de Alzheimer e diversos tipos de câncer (108, 109). De interessante, estudos recentes têm demonstrado a implicação dos CCVD em alguns tipos de câncer. A transformação de uma célula normal em uma célula com potencial cancerígeno está associada com o rearranjo da homeostase de Ca^{2+}, envolvendo a expressão alterada de proteínas de transporte e de canais permeáveis ao Ca^{2+}. Oscilações nas concentrações de Ca^{2+} têm sido diretamente relacionadas com a migração das células em gliomas (110, 111), sendo as flutuações nas concentrações do Ca^{2+} intracelular necessárias para a expressão de fenótipos malignos, como proliferação, migração, invasão e angiogênese sustentada neste tipo de tumor cerebral (109, 112-114).

Um estudo *in vitro* utilizando linhagens celulares de carcinoma hepatocelular demonstrou que o bloqueio dos canais de Ca^{2+} tipo-T diminuiu a proliferação celular neste tipo de tumor (115). Além disso, a inibição dos canais de Ca^{2+} tipo-T em modelo de melanoma promoveu a apoptose (116) e inibiu de forma importante a proliferação e viabilidade celular (117), demonstrando a importância deste tipo de canal na tumorigênese e progressão do melanoma. Com relação aos tumores do SNC, a inibição dos canais de Ca^{2+} tipo-T também atenuou a proliferação e migração em modelo utilizando células de GBM humano (118). Frente aos recentes achados, os CCVD surgem como potenciais alvos terapêuticos no manejo dos tumores malignos o desenvolvimento de bloqueadores potentes e seletivos para os diferentes subtipos torna esses canais de Ca^{2+} alvos moleculares promissores.

1.3.1 A *Phoneutria nigriventer*

Aranhas do gênero *Phoneutria* pertencem à família Ctenidae e são amplamente distribuídas nas regiões tropicais da América do Sul. No Brasil são descritas seis espécies do gênero *Phoneutria*: *P. boliviensis, P. reidyi, P. bahiensis, P. fera, P. keyserlingi* e a *P. nigriventer*, todas potencialmente perigosas a humanos e animais. A *P. nigriventer* tem hábitos noturnos e é extremamente agressiva, sendo predadora natural de uma variedade de insetos, de outras aranhas e pequenos roedores. Essa aranha não constrói teia e o seu sucesso como predadora pode ser explicado pela diversidade de toxinas presentes no seu veneno (119, 120). Popularmente a *P. nigriventer* é conhecida como “Armadeira”, devido à postura corporal que assume quando se sente ameaçada, ou antes do ataque à presa, onde ela mantém erguidos os dois pares de patas dianteiras

(Figura 7). A picada da *P. nigriventer* causa sintomas como dor intensa e irradiada, além de sinais de alta toxicidade, que incluem espasmos, tremores, convulsões tônicas, paralisia, taquicardia e disritmia, distúrbios visuais e sudorese, sintomas que se não tratados de forma adequada podem levar a vítima a óbito (121). Estes sintomas são causados por ações centrais e periféricas, em decorrência da liberação maciça de neurotransmissores nas terminações nervosas.

Figura 7. A aranha *Phoneutria nigriventer*.

Até o momento foram descritos cerca de 20 peptídeos ativos, com peso molecular que varia de 3500 a 9000 Daltons, obtidos das toxinas presentes no veneno da *P. nigriventer*, os quais interagem com canais de Na^+, Ca^{2+}, K^+ e em receptores presentes no SNC (120). Parte do efeito neurotóxico do veneno parece estar relacionado à sua ação sobre canais de Na^+ regulados por voltagem. Entretanto, outras atividades farmacológicas relacionadas à ação em canais iônicos, podem ser encontradas. Este fato pode ser facilmente compreendido com o fracionamento do veneno, onde é possível separar as frações e as ações farmacológicas distintas. Rezende Junior et al., (1991) (119) propuseram um método para o fracionamento do veneno da *P. nigriventer*, através do qual o veneno é processado por uma combinação de filtragem em gel e RFPLC gerando 3 frações neurotóxicas distintas: Phoneutriatoxina-1 (PhTx1), Phoneutriatoxina-2 (PhTx2) e Phoneutriatoxina-3 (PhTx3) (119). As frações PhTx1 e PhTx2 produzem sinais excitatórios e contração do íleo em cobaias. Por outro lado, a fração PhTx3 causa paralisia flácida (119), possivelmente em conseqüência da ação inibitória sobre a liberação de neurotransmissores (122, 123). Seis diferentes toxinas (PhTx3-1 a 6) foram purificadas a partir da fração PhTx3 (124) e três delas, PhTx3-3, PhTx3-4 e PhTx3-6, bloquearam o influxo de Ca^{2+} induzido por KCl em terminais

nervosos (123, 125, 126), demonstrando que estas toxinas bloqueiam canais de Ca^{2+} e apresentam, portanto, efeito semelhante às ω-conotoxinas.

As ω-conotoxinas foram descritas há mais de 30 anos (127, 128), isoladas a partir do veneno de moluscos marinhos do gênero *Conus*, sendo amplamente caracterizadas como potentes bloqueadores dos CCVDs (129). A especificidade destas toxinas aos diferentes subtipos de canais de Ca^{2+}, somada a características químicas e estruturais estáveis e à fácil manipulação e síntese, torna as ω-conotoxinas ferramentas farmacológicas atraentes. Ao longo do processo evolutivo, para exercer melhor efeito na captura das presas ou como ferramenta de defesa, os peptídeos presentes no veneno de diversas espécies tornaram-se potentes e especializados, e hoje representam uma importante fonte de diversidade química a ser estudada (129). Com relação ao gênero *Conus*, as 500 espécies deste gênero expressam aproximadamente 100 diferentes conopeptídeos, o que pode representar uma variedade de mais de 50.000 compostos farmacologicamente ativos a serem investigados (130).

Pesquisas realizadas na última década têm focado no estudo do papel de inibidores de canal de cálcio tipo-N para o desenvolvimento de novos fármacos analgésicos (131). Recentemente foi demonstrado o potencial terapêutico da ω-conotoxina MVIIA, bloqueadora específica dos CCVD tipo-N, capaz de prevenir e atenuar a dor. A versão sintética do peptídeo ω-conotoxina MVIIA é atualmente comercializada como Prialt® (Azur Pharma International, Filadélfia, EUA), um medicamento aprovado pelo FDA para o tratamento da dor em pacientes que necessitam de analgesia intratecal e são refratários à terapia com opióides (132). Este composto, também conhecido como ziconotida ou SNX-111, apresenta propriedades analgésicas e se mostrou eficaz no tratamento da dor crônica por bloquear os CCVD do tipo-N na medula espinhal. Além disso, a ω-conotoxina MVIIC, também extraída do molusco marinho *C. magus*, é um potente bloqueador dos CCVD tipo-P/Q (98, 133), e também vem sendo cuidadosamente analisada pelo seu potencial na aplicação clínica. Ambas as ω-conotoxinas, MVIIA e MVIIC, são extensivamente utilizadas como compostos de referência em estudos que envolvem os CCVD.

A isoforma PhTx3-6, extraída da fração PhTx3 do veneno da *P. nigriventer*, foi recentemente patenteada e denominada Phα1β, composto capaz de bloquear seletivamente os CCVD do tipo-N (134). Neste sentido, pesquisas demonstraram que a Phα1β é tão potente quanto a ω-conotoxina MVIIA na resposta antinociceptiva e

apresentou índice terapêutico maior do que ω-conotoxina MVIIA em experimentos pré-clínicos (135, 136). Além disso, o efeito neuroprotetor da fração PhTx3 foi recentemente descrito em estudos utilizando modelos de isquemia cerebral e morte neuronal (137, 138). De forma muito interessante, recentemente foi demonstrado o envolvimento do veneno da aranha *P. nigriventer* no aumento da expressão de VEGF (139) e na ativação do sistema calicreína-cininas, com a consequente liberação de BK (140). Estes eventos podem estar associados ao potencial do veneno em promover a abertura da BEH, além de destacar a possível interação entre os receptores B_1R e B_2R e os CCVD (140, 141). Frente aos recentes achados, especula-se que a sinalização dependente de Ca^{2+} pode desempenhar um papel importante via regulação dos CCVD no desenvolvimento e progressão do tumor e na migração das células tumorais.

2. JUSTIFICATIVA

Dentre os gliomas, o glioblastoma (grau IV) representa a forma mais comum e agressiva de tumores cerebrais primários. Os tratamentos disponíveis na prática clínica são limitados e constituem uma necessidade não atendida, já que contribuem pouco para a melhora da sobrevida dos pacientes. Diferentes vias de sinalização estão mais ativas no desenvolvimento de gliomas e podem ser utilizadas como ferramentas no combate às células malignas. A sinalização dependente de Ca^{2+} medeia ações importantes nas células tumorais, como proliferação, migração, invasão e metástase. A BK também é um importante mediador em tumores cerebrais e seus receptores podem estar associados ao grau de malignidade nestes tumores. Uma abordagem interessante é a combinação de estratégias, como o aumento da exposição de possíveis alvos citotóxicos, aliado à toxicidade específica e com baixos efeitos colaterais. O uso de receptores da família dos GPCRs como base para possíveis alvos do tratamento para o câncer é promissor e pode favorecer o desenvolvimento de novas abordagens terapêuticas. O efeito da sinalização desencadeada pela BK e da sinalização Ca^{2+}-dependente podem estar envolvidas na regulação do crescimento e desenvolvimento dos gliomas. Neste sentido, este trabalho visou explorar o efeito dos receptores B_1R e B_2R de cininas e da sinalização de Ca^{2+} via CCVD tipo-P/Q e -N em modelo de glioma *in vitro* e *in vivo*.

3. OBJETIVOS

3.1 Objetivo Geral

Avaliar o efeito dos receptores B_1 e B_2 de cininas e dos CCVD Tipo-P/Q e -N na proliferação e desenvolvimento dos gliomas *in vitro* e *in vivo*.

3.2 Objetivos Específicos

3.2.1 Parte I - *in vitro*

- Avaliar a expressão dos receptores B_1 e B_2 nas linhagens U-138MG e U-251MG de glioma humano;
- Avaliar a proliferação e a viabilidade celular das linhagens U-138MG e U-251MG após o tratamento com os agonistas dos receptores B_1 e B_2 (des-Arg^9-BK e BK);
- Verificar a citotoxicidade e caracterizar o tipo de morte celular desencadeada pelos antagonistas dos receptores B_1 e B_2 (SSR240612 e HOE-140) nas linhagens U-138MG e U-251MG de glioma humano;
- Determinar a relevância das diferentes vias de sinalização associadas ao desenvolvimento de gliomas nas linhagens U-138MG e U-251MG expostas aos agonistas dos receptores B_1 e B_2;
- Avaliar a proliferação e viabilidade celular das linhagens M059J, U-138MG e U-251MG de glioma humano após o bloqueio dos CCVD Tipo-P/Q e -N pelas frações da toxina da aranha *P. nigriventer* PhTx3-3 e Phα1β e pelos compostos de referência, ω-conotoxinas MVIIC e MVIIA;
- Caracterizar o tipo de morte celular desencadeada pelo bloqueio dos CCVD Tipo-P/Q e -N pelas frações da toxina da aranha *P. nigriventer* PhTx3-3 e Phα1β e pelos compostos de referência, ω-conotoxinas MVIIC e MVIIA;

3.2.2 Parte II – *in vivo*

- Avaliar o crescimento tumoral e alterações histopatológicas em animais *knockout* para os receptores B_1 e B_2 e em animais C57BL/6 tratados com antagonistas dos receptores B_1 e B_2 (SSR240612 e HOE-140);
- Avaliar a expressão dos receptores B_1 e B_2 em animais C57BL/6 tratados com os antagonistas SSR240612 e HOE-140;

- Verificar a imunodetecção dos receptores B_1 e B_2 e das células gliais no modelo de implante de glioma em camundongos;
- Avaliar o crescimento tumoral em animais C57BL/6 tratados com as frações PhTx3-3 e Phα1β da toxina da aranha *P. nigriventer* e com os compostos de referência, ω-conotoxinas MVIIC e MVIIA;
- Verificar a imunodetecção de astrócitos e microglia no modelo de implante de glioma após tratamento com as frações PhTx3-3 e Phα1β da toxina da aranha *P. nigriventer* e com os compostos de referência, ω-conotoxinas MVIIC e MVIIA.

Capítulo II

“Mechanisms involved in kinin-induced gliomas cells proliferation: the role of ERK1/2 and PI3K/Akt pathways”

Manuscrito publicado no periódico *Journal of neuro-oncology*, 2014.

J Neurooncol (2014) 120:235–244
DOI 10.1007/s11060-014-1549-4

LABORATORY INVESTIGATION

Mechanisms involved in kinin-induced glioma cells proliferation: the role of ERK1/2 and PI3K/Akt pathways

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Received: 6 February 2014 / Accepted: 6 July 2014 / Published online: 24 July 2014

Abstract Gliomas are the most common malignant brain tumors in adults. Bradykinin (BK) displays an important role in cancer, although the exact role of kinin receptors in the glioma biology remains unclear. This study investigated the role of kinin B_1 and B_2 receptors (B_1R and B_2R) on cell proliferation in human glioblastoma cell lineages. The mRNA expression of B_1R and B_2R was verified by RT-qPCR, whereas the effects of kinin agonists (des-Arg^9-BK and BK) were analyzed by cell counting, MTT assay and annexin-V/PI determination. The PI3K/Akt and ERK1/2 signaling activation was assessed by flow cytometry. Our results demonstrated that both human glioblastoma cell lines U-138MG and U-251MG express functional B_1R and B_2R. The proliferative effects induced by the incubation of des-Arg^9-BK and BK are likely related to the activation of PI3K/Akt and ERK 1/2 pathways. Moreover, the pre-incubation of the selective PI3Kγ blocker AS252424 markedly prevented kinin-induced AKT phosphorylation. Noteworthy, the selective B_1R and B_2R antagonists SSR240612 and HOE-140 were able to induce cell death of either lineages, with mixed apoptosis/necrosis characteristics. Taken together, the present results show that activation of B_1R and B_2R might contribute to glioblastoma progression in vitro. Furthermore, PI3K/Akt and ERK 1/2 signaling may be a target for adjuvant treatment of glioblastoma with a possible impact on tumor proliferation.

Keywords Bradykinin · Glioblastoma · Kinin receptors · PI3K/Akt · ERK1/2 signaling

Introduction

Gliomas constitute the largest group of primary intracranial tumors in both adults and children. The World Health Organization (WHO) distinguishes four grades of astrocytic tumors on the basis of histological characteristics [1]. Glioblastoma (GBM) is the malignant astrocytic glioma (WHO grade IV) considered the most common and lethal brain tumor. Glioblastoma displays high levels of

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proliferative, migratory and invasion activities [1, 2]. Patients with these tumors have a poor prognosis, and the treatment of malignant gliomas remained unchanged during the last years [3].

Kinins, including bradykinin (BK) and kallidin, are potent peptides which reproduce the classic signs of inflammation. In the central nervous system, BK is considered a potent inducer of neurogliosis, by stimulating the synthesis of other pro-inflammatory mediators [4]. Of interest, BK is recent described as an important molecule associated to brain tumors progression [5, 6]. The effects of BK and its bioactive natural metabolite des-Arg^9-BK are mediated by G-protein-coupled receptors called B_1 (B_1R) and B_2 (B_2R) [7]. B_2R are constitutively and widely expressed throughout the central and peripheral nervous system, mediating most of the physiological effects of kinins. Whereas, B_1R is not expressed to a great extent under normal conditions, but displays an essential role and it is rapidly upregulated following inflammatory, infectious, traumatic stimuli or cancer proliferation [4, 7, 8].

The precise function of B_1R and B_2R in the glioma progression remains unclear. It has been reported that BK induces astrocytic and microglial cell migration [9, 10]. Accumulating evidence suggests that B_1R activation in the brain tumor vasculature increases the delivery of chemotherapy agents [2, 11, 12]. Moreover, gliomas express B_1R [2] and B_2R and this expression was correlated with tumor grade [13]. Bradykinin has also been implicated with chemotactic invasion of gliomas towards blood vessels in situ [5, 14]. Of interest, a recent study suggested that BK enhances the migration of glioma cells through PI3K/Akt signaling cascade via B_1R [6] and the activation of this signaling pathway has been strongly reported in gliomas [15, 16].

Therefore, the aim of this study was to further evaluate the relevance of B_1R and B_2R for the proliferation of human glioma cells, attempting to determine the possible signaling pathways related to kinin receptors activation.

Materials and methods

Agonists and antagonists

Des-Arg^9-BK, BK and HOE-140 were obtained from Bachem Americas Inc. (Bachem, CA, USA.); SSR240612 was kindly donated by SANOFI (Sanofi Research, Paris, France). AS252424 was purchased from Tocris Bioscience (MO, USA).

Cell lines and cell culture

U-138MG and U-251MG human GBM cell lines were from American Type Culture Collection (ATCC-Rockville, Maryland, USA). Cells were cultured in Dulbecco's Modified Eagle Medium with 10 % fetal bovine serum (FBS) at a temperature of 37 °C, a minimum relative humidity of 95 %, and an atmosphere of 5 % CO2 in air.

Cell counting

Glioma cells were seeded at 15–20 $\times$ 10^3 cells per well in 24-well plates. Cells were blocked at G1-phase by initially reducing the concentration of 10 % FBS to 5 %, and subsequently to 0.5 %, for 24 h. Cells were treated for 24 h with the selective B_1R or B_2R agonists. After 24 h, the detach cells were counted in hemocytometer. The cell number of the control group FBS 0.5 % (not treated cells) was considered as 100 % and FBS 10 % served as a positive control for cell proliferation.

Cell viability

The number of cells with metabolically active mitochondria was determined based on the mitochondrial reduction of a tetrazolium bromide salt (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay) according to the method described by Gehring et al. [17]. The cells were treated with selective B_1R or B_2R ligands for 24 h, as described in the next section.

Functional effects of kinin receptors agonists and antagonists incubation

Cell lines U-138MG and U-251MG were incubated with selective agonists or antagonists kinin receptors, alone or in combination, for 24 h. Selective agonists for B_1R (des-Arg^9-BK) and B_2R (BK), were tested at the concentrations of 1, 3, 10, 30 and 100 nM, according to Molina et al. [18]. Selective kinin B_1R (SSR240612) or B_2R (HOE-140) antagonists were tested at the concentrations of 1, 10, 30 and 100 μM, as previously described by Andoh et al. [19]. In a separate series of experiments, cells were treated with the combination of des-Arg^9-BK (10 nM) plus SSR240612 (30 μM), or BK (10 nM) plus HOE-140 (10 μM).

Characterization of cell death by flow cytometry

U-138MG and U-251MG glioma cells were seeded at 2 $\times$ 10^4 cells per well in 24-well plates and grown for 24 h. Cells were treated with selective B_1R (SSR240612, 30 μM) or B_2R (HOE-140, 10 μM) antagonists for 24 h. Cell death was quantified by annexin V-FITC-propidium-iodide (PI) double staining, using Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, CA, U.S.A.), according to the manufacturer's instructions. Experiments were performed on FACSCanto II Flow Cytometer (BD

Biosciences) and the results were analyzed using FlowJo Software (Tree Star).

Molecular characterization of kinin receptors: RT-qPCR analysis

Total RNA from cultures of U-138MG and U-251MG was quantified by spectrophotometry and the cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from 1 μg total RNA. Quantitative PCR was performed using SYBR Green I (Invitrogen) to detect double-strand cDNA synthesis. PCR reactions and the cycling conditions were delineated was previously describe by Sgnaolin et al. [20]. Primers for the following targets were used: 18S and B2M, used as reference genes [17]; B_1R (forward 5′-GCAGCGCTTAACCATAGCGAAAT-3′ and reverse 5′-CCAGTTGAAACGGTTCCCGATGTT-3′); and B_2R (forward 5′-CAGCACCTTCCTGGATACGCTGCATC-3′ and reverse 5′-CACCTCCCAAGACTTCTTTCGGAAGC-3′) designed using Oligos 9.6. [20]. Relative expression levels were determined with 7,500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR 11.0 Software (http://LinRegPCR.nl). Relative RNA expression levels were determined using the $2\text{-}\Delta\Delta^{CT}$ method. The stability of the references genes 18S and B2M (M-value) and the optimal number of reference genes according to the pairwise variation (V) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/).

Determination of signaling pathways activation

The activation of MAP kinases ERK1/2 and p38, or PI3K/Akt in U-138MG and U-251MG was measured by FACScantoII using BD Phosflow Protocol for Adherent Cells [20]. Glioma cells were stimulated with des-Arg^9-BK or BK (10 nM) or FBS (10 %) for 3, 15 and 30 min. Cells were fixed in Phosflow Buffer I for 10 min at 37 °C. After washing, permeabilization was done with Phosflow Perm Buffer III for 30 min on ice. Then, U-138MG and U-251MG cell lines were washed and stained with Alexa 488 anti–phosphor-p38, PE anti–phosphor-AKT anti-phospho-ERK1/2 antibodies for 30 min at on ice.

Effects of the pharmacological inhibition of PI3Kγ by AS252424

In these experiments, the effects of incubation with the selective PI3Kγ blocker AS252424 (1, 3 and 10 μM; 20 min) were evaluated on the phosphorylation of AKT induced by des-Arg^9-BK or BK (10 nM, for 3 min) in U-138MG and U-251MG cells. This experimental set was accomplished as described in the item 2.8.

Statistical analysis

The number of experimental replications is provided in the figure legends. Data was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, using Graph-Pad Software (San Diego, CA, USA). $P < 0.05$ was indicative of statistical significance.

Results

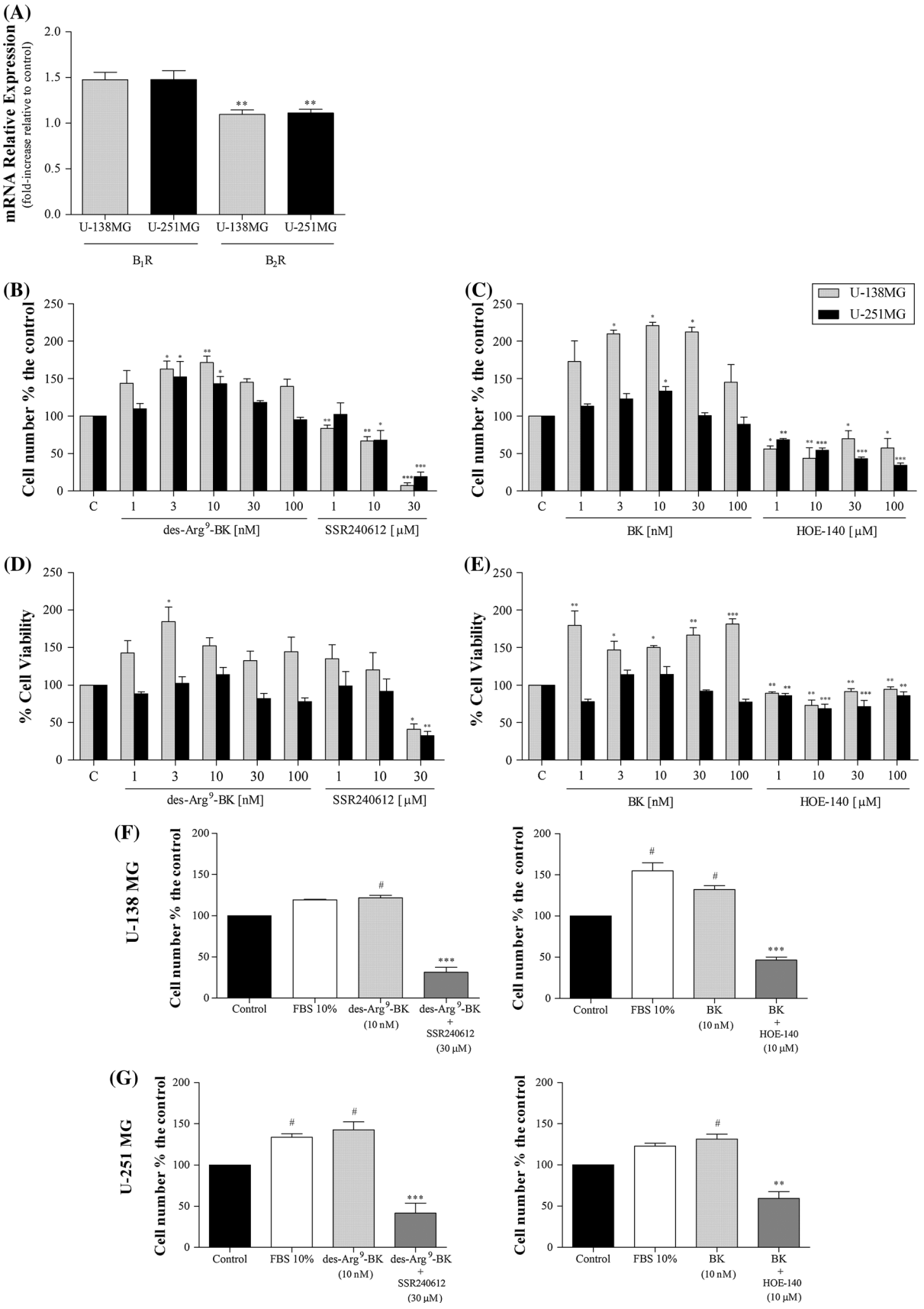
Kinin B_1R and B_2R are expressed in U-138MG and U-251MG human glioma cell lines

Firstly, we investigated the mRNA expression of B_1R and B_2R in U-138MG and U-251MG human GBM cells. We demonstrated the presence of both B_1R and B_2R in these cell lines, which is in agreement with previous reports that showed the expression of both kinin receptors in other glioma cells [2, 5, 6, 21] (Fig. 1a). Interesting, both cell lines tested expressed higher levels of B1R mRNA when compared of the B2R, which can be explained by the inducible feature of B_1R [4, 22].

B_1R and B_2R are related to proliferation in glioblastoma cell lines

The treatment with the selective B_1R des-Arg^9-BK (1–100 nM; Fig. 1b) and B_2R BK (1–100 nM; Fig. 1c) agonists induced a marked enhancement of cell proliferation and viability, according to evaluation of U-138MG and U-251MG cell lines. This effect was not clearly concentration-dependent, as no significant difference was found when comparing cell viability or proliferation among the different tested concentrations. Furthermore, the incubation of the selective B_1R SSR240612 (1–30 μM; Fig. 1b) or B_2R HOE-140 (1–100 μM; Fig. 1c) antagonists decreased cell counting in a concentration-dependent manner in both tested lineages, with maximal inhibitory effects at 30 and 10 μM, respectively. We observed a similar result for kinin agonists and antagonists by using the MTT cell viability assay (Fig. 1d, e).

In an independent series of experiments, cells were incubated with SSR240612 (30 μM) plus des-Arg^9-BK (10 nM) or HOE-140 (10 μM) plus BK (10 nM). The use of selective antagonists was able to significantly reverse the increased proliferation induced by kinin agonists in both cell lines analyzed, confirming the selectivity of kinin responses on cell proliferation (Fig. 1f, g). As depicted in Fig. 2, incubation of either B_1R SSR240612 (30 μM) or


(A)
mRNA Relative Expression
(fold-increase relative to control)
2.0
1.5
1.0
0.5
0.0
U-138MG
U-251MG
U-138MG
U-251MG
B1R
B2R
(B)
Cell number % the control
250
200
150
100
50
0
C
1
3
10
30
100
1
10
30
des-Arg9-BK [nM]
SSR240612 [μM]
(C)
U-138MG
U-251MG
Cell number % the control
BK [nM]
HOE-140 [μM]
(D)
% Cell Viability
des-Arg9-BK [nM]
SSR240612 [μM]
(E)
% Cell Viability
BK [nM]
HOE-140 [μM]
(F)
U-138 MG
Cell number % the control
Control
FBS 10%
des-Arg9-BK
(10 nM)
des-Arg9-BK
+
SSR240612
(30 μM)
BK
(10 nM)
BK
+
HOE-140
(10 μM)
(G)
U-251 MG
Cell number % the control

◀**Fig. 1** **a** Relative gene expression profile of B_1R and B_2R on human glioma cell lines. Data are expressed as mean ± SEM (n = 4) and were analyzed by one-way ANOVA followed by Tukey test as post hoc ($P < 0.05$). **b–e** Effect of treatment with des-Arg^9-BK (1–100 nM) and SSR240612 (1–30 μM) or BK (1–100 nM) and HOE-140 (1–100 μM) on cell counting and cell viability of U-138MG and U-251MG human glioma cells, after 24 h. The experiments were carried out at least three times in triplicate. Each column represents the mean ± SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ for comparison *versus* the control 0.5 % FBs, as determined by ANOVA with Tukey's post hoc test. **f–g** Effects of treatment with the selective B_1R antagonist SSR240612 (30 μM) plus des-Arg^9-BK (10 nM) and B_2R antagonist HOE-140 (10 μM) plus BK (10 nM) on cell counting of U-138MG (**f**) and U-251MG (**g**) cell lines, after 24 h. **f–g** The experiments were carried out at least three times in triplicate. Each column represents the mean ± SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ for comparison versus agonist des-Arg^9-BK or BK and $^{\#}P < 0.05$ for comparison versus the control 0.5 % FBS, as determined by ANOVA with Tukey's post hoc test

B_2R HOE-140 (10 μM) antagonists induced cell death with mixed apoptosis/necrosis characteristics in U-138MG (Fig. 2a) and U-251MG (Fig. 2b) cell lines, according to assessment of AnnexinV/PI positivity, by flow cytometry.

Proliferative effects induced by kinins are related to ERK 1/2 and PI3K/Akt pathways activation

We also verified some of the potential signaling pathways implicated in the proliferative effects of kinin agonists in human GBM cells. The phosphorylation status of PI3K/Akt, p38 and ERK1/2 in response to FBS 0.5 %, des-Arg^9-BK (10 nM) or BK (10 nM) incubation were evaluated by flow cytometry. The stimulation of U-138MG (Fig. 3a, b) and U-251MG (Fig. 3c, d) cell lines with des-Arg^9-BK or BK (at 10 nM) induced a sustained enhancement of either ERK1/2 or PI3K/Akt phosphorylation, which was not significantly different when comparing the distinct time-points evaluated (3, 15 and 30 min). In contrast, the incubation of des-Arg^9-BK or BK failed to induce any significant change of MAP kinase p38 phosphorylation in either tested cell lines.

The role of PI3Kγ in PI3K/Akt phosphorylation by kinin agonists was demonstrated through the inhibition of γ-isoform by AS252424 (1–10 μM). Remarkably, the pre-treatment with the selective PI3Kγ inhibitor AS252424 prevented PI3K/Akt phosphorylation elicited by either des-Arg^9-BK or BK (10 nM) in a concentration-dependent manner, in U-138MG (Fig. 4a) and U-251MG (Fig. 4b) cell lines.

Discussion

The concept that kinins display an important role in tumor growth by regulating cell motility, invasion and angiogenesis has gained great attention over the last years [23]. Recent data has demonstrated that glioma cells express B_1R [2] and B_2R [5] and this expression has a direct relation with the tumor grade malignancy [13]. Importantly, B_2R exhibits constitutive expression and it is thought to be responsible for most of kinin activities under physiological conditions, while B_1R is overexpressed during tissue injury [4]. In agreement with previous reports with other cell lines [2, 5, 6, 21], we demonstrated by RT-qPCR, the presence of B_1R and B_2R in both human glioma tested cell lines. Accordingly, evidence demonstrates that B_1R is generally absent from normal tissues, but it is rapidly induced under stressful situations, such as cancer [4, 22], what might justify the higher levels of B_1R mRNA in comparison to B_2R.

Our data demonstrated that des-Arg^9-BK and BK induced a marked cell growth of U-138MG and U-251MG

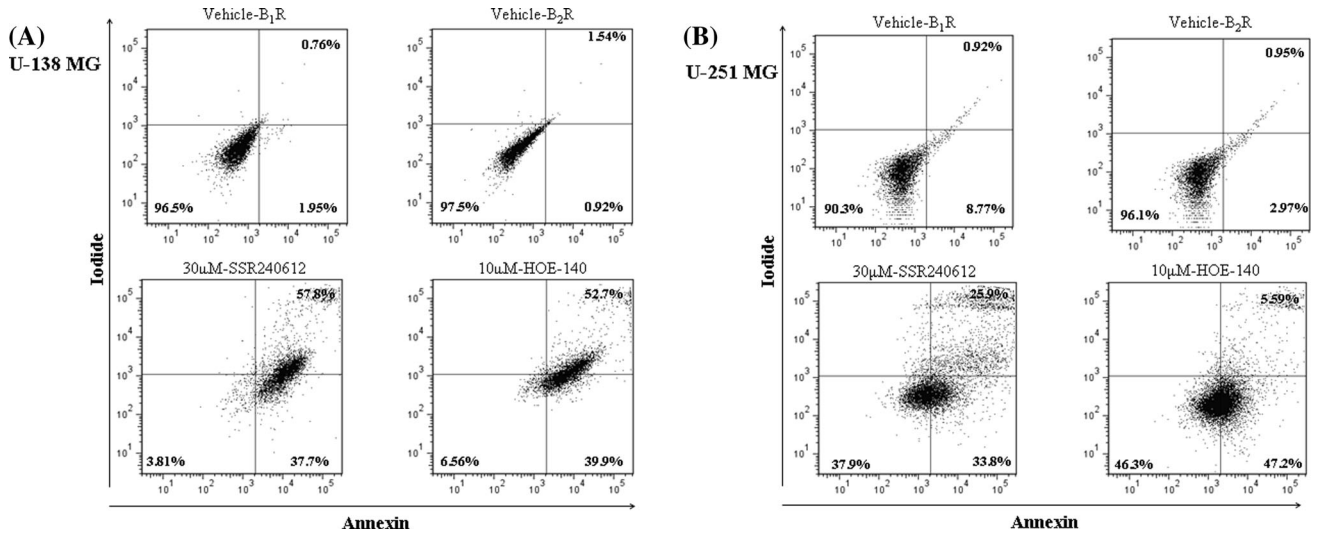


Fig. 2 *Dot* plot with percentage of Annexin V/PI positive U-138MG (**a**) and U-251MG (**b**) cells, 24 h after treatment. Each sample has 50,000 cells. *Data* shown is representative of at least two independent experiments in duplicate

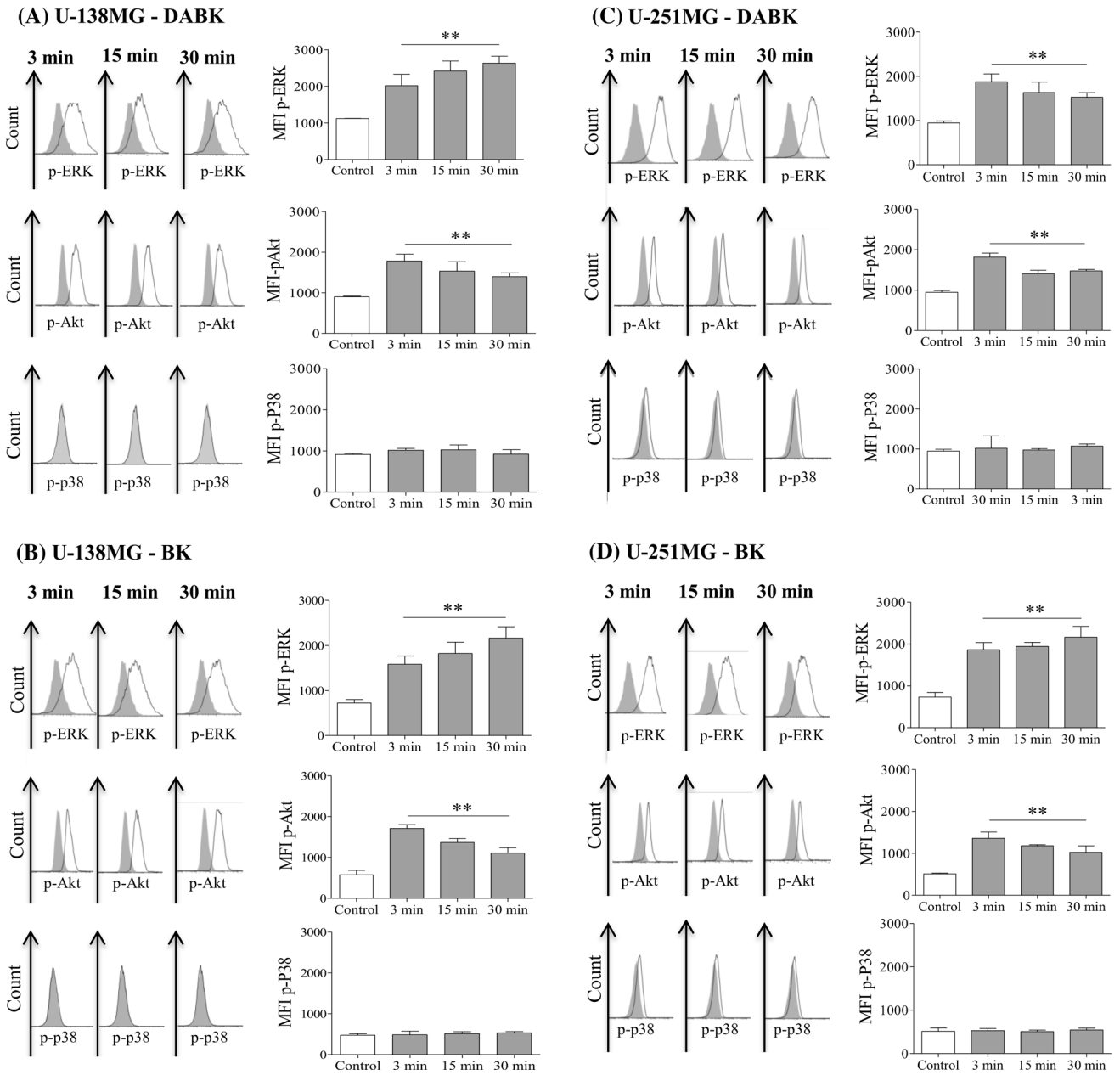


Fig. 3 Effects of stimulation with FBS (0.5 %), des-Arg^9-BK (10 nM) and BK (10 nM) (at 0, 15 and 30 min) on the phosphorylation of PI3K/Akt and ERK1/2 in U-138MG (**a, b**) and U-251MG (**c, d**) cell lines. *Graph bars* represent the mean ± SEM of four independent experiments. $*P < 0.05$ $**P < 0.01$ versus control. ANOVA followed by Tukey's post hoc test

lineages, although this effect was not proportional to the tested concentrations. Accordingly, our research group recently demonstrated that stimulation of B_1R and B_2R through des-Arg^9-BK or BK induced proliferation of bladder cancer cells in an independent-concentration manner [20]. Previous reports showed that kinins are able to enhance the migration of C6 rat glioma cells and U-251MG human glioma cells, as well as COX-2 expression via B_1R activation [6]. Moreover, BK likely contributes to the invasive migration and dispersal of astrocyte-derived tumors through activation of B_2R [5]. These findings are in agreement with a recent study in human chondrosarcoma [24], suggesting that this effect may apply to other cancer types as well. Otherwise, we must highlight the ability of B_1R agonists as selective modulators of the brain vascular permeability, facilitating the access of chemotherapeutic agents into the brain [2, 11, 12].

The inhibition of B_1R and B_2R by antagonists reduced glioma cells proliferation and viability, and induced cell death. We confirmed the selectivity of kinin responses in

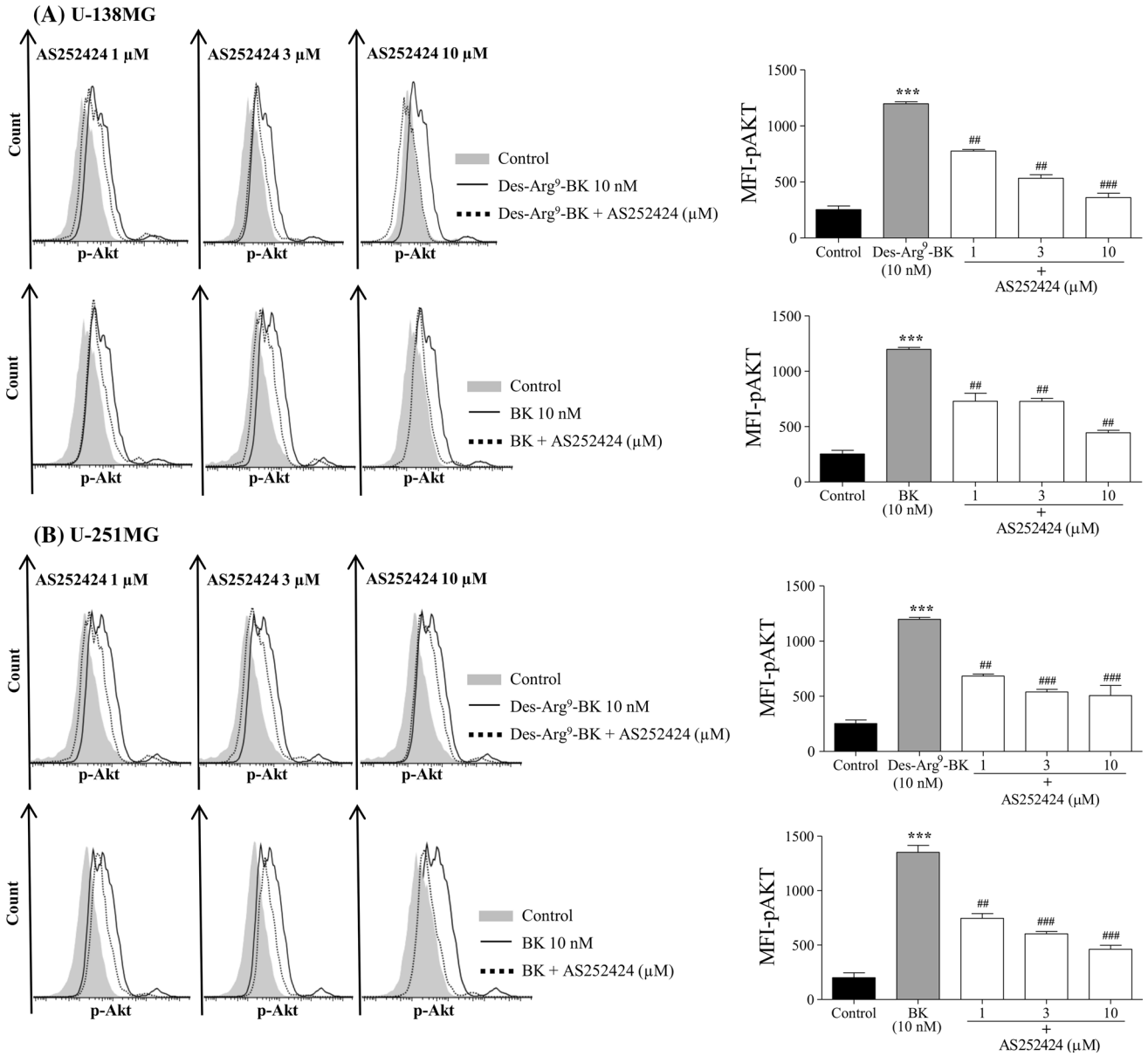


Fig. 4 Effects of incubation with the selective PI3Kγ inhibitor AS252424 (1, 3, 10 µM; for 20 min) on AKT phosphorylation induced by des-Arg^9-BK (10 nM) or BK (10 nM) in U-138MG (**a**) and U-251MG (**b**) cell lines. *Graph bars* represent the mean ± SEM of four independent experiments. $^{***}P < 0.001$ versus control and $^{\#\#}P < 0.01$ $^{\#\#\#}P < 0.001$ for comparison versus agonist des-Arg^9-BK or BK. ANOVA followed by Tukey's post hoc test

cell proliferation by exposing the cell lines to a combination of SSR240612 plus des-Arg^9-BK or HOE-140 plus BK. It has been demonstrated that GBM cell line U-373 has the ability of producing kinins, and this might help to explain the over-negative effect of the antagonists [25]. Finally, we have demonstrated that cell death caused by SSR240612 or HOE-140 displayed a dual positive staining (Annexin V^+/PI^+). A similar profile of cell death was demonstrated with the incubation of nanosponge-encapsulated-camptothecin, which produced cytotoxicity showed double AnnexinV/PI positivity in prostate tumor cells [26]. Both cell lines tested herein are resistant to radiotherapy, and are not sensitive to standard chemotherapy treatment using temozolamide [27, 28]. Therefore, data showing the ability of kinin antagonists to reduce the proliferation of both glioma cell lines might be of high clinical interest.

We provide novel evidence indicating that proliferation of U-138MG and U-251MG cells, induced by des-Arg^9-BK or BK is related to PI3K/Akt pathway activation. The incubation of des-Arg^9-BK or BK led to a rapid and marked phosphorylation of PI3K/Akt as early as 3 min, which lasted for up 30 min after incubation. The isoform

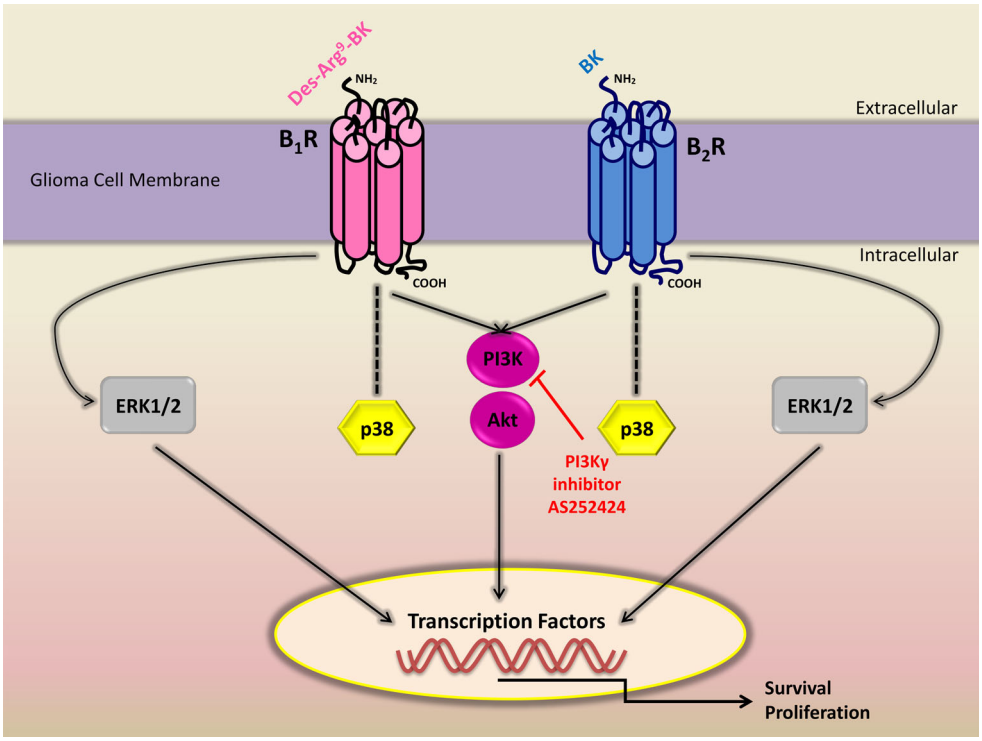


Fig. 5 Schematic representation of the signaling pathways activated by B_1R (des-Arg^9-BK) and B_2R (BK) kinin agonists in glioma cells

PI3Kγ is activated by ligands of G-protein-coupled receptors [29] and it integrates multiple inputs during tumorigenesis. Notably, the selective inhibitor of PI3Kγ AS252424 clearly reduced PI3K/Akt pathway activation induced by kinin agonists, confirming the relevance of this pathway following B_1R and B_2R activation. Phosphatase and tensin homolog (PTEN) is the major control mechanism in the PI3K pathway and PTEN mutations are commonly seen in de novo formation of GBM [30]. A recent report suggests that BK enhances migration of C6 rat glioma cells and U-251MG glioma cells in vitro through PI3K/Akt signaling cascade via B_1R activation [6]. Accordingly, Sgnaolin et al. [20] provided evidence that proliferation of bladder cancer cells induced by des-Arg^9-BK and BK was reduced by treatment with AS252424 [20].

The activation of ERK1/2 could lead to the phosphorylation of transcriptional factors, leading to cell proliferation and progression of GBM [31, 32]. Noteworthy, it was demonstrated that B_1R activation induces proliferation of estrogen-sensitive breast cancer cells by activation of ERK1/2 signaling [18]. Moreover, incubation of BK caused proliferative effects in epithelial breast cells, via B_2R activation, resulting in sustained stimulation of ERK1/2 [33]. Furthermore, T24 cell proliferation induced by kinin agonists was associated to a marked raise in ERK1/2 phosphorylation, confirming the significance of this intracellular signaling to cancer cell proliferation [20]. In agreement with previous studies, we show that incubation of des-Arg^9-BK and BK, at 10 nM, promoted a sustained phosphorylation of ERK1/2 pathway.

In our in vitro cell model, kinin agonists did not induce any significant change of MAPK p38 phosphorylation in either tested cell lines. A recent study using kinin agonists also failed to stimulate the p38 pathway [20]. Moreover, Iyoda et al. [34] demonstrated that reduction of p38MAPK cascade was related to apoptosis resistance and unrestricted cell growth of human hepatocellular carcinoma [34], whereas the sustained ERK activation and p38MAPK inhibition conferred drug resistance in AT3 prostate cancer cells [35].

A number of clinical trials have been performed with peptide and non-peptide B_1R and B_2R ligands for the treatment of different tumor types. A review published by Costa et al. [36–38] emphasizes the favorable profile of kinin antagonists in several pre-clinical models of cancer, particularly lung, prostate and breast tumors. Nonetheless, a phase II trial using the agonist B_2R RMP-7 and carboplatin have failed in childhood gliomas [39, 40]. Regarding the antagonists used in our study, HOE-140 is currently approved for angioedema treatment, what can be of interest for future application of its in oncology [41, 42].

The present findings extend previous literature data, showing that both B_1R and B_2R can be involved in the human glioma proliferation. The enhancement of U-138MG and U-251MG cell proliferation induced by des-Arg^9-BK and BK is likely mediated via PI3K/Akt and ERK1/2 signaling (Fig. 5). We might suggest that

pharmacological inhibition with selective kinin antagonists could represent an alternative for the treatment of gliomas in the future. Nevertheless, the recent notion indicating the possible clinical applicability of B_1R agonists as adjuvants on modulation of brain vascular permeability in glioma treatment cannot be disregarded [2]. Studies are in progress in our laboratory to elucidate the in vivo mechanisms by which kinin receptors regulate glioma progression, what can help to further define the role of kinins in this cancer type.

Acknowledgments We are grateful to Dr Ana Maria Battastini, from Federal University of Rio Grande do Sul (Brazil), for donating both tested cell lines.

Conflict of interest The authors declare that they have no conflict of interest.

Financial support This study was Supported by the FINEP research grant "Implantação, Modernização e Qualificação de Estrutura de Pesquisa da PUCRS" (PUCRSINFRA) # 01.11.0014-00, CNPq, CAPES and FAPERGS. NFN is a PhD student in Cellular and Molecular Biology receiving a grant from CAPES/Edital 63/Toxinologia and PROBOLSAS/PUCRS; TCE is an undergraduate student in Pharmacy supported by PIBITI/CNPq and BPA/PUCRS; RFZ is a post-doc fellow supported by PNPD/CAPES; TBP is a PhD student receiving grants from CAPES/PROEX.

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Capítulo III

"Impact of genetic deletion and pharmacological blockade of kinin receptors on tumor proliferation in a mouse glioma model "

Manuscrito a ser submetido ao periódico *Glia*.

IMPACT OF GENETIC DELETION AND PHARMACOLOGICAL BLOCKADE OF KININ RECEPTORS ON TUMOR PROLIFERATION IN A MOUSE GLIOMA MODEL

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Abbreviated title: Kinin receptors in a mouse glioma model.

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Abstract

This study investigated the role of kinins and their receptors (B_1R and B_2R) in malignant brain tumors *in vivo*. GL-261 glioma cells were injected (2 µl/2 min) into the right striatum of adult C57/BL6, KOB_1R, KOB_2R or KOB_1B_2R mice. In some cases, the animals received the selective B_1R SSR240612 and/or B_2R HOE-140 antagonists by intracerebroventricular (i.c.v.) route at 5, 10 and 15 days. The tumor size quantification, mitotic index, western blot analysis, quantitative autoradiography, immunofluorescence and confocal microscopy were carried out in brain tumor samples, 20 days after tumor induction. Our results revealed an uncontrolled tumor growing in KOB_1R or SSR240612-treated mice, which was blunted by B_2R blockade with HOE-140, suggesting a crosstalk between B_1R and B_2R in tumor growing. In addition, the combined treatment with B_1R and B_2R antagonists, SSR240612 plus HOE-140, normalized the upregulation of tumor B_1R and decreased the tumor size and the mitotic index. A similar reduction was seen in double KOB_1B_2R. B_1R was detected on astrocytes in the tumor indicating a close relationship between this receptor and astroglial cells in this glioma model. Taken together, our results show that the combined suppression of B_1R and B_2R could markedly prevent tumor proliferation, which might represent an attractive alternative for the treatment of malignant gliomas in the future.

Key Words: Glioblastoma, kinins, B_1R and B_2R.

Introduction

Primary intracranial tumors are considered a challenge in oncology due to the highly aggressive nature, and the elevated mortality rates. The World Health Organization (WHO) classifies four grades (grade I-IV) of astrocytic tumors on the basis of histological characteristics. High-grade astrocytomas are considered malignant and include anaplastic astrocytoma (WHO grade III) and glioblastoma (WHO grade IV) (Louis et al. 2007; Wen and Kesari 2008). As other cancer types, malignant gliomas display self-initiated proliferation, diminished apoptosis, evasion of external growth control and immunosurveillance, tissue invasion and ability to form and sustain new blood vessels (Wen and Kesari 2008). Glioblastoma is the most common and agressive form of malignant glioma and is characterized by the presence of areas of microvascular proliferation and/or necrosis, elevated cellularity and mitotic activity. Although malignant gliomas are highly invasive, they rarely metastasize outside of the central nervous system (CNS) (Omuro and DeAngelis 2013).

Standard-of-care therapy includes surgical resection followed by radiotherapy and chemotherapy. Nevertheless, the current gold standard treatment has not been effective to prevent the tumor evolution, as indicated by the poor survival rates. The location of the tumor and its infiltrative nature avoid total surgical resection, whereas radiotherapy dosage is limited due to the inevitable damage to the normal brain parenchyma (Omuro and DeAngelis 2013). Chemotherapeutic agents currently available in clinic hardly cross the blood–brain barrier (BBB) efficiently, and glioma cells commonly develop resistance against these agents (Agarwal et al. 2011).

Bradykinin (BK) and related kinins are a family of vasoactive peptides, formed by the cleavage of kininogen precursors, via kallikrein enzymes. The biological effects of kinins are mediated by the activation of two G protein-coupled receptors, classified as B_1R and B_2R. BK

displays a high affinity for B_2R, which are constitutive throughout most tissues. Alternatively B_1R preferentially binds to the active metabolite des-Arg^9-BK; this receptor is absent or weakly expressed under basal conditions, but it can be upregulated by pro-inflammatory mediators and the oxidative stress (Couture et al. 2014; Leeb-Lundberg et al. 2005; Regoli et al. 2012).

The expression of both B_1R and B_2R has been described in different regions of the mammalian brain and spinal cord (Couture and Lindsey 2000). Some previous reports indicate that pharmacological activation of kinin receptors might facilitate the transvascular drug delivery into brain tumors (Borlongan and Emerich 2003; Cote et al. 2012; Cote et al. 2010; Liu et al. 2010). In addition, recent data using human tumor samples demonstrated that gliomas express B_1R and B_2R, and this expression positively correlates with WHO tumor grade (Zhao et al. 2005). *In vitro* studies indicate that B_2R activation by BK leads to chemotactic invasion of gliomas towards blood vessels (Montana and Sontheimer 2011; Watkins and Sontheimer 2012), and BK is able to induce astrocytic and microglial cell migration (Hsieh et al. 2008; Ifuku et al. 2007). More recently, our research group showed that stimulation of B_1R and B_2R with des-Arg^9-BK and BK enhanced proliferation of human glioma cell lineages, whereas their inhibition with selective kinin receptor antagonists led to a marked cell death (Nicoletti et al. 2014).

Based on the above-mentioned findings, the present study was aimed to investigate to what extent the genetic ablation or the pharmacological antagonism of B_1R and B_2R can modulate the glioma development, by using an *in vivo* glioma model in mice. Our results shed new light on the relevance of kinin receptors in the progression of brain tumors.

Materials and methods

Cell culture

Mouse GL-261 glioma cell line was kindly donated by Dr. Ilker Eyüpoglu, University of Zurich, or obtained from Dr. Melinda Hollingshead at the National Cancer Institute, Frederick National Laboratory for Cancer Research (Frederick, Maryland, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) at a temperature of 37 °C, a minimum relative humidity of 95%, and an atmosphere of 5 % CO_2 in air.

Animals

Male and female C57/BL6 wild-type, kinin B_1 and B_2 receptor knockout mice (KOB_1R and KOB_2R) and B_1 and B_2 receptor double knockout mice (KOB_1B_2R) were used throughout this study (8 weeks old, 25–30 g). The animals were housed under conditions of optimum light, temperature and humidity (12 h light-dark cycle, 22±1°C, under 60 to 70 % humidity), with food and water provided *ad libitum*. The knockout mice used in this study were C57/BL6 inbred and were supplied by the Department of Biophysics, Universidade Federal de São Paulo (UNIFESP-EPM, São Paulo, Brazil). Control C57/BL6 mice used for autoradiography, immunohistochemistry and pharmacological studies were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). All the experimental procedures were in accord to the Principles of Laboratory Animal Care from NIH, and were approved by the Animal Ethical Committee of the Pontifícia Universidade Católica do Rio Grande do Sul, Brazil (protocol number: 11/00258) and Université de Montréal, Canada (protocol number 13-040).

Mouse model of glioma

The *in vivo* mouse model of GL-261 glioma cells implantation was the same described before with minor adaptations (Szatmari et al. 2006). Briefly, the GL-261 glioma cells were cultured to approximately 70% confluence and resuspended at 2 x 10^5 cells/2 µl DMEM. The cells were injected using a 10-µl Hamilton microsyringe coupled to an infusion pump (1 µl/min x 2 min) at a depth of 3.0 mm into the right striatum (coordinates with regard to bregma: 2.0 mm lateral) of C57/BL6 wild-type, KOB_1R ($B_1R^{-/-}$), KOB_2R ($B_2R^{-/-}$) and KOB_1B_2R ($B_1RB_2R^{-/-}$) mice, previously anesthetized by an intraperitoneal administration of ketamine and xylazine (75 and 7.5 mg/kg, respectively). Sham-operated animals received the same volume of cell culture medium (DMEM; 2 µl), without glioma cells.

Experimental groups and treatments

The animals were divided into twelve experimental groups, according to the procedures: (i) C57/BL6 sham-operated; (ii) C57/BL6 control-tumor; (iii) KOB_1R-tumor; (iv) KOB_2R-tumor; (v) KOB_1R-tumor + B_2R antagonist; (vi) KOB_2R-tumor + B_1R antagonist; (vii) KOB_1B_2R-tumor; (viii) C57/BL6-tumor + B_1R agonist; (ix) C57/BL6-tumor + B_2R agonist; (x) C57/BL6-tumor + B_1R antagonist; (xi) C57/BL6-tumor + B_2R antagonist; (xii) C57/BL6-tumor + B_1R antagonist + B_2R antagonist. After glioma implantation, the animals received the compounds by intracerebroventricular (i.c.v.) injection at three periods on day 5, 10 and 15, in the following doses: (a) B_1R agonist: des-Arg^9-BK (1 nmol/site); (b) B_2R agonist: BK (1 nmol/site); (c) B_1R antagonist: SSR240612 (25 nmol/site); (d) B_2R antagonist: HOE-140 (50 pmol/site). The doses of kinin agonists (Ongali et al. 2004) and antagonists (Costa et al. 2011; Quintao et al. 2008) were chosen in accordance with previous publications. After 20 days of glioma implantation, the mice were euthanized by isoflurane inhalation and decapitated (Figure 1). The entire brain was

removed for further analysis, as described in the next sections. Mice that displayed severe neurological symptoms (seizures, paresis, and awareness disturbance) before the 20^{th} day were killed by isoflurane inhalation and decapitated earlier. In this case, the entire brain was also collected.

Determination of tumor size

For the quantification of tumor volume, at least three hematoxylin-eosin (HE) coronal sections (20-µm thick) from each animal were analyzed. Images were captured (x8 magnification) using a digital camera (Canon PowerShot C9 – PC1250 7.4V, Canon Inc, Japan) connected to a stereotaxic microscope (Stemi DV4, Carl Zeiss MicroImaging, Germany), and analyzed by Axion Vision version 4.7.1 (Imaging Systems, Carl Zeiss Vision program group, Germany). The total volume (mm^3) of the tumor was computed by the multiplication of the slice sections and by summing the segmented areas.

Mitotic Index

The pathological analysis of the slides sections stained with HE was carried out by a blinded experimented pathologist. Glioma cell proliferation was assessed by counting the number of mitotic glioma cells in ten fields per tumor, selected randomly (×200 magnification) in a microscope (Zeiss Axioskop 40, Zeiss Oberkochen, Germany) equipped with a camera (Qimaging Retiga 2000R, Surrey, Canada).

Western blot analysis

The complete protocol has been described elsewhere (Lin et al. 2010). After the above-mentioned treatments, the brain slices were homogenized in phosphate buffer saline (PBS) containing a

cocktail of protease inhibitors (Sigma–Aldrich, Canada). The blots were cut in pieces according to the molecular weight of the protein and then incubated with the specific antibodies for B_1R and B_2R and dynein (internal control) in PBS-Tween 20 solution at 4°C overnight. The secondary antibody for B_1R and B_2R identification was an HRP-linked goat anti-rabbit (Table 1). B_1R and B_2R proteins were revealed by using an enhanced chemiluminescence detection kit (Super-Signal, Thermo Scientific, Rockford, USA). The semi-quantitative analysis of the detected protein was performed by densitometry using an MCID^{TM} image analysis system (Imaging Research, St. Catharines, ON, Canada).

Tissue preparation for autoradiography, immunofluorescence and confocal microscopy

After euthanasia, the brains were immediately frozen in 2-methyl butane cooled at – 45ºC to – 55ºC in liquid nitrogen, and then stored at – 80ºC until use. Matched whole brains were mounted in a gelatin block and serially cut into 20-µm thick sections on a cryostat with temperature varying between – 10 to – 12ºC. The sections were thaw-mounted on 0.2% gelatin/0.033% chromium potassium sulfate-coated slides and stored at – 80ºC. The slides were used for the autoradiography and microscopy analysis.

Quantitative Autoradiography

The autoradiography procedure and concentrations of radioligands were conformed to previous studies (Campos et al. 2005; Cloutier et al. 2002; Ongali et al. 2003; Talbot et al. 2010; Talbot et al. 2012). The sections were thawed at room temperature and pre-incubated for 30 s in 25 mM PIPES buffer (pH 7.4; 4°C). Subsequently, the slides were incubated for 90 min at room temperature in 25 mM PIPES buffer containing: 1 mM 1,10-phenanthroline, 1 mM DTT, 0.014% bacitracin, 0.1 mM captopril, 0.2% BSA (protease-free), and 7.5 mM magnesium chloride in the

presence of 150 pM [^{125}I]HPP-desArg^{10}-Hoe 140 (for B_1R) or 200 pM [^{125}I]HPP-HOE-140 (for B_2R). The nonspecific binding was determined in the presence of 1 µM of antagonists (R-715 for B_1R and HOE-140 for B_2R). At the end of the incubation period, the slides were transferred sequentially through four rinses of 4 min each in 25 mM PIPES (pH 7.4; 4°C) and dipped for 15 s in distilled water (4°C) to remove the excess salts and air-dried. Kodak Scientific Imaging Films BIOMAX MS were juxtaposed onto the slides in the presence of ^{14}C-labeled microscales (calibrated in ^{125}I equivalent) and exposed at room temperature for 7 days. Densitometric readings were measured with an image analysis system (MCID, Imaging Research, ON, Canada) and expressed in nanoCurie per milligram of tissue (nCi/ mg tissue). The specific binding was determined by subtracting superimposed digitalized images of nonspecific labeling from total binding. At least three different regions of the tumor area were considered for quantification purposes for each animal.

Immunofluorescence for B_1R and GFAP and confocal microscopy

For immunofluorescence staining, the brain tumor sections were incubated with rabbit anti-B_1R (1:100) or chicken anti-GFAP (1:1000), followed by conjugated secondary antibodies anti-rabbit AlexaFluor 568 (1:200) and anti-chicken FITC (1:200), respectively, for the detection of activated B_1R and astrocytes. For the confocal microscopy analysis, the sections were incubated with rabbit anti-B_1R (1:100) or chicken anti-GFAP (1:1000), followed by conjugated secondary antibodies anti-rabbit FITC (1:200) and anti-chicken AlexaFluor 647 (1:200), respectively, for the detection of activated B_1R and astrocytes. The sections were observed under a microscope (Leica Confocal microscope, Richmond Hill, ON, Canada) using bright field, or a FITC filter and epifluorescence. For double immunofluorescence labeling, the sections were simultaneously incubated with rabbit anti-B_1R antibody and chicken anti-GFAP. The specificity of B_1R and B_2R antibodies was confirmed

in tissues isolated from B_1R and B_2R knockout mice. B_1R pre-immune serum was also used as a supplementary specificity control (Lacoste et al., 2013; Lin et al., 2010; Talbot et al., 2012).

Immunohistochemistry for B_1R

For these experiments, the sections were pretreated with 3% H_2O_2 (20 min) and incubated overnight at room temperature with either rabbit anti-B_1R antibody diluted in a blocking buffer (1:100), followed by biotinylated anti-rabbit IgG (1:200) and the ABC kit; labeling was revealed with 0.05% DAB.

Drugs and Reagents

The B_1R antagonist, SSR240612 [(2R)-2-[((3R)-3-(1,3-benzodioxol-5-yl)-3-[[(6-methoxy-2-naphthyl)sulfonyl]amino]propanoyl)amino]-3-(4-[[2R,6S)-2,6-dimethylpiperidinyl]methyl]phenyl)-N-isopropyl-N-methylpropanamide,fumarate], was kindly provided by Sanofi-Aventis (Montpellier, France) (Gougat et al. 2004). HPP-des-Arg^{10}-H0E-140 and HPP-H0E-140 were synthesized at the Research Institute of Biotechnology, National Research Council of Canada (Montreal, Qc, Canada). R-715 (AcLys[D-βNal^7, Ile^8]des-Arg^9-BK) was kindly provided by Dr Fernand Gobeil (Pharmacology, University of Sherbrooke, Sherbrooke, Qc, Canada) (Gobeil et al. 1996). Des-Arg^9-BK, BK and HOE-140 (D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]BK were of commercial source (Bachem, USA). All the reagents used for *in vivo* treatments were dissolved in sterile PBS.

Statistical analysis

The results are presented as the mean ± s.e.m. of 4 to 5 animals per group, depending on the experimental protocol. Data was analyzed by one-way analysis of variance followed by Dunnett's

or Bonferroni's post-hoc tests, depending on the experimental set. Comparison of the survival curves was performed using the Log-rank test. Statistical comparisons were performed with Graph-Pad Software (San Diego, CA, USA). $P< 0.05$ was indicative of statistical significance.

Results

The i.c.v. injection of GL-261 glioma cells into the right striatum of C57/BL6 wild-type mice resulted in the development of a tumor mass with histological features similar to that seen in humans, with mitotic activity (Figure 2A, a). Interestingly, the tumor invasiveness was markedly increased following the implantation of the same number of GL-261 glioma cells into the brain of KOB_1R mice (Figure 2A, panel b). In KOB_2R mice, the glioma histological characteristics were similar to that observed in wild-type animals (Figure 2A, c). The histological evaluation also revealed a reduction of the tumor malignant characteristics in double KOB_1B_2R mice, or in KOB_1R or KOB_2R, that had been treated with the selective B_2R HOE-140 or B_1R SSR240612 receptor antagonists, respectively (Figure 2A, d-f). Extending this evidence, either the tumor volume in mm^3 (Figure 2B, a), or the mitotic indexes (Figure 2B, b) were markedly increased (about 2-fold) in KOB_1R mice, whereas these parameters were significantly diminished in KOB_1B_2R, HOE-140-treated KOB_1R or SSR240612-treated KOB_2R mice, when compared to wild-type animals. Conversely, there was no significant change of the tumor volume or the mitotic index in KOB_2R mice (Figure 2B, a-b).

From separated experimental groups (Figure 2C), it was possible to observe that treatment of C57/BL6 wild-type mice with the selective B_2R receptor antagonist HOE-140 was not able to modify the glioma clinical progression. However, the clinical aspect of glioma was markedly enhanced in wild-type animals pre-treated with the B_1R receptor antagonist SSR240612, while the tumor development was clearly reduced in mice that received HOE-140 plus SSR240612. This set of data was confirmed by the measurement of the tumor volume (Figure 2B, c). In addition, the percentage of survival was significantly reduced by the administration of SSR240612 in C57/BL6 wild-type mice submitted to the glioma model, although this parameter was not modified by the administration of HOE-140 alone, or HOE-140 plus SSR240612 (Figure

2D). It is worth noting that i.c.v. injection of the selective B_1R des-Arg^9-BK or B_2R BK receptor agonists did not significantly modify the tumor size (results not shown).

Next, the protein expression of B_1R and B_2R was analyzed in the brain tumor samples of animals treated with kinin antagonists, by western blotting. The protein expression of B_1R was slightly increased in the control group (with tumor induction, but no treatment), when compared to the sham-group (no tumor induction), although this effect was not significant. The B_1R protein levels were significantly decreased in the brain tumor samples of SSR240612- or HOE-140-treated groups, when compared to control tumor-bearing mice. Finally, the B_1R protein expression in the brain tumors of animals that received SSR240612 plus HOE-140 was normalized to that observed in the sham-group (Figure 3A). Noteworthy, marked immunolabelling for B_1R was detected in both the core (panel a) and the peritumoral region (panel b) of mouse glioma (Figure 3C). Furthermore, B_1R specific binding sites were observed in the center of the tumor, by using *in vitro* autoradiography with the radioligand [$^{(125)}$I]HPP-des-Arg^{10}-Hoe 140. Pretreatment with HOE-140 or HOE-140 plus SSR240612 significantly decreased B_1R densities in the tumor sections, yet the reduction seen with SSR240612 alone did reach statistical significance (Figure 3D). In contrast to B_1R, B_2R protein expression and binding sites, as assessed by western blot (Figure 3B) and by autoradiography (data not shown) were not significantly altered in any of the evaluated experimental groups.

The induction of B_1R in the tumor tissues was also evidenced by immunofluorescence analysis, which demonstrated an increase of B_1R immunolabelling in the tumor core, when compared to sham tissues. This was associated with marked astrocytic activation, as revealed by positive immunolabelling for GFAP (Figure 4A). The merged images revealed that B_1R is co-localized with astrocytes in the tumor (Figure 4A). The co-localization of B_1R and activated astrocytes in the center of the tumor was confirmed by confocal microscopy (Figure 4B).

Discussion

The notion that kinin receptors play an important role in tumor growth by regulating cell motility, invasion and angiogenesis has gained great attention over the last few years (Figueroa et al. 2012). Gliomas are often heterogeneous tumors, regarding either the phenotype or the genotype (Watkins and Sontheimer 2012). In this type of solid tumor, specific subpopulations of tumor cells have a great potential of cancer repopulation (Huang et al. 2010), which hampers the complete removal of tumor cells through surgical resection. Recent data demonstrated that glioma cells express B_1R (Cote et al. 2012) and B_2R (Montana and Sontheimer 2011), and this expression has a positive correlation with *in vitro* cancer cell proliferation, or tumor grade malignancy in patients (Zhao et al. 2005). Of note, it has been recently proposed that B_1R-agonist can be used to open the blood-brain-barrier to allow access of chemotherapeutic agents to the glioma (Cote et al. 2012). Furthermore, some authors have used B_2R agonists, in combination with B_1R agonists, as an adjunctive therapy to favor drug delivery to brain tumors (Cote et al. 2010; Cote et al. 2013; Sarin et al. 2009). Since the i.c.v. injection of the selective B_1R des-Arg^9-BK or B_2R BK receptor agonists did not significantly modify the tumor size in our study, the use of kinin receptor agonists to give access of chemotherapeutic agents to the glioma is not expected to stimulate the growing of the tumor and then this approach looks quite safe.

In the present study, we report that genetic or pharmacological modulation of B_1R and B_2R triggered marked modulatory effects on tumor growth and progression, as assessed in a mouse *in vivo* glioma model. The reduction of tumor size and macroscopic aggressiveness parameters, such as necrosis and hemorrhage, together with the increased survival rate in animals pretreated with B_1R plus B_2R antagonists (namely, SSR240612 and HOE-140) suggest that inhibition of kinin receptors might well represent a potential tool to prevent glioma progression. It is important to emphasize that B_1R and B_2R modulation was able to modify the malignancy

grade of gliomas in our experimental model, leading to the proposal that modification of histopathological parameters by simultaneous blockade of B_1R and B_2R could positively affect the tumor prognosis in clinical setting (Wen and Kesari 2008). Assuming that treatment of malignant gliomas remained unchanged during the last years (Mathieu and Fortin 2006; Nieder et al. 2009), a possible alteration of malignancy grade after surgical glioblastoma resection (grade IV) could be a feasible approach to enhance the patient survival greater than 12-15 months. In agreement with pharmacological inhibition of both kinin receptors, the tumor size and mitotic index were markedly blunted in double B_1RB_2R knockout mice, in KOB_1R treated with the selective B_2R antagonist HOE-140, and in KOB_2R treated with the selective B_1R inhibitor SSR240612. Collectively, these findings reinforce the primary role of kinin receptors in the proliferation of glioma cells *in vivo*. Our data are in agreement with previous evidence indicating favorable effects for kinin receptor antagonists in CNS injuries, such as traumatic brain injury or ischemic stroke (Austinat et al. 2009; Thornton et al. 2010).

B_2R exhibits constitutive expression and it is thought to be responsible for most of the kinin activities under physiological conditions, while B_1R is overexpressed during tissue injury (Marceau and Regoli 2004). Accordingly, compelling evidence shows that B_1R is rarely expressed in normal tissues, but it can be overexpressed under stressful situations, such as cancer (Calixto et al. 2000; da Costa et al. 2014; Marceau and Regoli 2004), which might explain the higher protein levels of B_1R, when compared to B_2R, in the mouse brain tumors analyzed. Extending this notion, in the present study, the B_1R immunostaining was almost undetectable in brain samples obtained from sham-injected animals, whereas it was markedly enhanced in mouse gliomas induced by implantation of GL-261 cells, either in the tumor core or its periphery. Of interest, B_1R was found co-localized with GFAP in mouse tumors, providing clear evidence about the presence of B_1R on glial cells. Astrocytes are the most abundant non-neuronal cells in

the brain, and they have multiple roles in the CNS, being rapidly activated in response to insults, such as infection and neurodegeneration. In brain tumors, the activated astrocytes are likely related to cell proliferation and metastasis (Fidler et al. 2010). Most importantly, we showed that co-localization was almost absent in brain samples of sham-operated control mice, confirming a close relationship between B_1R and astroglial cells in this tumor model. In agreement with our results, it was demonstrated before that B_1R are upregulated in reactive astrocytes around Aβ plaques, in a genetic mouse model of Alzheimer's disease (Lacoste et al. 2013).

Data presented herein revealed the occurrence of uncontrolled tumor growing in KOB_1R mice. This might be secondary to the compensatory B_2R up-regulation, similarly to that described previously, in other experimental models employing the knockout mice for kinin receptors (Duka et al. 2008; Rodi et al. 2013; Seguin et al. 2008). Similarly, the selective pharmacological inhibition of B_1R by SSR240612 in wild-type mice also resulted in severe tumor growth, yet in this case, B_2R expression remained unaltered, according to western blot analysis. In addition, B_1R protein expression was significantly decreased in tumor samples obtained from mice pretreated with the B_1R antagonist SSR240612, and the same pattern was seen in the mouse groups that received the B_2R antagonist HOE-140. Although one cannot exclude that HOE-140 is partly converted *in vivo* into des-Arg^9-HOE-140, a molecule which behaves as a B_1R antagonist (Gobeil et al. 1996), this possibility is unlikely as HOE-140 had different profile than SSR240612 on tumor volume and proliferation. Also, the combined treatment with SSR240612 and HOE-140, in mice injected with GL-261 cells, normalized B_1R expression to the levels observed in the sham-injected group. Nevertheless, the genetic deletion of B_2R in the presence of B_1R did not alter the tumor development as seen with the blockade of B_2R with HOE-140 alone. Thus, it is reasonable to propose that B_2R displays a marked stimulatory effect on glioma progression in the absence of B_1R as compensatory mechanism, and that both kinin receptors must be inhibited in

order to stop tumor growing. Supporting our data, Rodi and collaborators (Rodi et al. 2013) reported that KOB_1R are more susceptible to seizures, in two mouse models of epilepsy; most importantly, the exacerbation of the epileptic status in KOB_1R was widely prevented by the administration of the selective B_2R antagonists LF16-0687 or HOE-140 (Rodi et al. 2013). In contrast to our findings, it was recently shown that B_1R stimulation with the B_1R agonist des-Arg^9-BK provides protective effects in a mouse model of melanoma *in vivo* (Dillenburg-Pilla et al. 2013). At this moment, we are not able to explain this divergent data, but this might be related to the different tumor types and microenvironment.

Using genetic and pharmacological approaches, our data provide clear evidence on the beneficial effects of the simultaneous inhibition of both B_1R and B_2R on glioma progression, further contributing to understand the role of kinins and their receptors in cancer. Hence, it is tempting to propose that kinin receptors are a promising therapeutic target for the treatment of glioblastomas.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

The authors thank Mr Youssef Haddad for him excellent technical assistance in western blot analysis and Mrs Julie Verner for her technical support in cell culture.

Financial support

This study was supported by the FINEP research grant “Implantação, Modernização e Qualificação de Estrutura de Pesquisa da PUCRS” (PUCRSINFRA) # 01.11.0014-00, CNPq, CAPES and FAPERGS and by the Canadian Institutes of Health Research to RC (MOP-79471). NFN is a PhD student in Cellular and Molecular Biology receiving a grant from CAPES (AUX-PE/Toxinologia), CAPES/PDSE and PROBOLSAS/PUCRS.

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Table

Table 1. Western Blot primary and secondary antibodies

Primary Antibody	Molecular weight	Dilution	Source
Kinin B_1R, rabbit polyclonal	37 kDa	1:1000	Dr. Réjean Couture's Laboratory*
Kinin B_2R, rabbit polyclonal	42 kDa	1:1000	Dr. Réjean Couture's Laboratory*
Dynein, mouse monoclonal	74 kDa	1:4000	SantaCruz Biotechnology (Sc-13524)
Secondary Antibody	**Protein detected**	**Dilution**	**Source**
HRP-linked goat anti-rabbit	B_1R	1:25000	SantaCruz Biotechnology (Sc-2077)
HRP-linked goat anti-rabbit	B_2R	1:25000	SantaCruz Biotechnology (Sc-2077)
HRP-linked goat anti-mouse	Dynein	1:5000	SantaCruz Biotechnology (Sc-2005)

* Lin, J.C. et al., 2010

Figures

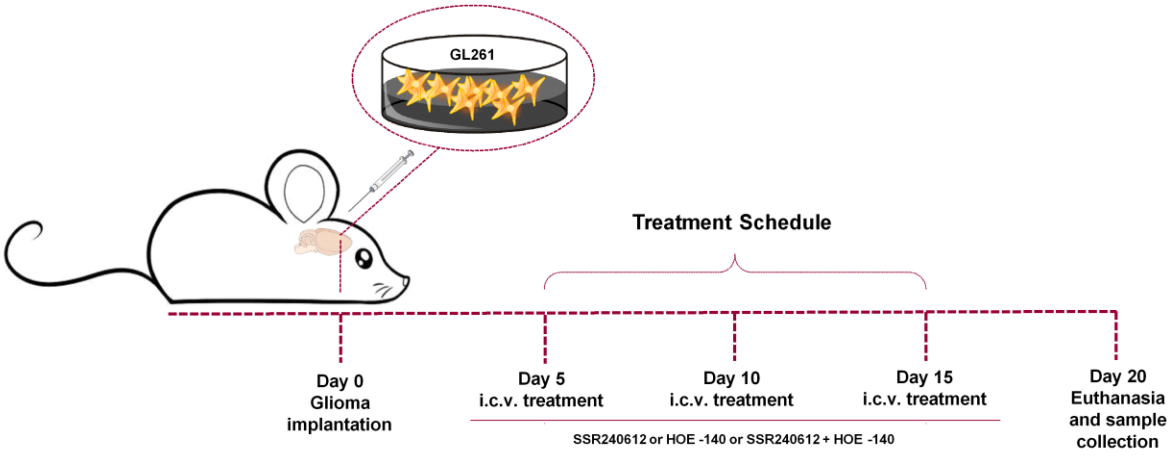


Figure 1. Schematic *in vivo* GL-261 glioma model and treatment schedule. GL-261 glioma cells were injected at 2 x 10^5 cells/2 µl density into the right striatum of adult mice C57/BL6 wild-type, KOB_1R ($B_1R^{-/-}$), KOB_2R ($B_2R^{-/-}$) and KOB_1B_2R ($B_1RB_2R^{-/-}$). The animals received the compounds by intracerebroventricular (i.c.v.) injection at three periods on day 5, 10 and 15 in the following doses: (a) agonist B_1R: Des-Arg^9-BK (1 nmol/site); (b) agonist B_2R: BK (1 nmol/site); (c) antagonist B_1R: SSR240612 (25 nmol/site); (d) antagonist B_2R: HOE-140 (50 pmol/site). On day 20 after glioma implantation the mice were euthanized.

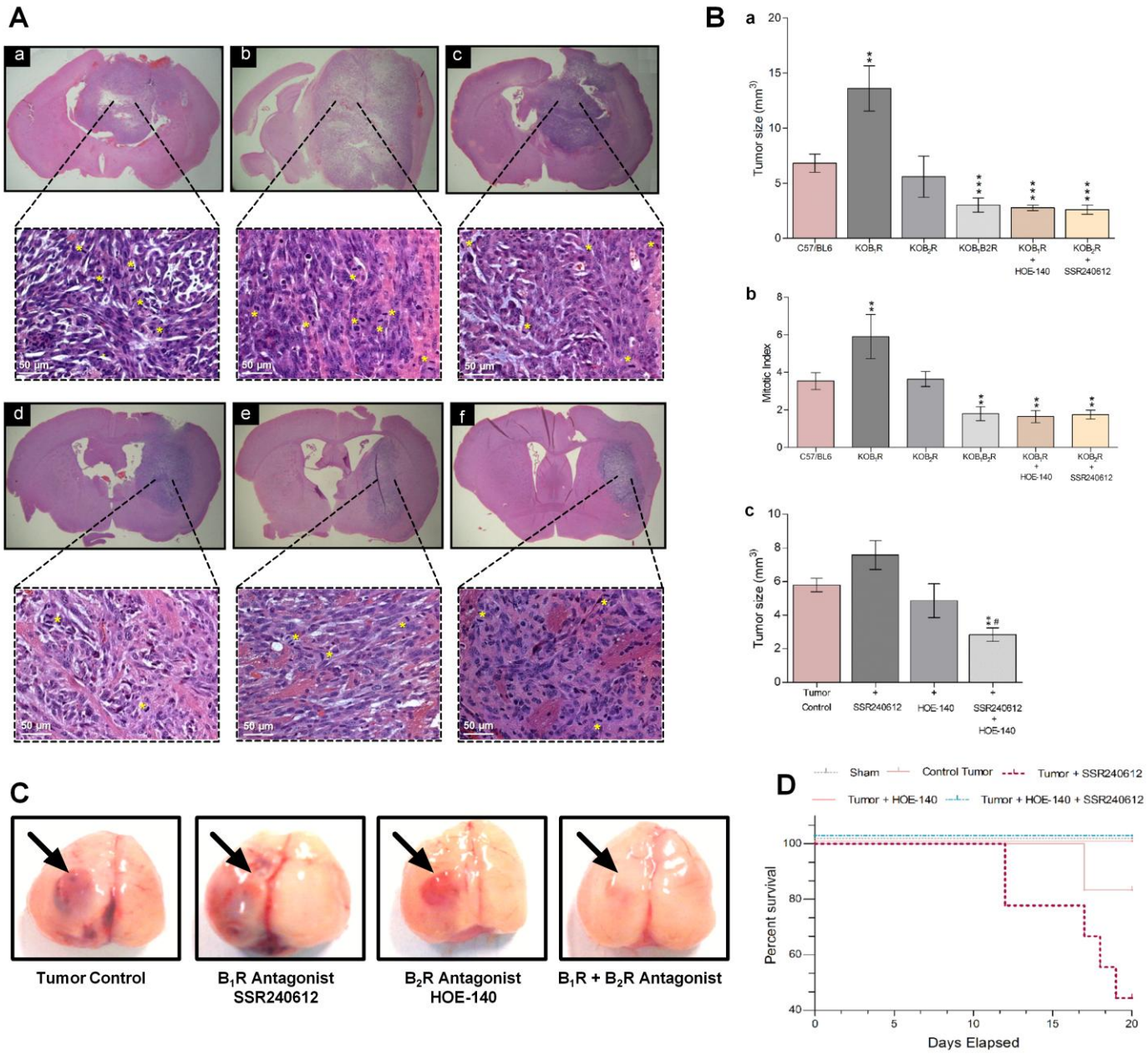
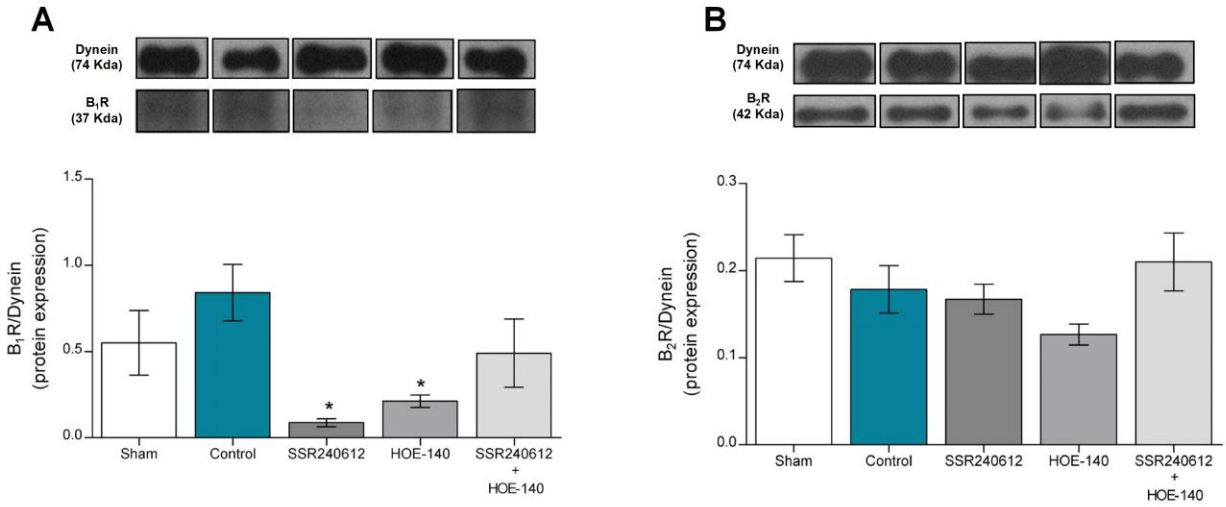
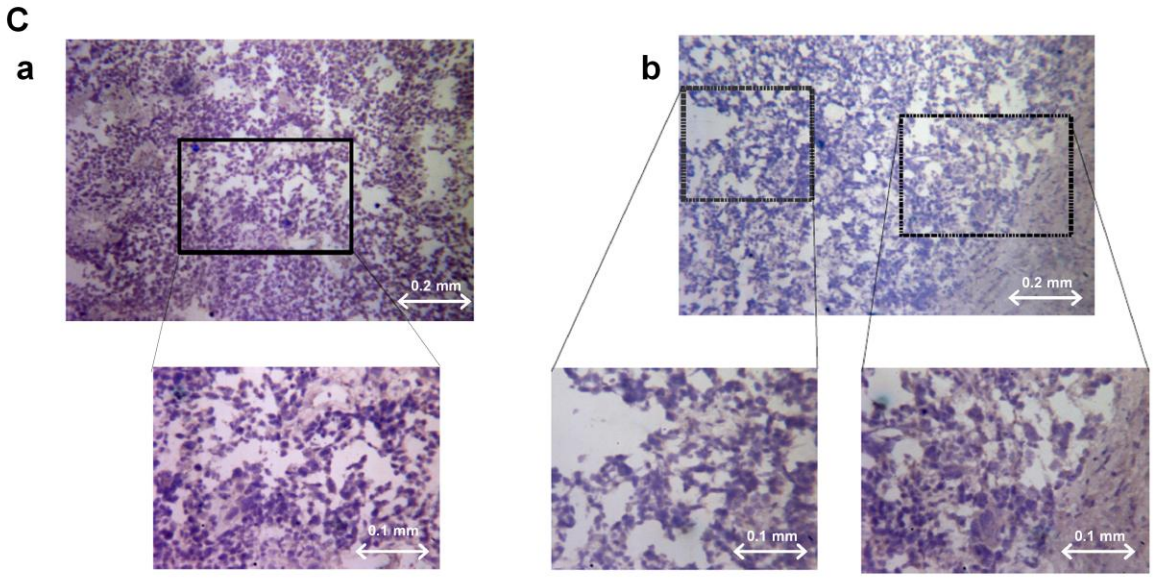

A
a
b
c
d
e
f
50 µm
B
a
Tumor size (mm3)
C57/BL6
KOB1R
KOB2R
KOB1B2R
KOB1R + HOE-140
KOB2R + SSR240612
b
Mitotic Index
c
Tumor size (mm3)
Tumor Control
+ SSR240612
+ HOE-140
+ SSR240612 + HOE-140
C
Tumor Control
B1R Antagonist SSR240612
B2R Antagonist HOE-140
B1R + B2R Antagonist
D
Sham
Control Tumor
Tumor + SSR240612
Tumor + HOE-140
Tumor + HOE-140 + SSR240612
Percent survival
Days Elapsed

Figure 2. Blockade of kinin B_1R and B_2R reduce glioblastoma growth *in vivo*. Animals were treated, and samples were analyzed as described in Materials and Methods. (**A**) Representative H&E-stained brain coronal sections of GL-261-induced tumors of five animals of the (a) C57/BL6 control-tumor; (b) KOB_1R-tumor; (c) KOB_2R-tumor; (d) KOB_1B_2R-tumor; (e) KOB_1R-tumor + antagonist B_2R HOE-140 (50 pmol/site); (f) KOB_2R-tumor + antagonist B_1R SSR240612 (25 pmol/site); and the respective mitotic index (asterisk indicate mitotic cells), magnification 20x. (**B**) (a, c) Tumor size quantification (mm^3) in mouse brain and (b) mitotic index quantification after glioma GL-261 cell implantation. Each column represents the mean ± s.e.m. of 5 animals. Scale bars = 50 µm. (a, b) ***$p<0.01$; ** $p<0.05$ for comparison versus C57/BL6 control group; (c) ** $p<0.05$ versus control; # $p<0.05$ versus SSR240612 as determined by ANOVA with Bonferroni's post-hoc test. (**C**) Representative macroscopic images of GL-261 brain tumors (arrows) in C57/BL6-treated mice. (**D**) Kaplan-Meier survival curve in the tumor-bearing C57/BL6 mice.


A
Dynein
(74 Kda)
B1R
(37 Kda)
B1R/Dynein
(protein expression)
1.5
1.0
0.5
0.0
Sham
Control
SSR240612
HOE-140
SSR240612
+
HOE-140
B
Dynein
(74 Kda)
B2R
(42 Kda)
B2R/Dynein
(protein expression)
0.3
0.2
0.1
0.0
Sham
Control
SSR240612
HOE-140
SSR240612
+
HOE-140


C
a
0.2 mm
0.1 mm
b
0.2 mm
0.1 mm
0.1 mm

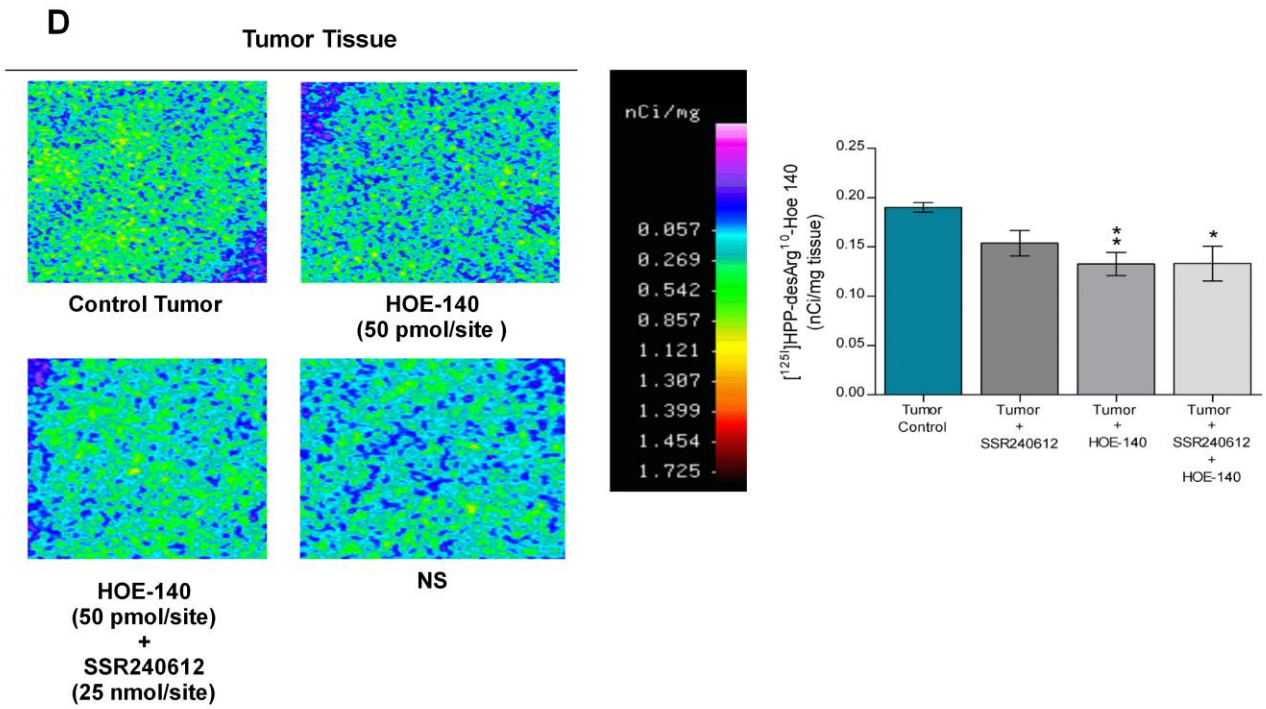

D
Tumor Tissue
Control Tumor
HOE-140
(50 pmol/site)
HOE-140
(50 pmol/site)
+
SSR240612
(25 nmol/site)
NS
nCi/mg
0.057
0.269
0.542
0.857
1.121
1.307
1.399
1.454
1.725
[125I]HPP-desArg10-Hoe 140
(nCi/mg tissue)
0.25
0.20
0.15
0.10
0.05
0.00
Tumor
Control
Tumor
+
SSR240612
Tumor
+
HOE-140
Tumor
+
SSR240612
+
HOE-140

Figure 3. **Kinin B_1R and B_2R expression and density.** Effects of i.c.v administration of kinin antagonists at (**A**) B_1R protein expression and (**B**) B_2R protein expression in the mouse brain tumor 20 days after GL-261-induced glioma. Each column represents the mean ± s.e.m. of 5 animals. *$p<0.05$ for comparison versus control, as determined by ANOVA with Bonferroni's post-hoc test. **(C)** Representative immunohistochemistry analysis for B_1R expression in the (a) core and the (b) peritumoral regions of mouse gliomas. Scale bars = 0.2 and 0.1 mm. **(D)** Representative autoradiograms and quantitative densitometric analysis of B_1R distribution in the glioma tumor of mice treated with kinin antagonists by i.c.v. injection at 5, 10 and 15 days. Each column represents the mean ± s.e.m. of 5 animals. *$p<0.05$ and **$p<0.01$ for comparison versus control, as determined by ANOVA with Dunnet's post-hoc test.

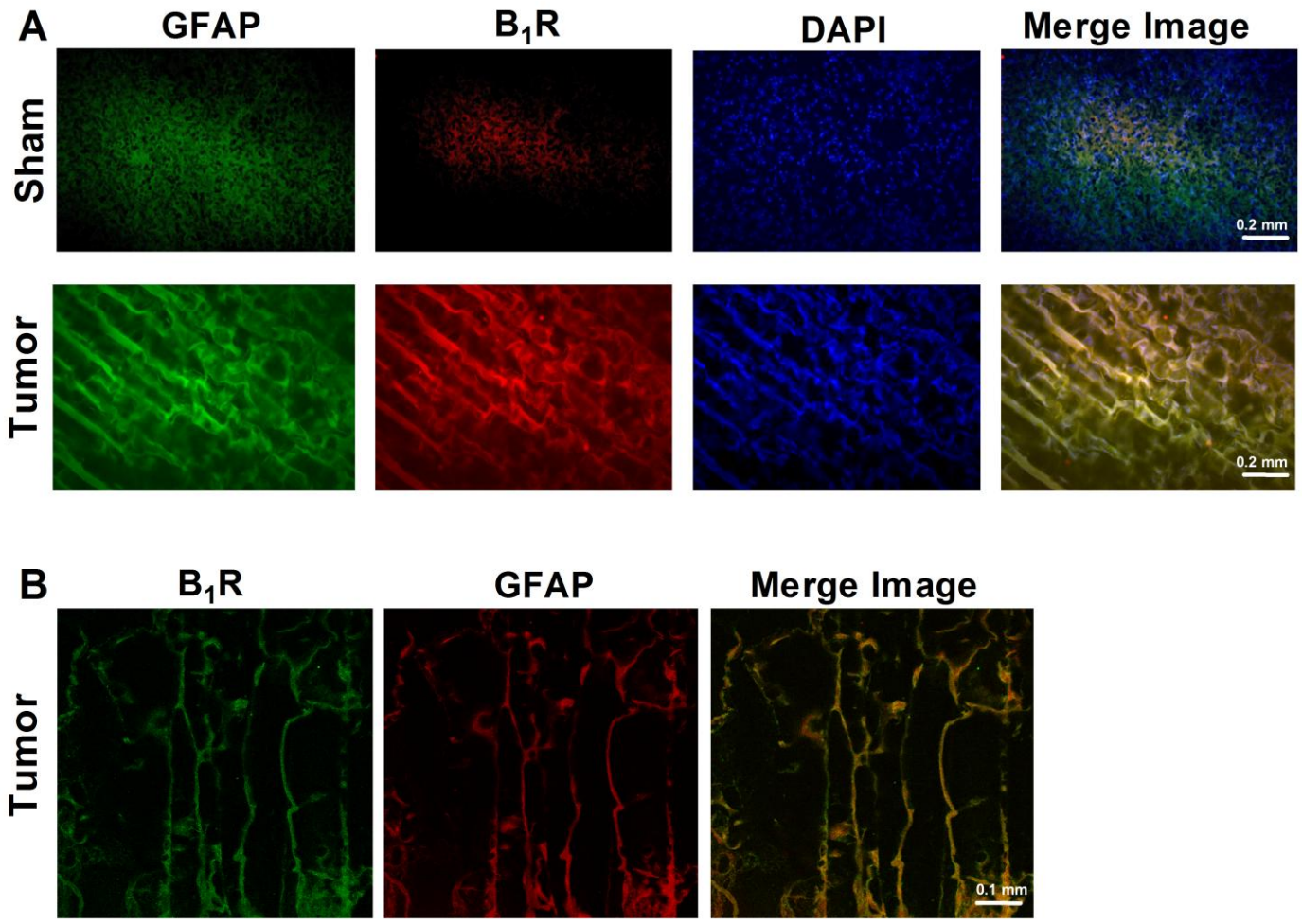


Figure 4. Immunolocalization of B_1R and GFAP. (A) Triple-fluorescence labeling with anti-GFAP, anti-B_1R and DAPI to label nuclei was performed as described in material and methods in a coronal section taken from sham brain or GL-261-induced glioma. The merge image demonstrates co-localization of B_1R and GFAP just in the tumor core. Scale bar = 0.2 mm. **(B)** Confocal microscopy pictures of coronal section of GL-261 glioma tumor labeled with anti-B_1R and anti-GFAP. The merge image shows the co-localization of B_1R and GFAP. Scale bar = 0.1 mm.

Capítulo IV

"Pre-clinical evaluation of N- and P/Q-type voltage-gated calcium channel blockers in glioma progression"

Manuscrito a ser submetido ao periódico *British Journal of Pharmacology*.

PRE-CLINICAL EVALUATION OF N- AND P/Q-TYPE VOLTAGE-GATED CALCIUM CHANNEL BLOCKERS IN GLIOMA PROGRESSION

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Running title: Calcium-channel blockers and glioblastomas.

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List of Authors Contribution

N.F.N, M.M.C. and F.B.M. designed the experiments, analyzed the data and wrote the manuscript; T.C.E. contribute in cell culture experiments; R.F.Z. assisted in cytometry experiments; M.V.G. provided all toxins; M.M.C., F.B.M. and M.V.G. made a critical review of the manuscript.

Summary

Background and purpose: Gliomas are among the most deadly and prevalent brain tumors. This study investigated the implication of P/Q- and N-type high-voltage-gated calcium channels (VGCC) in the mechanisms of gliomas progression.

Experimental approach: Human glioma cells M059J, U-138MG and U-251MG were used to evaluated the role of the selective P/Q- and N-type VGCC inhibitors PhTx3-3 and Phα1β from *P. nigriventer* (0.3 - 100 pM) or MVIIC and MVIIA from *C. magus* (0.3 - 100 pM) in cell death and proliferation. The effects of toxins were also analyzed in a mouse glioma model induced by implantation of GL261 cells.

Key results: PhTx3-3, Phα1β and MVIIA displayed a significant inhibitory effect on proliferation and viability of all tested cell lines, and evoked cell death mainly with apoptosis characteristics, assessed by AnnexinV/PI positivity. The antiproliferative effects of toxins were confirmed by flow cytometry with Ki67 stainning. In the *in vivo* model, the N-type VGCC blockade by either Phα1β (50 pmol/site; i.c.v. and i.t.) or MVIIA (10 pmol/site; i.c.v.) caused significant reductions of glioma tumor area. N-type inhibition by Phα1β and MVIIA led to a marked increase of GFAP-activated astrocytes and Iba-1-positive microglia in the peritumoral area, which might be related to the inhibitory effects of the toxins in tumor development.

Conclusions and implications: This study provides novel evidence on the role of P/Q-, and especially N-type high-VGCC in gliomas. It is feasible to suggest that N-type VGCC inhibitors could be an attractive alternative for the treatment of malignant gliomas in the future.

Key words: Malignant gliomas; *Phoneutria nigriventer*; *Conus magus*; High-voltage-gated calcium channels.

Abbreviations: Central nervous system (CNS); Glioblastoma (GBM); high-voltage-gated calcium channels (VGCC); propidium iodide (PI).

Introduction

P/Q- and N-type high-voltage-gated calcium channels (VGCC) are widely distributed in the membrane of excitable cells, such as neurons (Dolphin, 2006). In neuronal cells, VGCC are implicated in several relevant pathophysiological processes, including the release of neurotransmitters and pain transmission (Dolphin, 2006; Chen *et al.*, 2013; Prevarskaya *et al.*, 2013). The potent toxins PhTx3-3 and PhTx3-6 (re-named as Phα1β) obtained from the venom of the Brazilian spider *Phoneutria nigriventer* have been described as preferential P/Q- and N-type VGCC blockers, respectively (Gomez *et al.*, 2002). Similarly, the toxins isolated from *Conus magus*, namely MVIIC and MVIIA, have also been described as selective inhibitors of P/Q- and N-type calcium channels (Lewis *et al.*, 2012). Of note, a series of previous studies provided compelling evidence on the beneficial effects of such animal-derived toxins against neuronal ischemic injury, neurogenic inflammation, as well as pain and pruritus (Sarin *et al.*, 2009; Agostini *et al.*, 2011; Maciel *et al.*, 2014; Silva *et al.*, 2015).

Glioblastoma (GBM) is the most malignant and frequent primary brain tumor arising from glial cells. Glioma cells display remarkable migration ability, which is associated with rapid tumor growth and invasiveness. The complete surgical resection of this tumor remains a challenge, and the current chemotherapy and radiotherapy schemes are only partially effective. Unfortunately, the median survival for GBM has not been increased over the last years (Omuro *et al.*, 2013). Accumulating evidence indicates a direct relationship between intracellular calcium concentrations and glioma cell migration that might be positively correlated with tumor aggressiveness (Montana *et al.*, 2011; Watkins *et al.*, 2012). Supporting this notion, it has been suggested that disruption of

calcium signaling and/or altered expression of VGCC might contribute with pivotal mechanisms underlying cancer progression, such as cell proliferation, migration, invasion and metastasis (Chen *et al.*, 2013; Azimi *et al.*, 2014; Borowiec *et al.*, 2014). Interestingly, a recent study demonstrated that the selective inhibition of T-type low-VGCC, by the pharmacological blocker mibefradil or siRNA knockdown, markedly reduced the cell viability of three different human GBM cell lines, via modulation of apoptosis pathways (Valerie *et al.*, 2013). Hence, selective inhibitors of T-type VGCC have been pointed out as novel interesting alternatives for GBM treatment (Zhang *et al.*, 2012). Nevertheless, the effectiveness of P/Q- or N-type VGCC blockers in GBM remains to be investigated.

The present study was aimed to evaluate the potential anti-tumor effects of the animal-derived inhibitors of P/Q- and N-type VGCC, namely PhTx3-3 and Phα1β from *P. nigriventer*, or MVIIC and MVIIA from *C. magus* in pre-clinical *in vitro* and *in vivo* glioma models. Our data shed new light on the functional relevance of VGCC in glioblastoma.

Methods

Drugs

The ω-conotoxins MVIIA and MVIIC were obtained from Latoxan (Valence, France). Phα1β and PhTx3-3 were purified as described before (Cordeiro Mdo *et al.*, 1993; Gomez *et al.*, 2002). The toxins (MVIIA, MVIIC, Phα1β and Tx3-3) were prepared in phosphate-buffered saline (PBS) in siliconized plastic tubes and maintained at -18 °C. All the drugs and reagents used in the present study held purity superior to 95%.

Cell lines and cell culture

The M059J, U-138MG and U-251MG human glioblastoma cell lines were obtained from American Type Culture Collection (ATCC-Rockville, Maryland, USA), and used for *in vitro* protocols. Mouse GL261 glioma cells were kindly donated by Dr. Ilker Eyüpoglu, University of Zurich, and this cell lineage was used for *in vivo* experiments. All the cell lineages were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) at a temperature of 37 ° C, a minimum relative humidity of 95 %, and an atmosphere of 5 % CO2 in air. For *in vitro* assays, at least three independent experiments were performed in triplicate.

Cell viability assay

The number of viable cells with metabolically active mitochondria were determined based on the mitochondrial reduction of a tetrazolium bromide salt (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay) according to the

method described by Nicoletti et al. (Nicoletti *et al.*, 2014). The cells were treated for 18 h with PhTx3-3 and Phα1β from *P. nigriventer*, or MVIIC and MVIIA from *C. magus*, at different concentrations, ranging from 0.3 to 100 pM. The cell viability was calculated using the equation: *Cell viability (%) = (*$Abs_s/Abs_{control}$*)100*; where Abs_s is the absorbance of cells treated with different formulations and $Abs_{control}$ is the absorbance of control cells (incubated with cell culture medium only).

Cell counting

To assess the proliferation grade, the human glioma cells were seeded at 15-20×10^3 cells per well in 24-well plates for 24 h. Then, the cells were treated for 18 h with the toxins PhTx3-3 and Phα1β, or MVIIC and MVIIA (0.3 to 100 pM). After this period of incubation, the medium was collected and 200 µl of trypsin/EDTA solution was added to detach the cells, which were counted in a hemocytometer. The cell number of the control group (non-treated cells) was considered 100 %.

Annexin V-FITC (fluorescein isothiocyanate)/propidium iodide staining assay

The qualitative analysis of apoptotic or necrotic cells was carried out by flow cytometry. Briefly, the M059J, U-138MG and U-251MG glioma cells were seeded at 5×10^4 cells per well in 24-well plates, and grown for 18 h. The cells were treated with the toxins PhTx3-3 and Phα1β, or with MVIIC and MVIIA, at the concentration of 10 pM, for 18 h. Dead cells were quantified by annexin V-FITC–propidium iodide (PI) double staining, using Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, U.S.A) 24 h after treatment, according to the manufacturer's instructions. The

experiments were performed on FACSCanto II Flow Cytometer (BD Biosciences, San Jose, CA, U.S.A) and the results were analyzed using FlowJo Software (Tree Star, Inc, Ashland, OR, USA).

Flow cytometry for Ki67expression

The verify the expression of the proliferation-related molecule Ki67, the human glioma cells were plated in 12-well plates at 6×10^4 and grown for 24 h. The cell lines were treated with PhTx3-3 and Phα1β toxins, or MVIIC and MVIIA, all at 10-pM concentration. For intracellular staining, the cells were fixed with a freshly prepared fixation/permeabilization working solution (eBioscience, San Diego, CA, U.S.A) at 37°C, for 30 min. After washing with the permeabilization buffer (eBioscience, San Diego, CA, U.S.A), the cells were stained with the intracellular marker Ki67-FITC (4°C of temperature), according to the manufacturer's instructions. The experiments were performed on a FACSCanto II Flow Cytometer (BD Biosciences, San Jose, CA, U.S.A).

Animals

Male and female C57/BL6 wild-type were used in this study (8 weeks old, 25–30 g; total N=35 mice). The animals were housed under conditions of optimum light, temperature and humidity (12 h light-dark cycle, 22±1°C, under 60 to 80 % humidity), with food and water provided *ad libitum*. The animals were supplied by Federal University of Pelotas (UFPEL), Brazil. All the experimental procedures were in accord to the Principles of Laboratory Animal Care from NIH, and were approved by the local Animal Ethical Committee (CEUA-PUCRS; protocol number: 11/00258).

In vivo mouse glioma model

The anti-tumor effects of the toxins were tested *in vivo*, by using an orthotopic mouse glioblastoma model, according to the methodology previously described (Szatmari *et al.*, 2006). For this purpose, the GL261 glioma cells were cultured to approximately 70% confluence and resuspended at 2 x 10^5 cells/2 µl DMEM. The cells were injected using a 10-µl Hamilton microsyringe coupled to an infusion pump (1 µl/min x 2 min) at a depth of 3.0 mm into the right striatum (coordinates with regard to bregma: 2.0 mm lateral) of C57/BL6 mice, previously anesthetized with ketamine plus xylazine (75 and 7.5 mg/kg, respectively), dosed by intraperitoneal route.

Experimental groups and treatments

The animals received the toxins, given by intracerebroventricular (i.c.v.) or intratecal (i.t.) routes, at three periods of administration (5, 10 and 15 days after tumor induction), in the following doses: (a) PhTx3-3 (50 pmol/site); (b) Phα1β (50 pmol/site); (c) MVIIC (30 pmol/site); (d) MVIIA (10 pmol/site). The doses of toxins were chosen on the basis of previous publications (Maciel *et al.*, 2014; Silva *et al.*, 2015).

The animals were divided into the following experimental groups (N=5 per group), according to the treatment schemes: (i) i.c.v. control (mice implanted with GL261 cells and treated with vehicle i.c.v.); (ii) i.c.v. PhTx3-3 (mice implanted with GL261 cells and treated with PhTx3-3 i.c.v.); (iii) i.c.v. Phα1β (mice implanted with GL261 cells and treated with Phα1β i.c.v.); (iv) i.c.v. MVIIC (mice implanted with GL261 cells and treated with MVIIC i.c.v.); (v) i.c.v. MVIIA (mice implanted with GL261 cells and

treated with MVIIA i.c.v.); (vi) i.t. control (mice implanted with GL261 cells and treated with vehicle i.t.); (vii) i.t. Phα1β (mice implanted with GL261 cells and treated with Phα1β i.t.). After 20 days of glioma implantation, the mice were euthanized by isoflurane inhalation and decapitated. The entire brain was removed for further analysis as described in the next sections. For this, the brain samples were immediately fixed in 4%-buffered formaldehyde solution.

Determination of tumor size

To determine the tumor areas, at least three hematoxylin-eosin (HE) coronal sections (20-µm thick) from each animal were analyzed. Images were captured (x8 magnification) using a digital camera (Canon PowerShot C9 – PC1250 7.4V, Canon Inc, Tokyo, Japan) connected to a stereoscopic microscope (Stemi DV4, Carl Zeiss MicroImaging, Gottingen, Germany), and analyzed by using Axion Vision version 4.7.1 (Imaging Systems, Carl Zeiss Vision program group, Gottingen, Germany). The results were calculated in percentage by measuring the tumor area (*TA b x h*) in relation to the total brain area (*TBA b x h*, considered as 100%).

Immunohistochemistry for GFAP and IBA-1

Immunopositivity for activated astrocytes and microglia was assessed on paraffin tissue sections (3-µm thick) by using the monoclonal rabbit anti-GFAP (1:250, Cat. #04-1062; Lot #2145973; Merck Millipore, Darmstadt, Germany), and the monoclonal mouse anti-Iba1/AIF1 (1:300; Cat. #MABN92; Lot #2172784; Merck Millipore, Darmstadt, Germany), respectively. The general procedures were the same adopted before (Maciel *et*

al., 2014). High-temperature antigen retrieval was performed by immersion of the slides in a water bath at 98–100 °C in 10 mM trisodium citrate buffer, pH 6.0 (anti-Iba-1), Tris-EDTA buffer pH 9.0 (anti-GFAP and anti-GPR40) for 40 min. The peroxidase was blocked by incubating the sections with perhidrol 5% for 30 min. The nonspecific protein binding was blocked with milk serum solution 5% for 30 min. After overnight incubation at 4 °C with primary antibodies, the slides were washed with PBS and incubated with the secondary antibody HRP conjugate (Invitrogen, Invitrogen, Carlsbad, CA, U.S.A.), ready-to-use, for 20 min at room temperature. The sections were washed in PBS, and the visualization was completed by using 3,3′-diaminobenzidine (Dako Cytomation, Carpinteria, CA, U.S.A.) in chromogenic solution and counterstained lightly with Harris's Hematoxylin solution. Images were examined with a Zeiss AxioImager M2 light microscope (Carl Zeiss, Gottingen, Germany). The images were captured in x200 magnification, and evaluated by using the Image NIH Image J 1.36b Software (NIH, Bethesda, MD, USA). The number of GFAP-positive astrocytes and Iba1-positive microglia cells was quantified by two independent examiners in a blinded manner, in the tumor core and peritumoral region.

Statistical analysis

The number of experimental replications is provided in the figure legends. The results are presented as the mean ± standard error mean of five animals per group. The statistical comparison of the data was analyzed by one-way analysis of variance (Huang *et al.*) followed by Tukey's post-hoc test, using Graph-Pad Software (San Diego, CA, U.S.A.). $P<0.05$ was indicative of statistical significance.

Results

The effects of P/Q- and N-type VGCC blockers were initially evaluated in three distinct human GBM cell lineages, namely M059J (radiosensitive cells), or U-138MG and U-251MG (radiresistant cells), in viability and proliferation *in vitro* assays. The preferential P/Q-type VGCC inhibitor MVIIC obtained from *C. Magus* (0.3 to 100 pM) failed to significantly alter either the cell viability or the proliferation of all the tested cell lines, regardless of minor inhibitory effects (Figures 1 and 2, lanes b). However, the P/Q-type VGCC blocker PhTx3-3 originated from *P. Nigriventer*, was able to significantly affect the viability and the proliferation of all the cell lines, when tested at 3 and 10 pM (Figures 1 and 2, lanes a). The viability and the proliferation of the radiosensitive cell line M059J were also significantly reduced by PhTx3-3, at the concentrations of 1 and 30 pM, with a partial inhibition of cell proliferation at 100-pM concentration (Figures 1 and 2A, lanes a). The selective N-type VGCC blocker *C. magus*-derived MVIIA triggered a significant decrease of the cell viability and proliferation in all the tested cell lines, except for an absence of significant inhibition of M059J viability (Figures 1 and 2, lanes d). The cytotoxicity grade of MVIIA was variable among the different cell lines, with the maximal effects observed at 10-pM concentration. The preferential N-type VGCC blocker Phα1β from *P. nigriventer* displayed significant cytotoxic effects in all the glioblastoma cell lines, by reducing both the viability and proliferation rates (Figures 1 and 2, lanes c). For Phα1β, the most effective concentrations were 10 and 30 pM. Remarkably, none of the tested VGCC inhibitors presented classical concentration-dependent effects (Figures 1 and 2).

Based on data described above, the concentration of 10 pM of the toxins was selected for the next *in vitro* assays. The qualitative flow cytometry analysis revealed distinct patterns of death for the P/Q- and N-type VGCC blockers depending on the cell line tested. Regarding the radiosensitive human cell line M059J, it was possible to observe the induction of death with mixed apoptosis and necrosis features, following the *in vitro* treatment with MVIIC and MVIIA from *C. magus*, or PhTx3-3 and Phα1β from *P. nigriventer* (Figure 3A, lane a). Alternatively, the radioresistant human cell line U-138MG showed cell death with predominant apoptosis characteristics, after the incubation of MVIIA, PhTx3-3 or Phα1β, whereas MVIIC caused cell death mostly via necrosis (Figure 3A, lane b). A similar profile was observed for the radioresistant human cell line U-251MG, but in this case all the tested toxins led to cell death mainly via apoptosis ((Figure 3A, lane c).

The effects of P/Q- and N-type VGCC blockers were also investigated by using the cell proliferation marker Ki67. The incubation with MVIIC and MVIIA from *C. magus*, or PhTx3-3 and Phα1β from *P. nigriventer* caused a marked reduction of cell proliferation of all the tested GBM cell lines, as qualitatively evaluated by flow cytometry Ki67 staining (Figure 3B, lanes a-c). In this experimental set, all the toxins displayed a similar profile, apart from a less pronounced effect for MVIIC in the radioresistant cell lines U-138MG and U-251MG (Figure 3B, lanes b and c).

To gain further insights on the anti-tumor effects of the P/Q- and N-type VGCC blockers tested in the present study, we have also employed an *in vivo* model of mouse GBM, induced by the implantation of GL261 cell line. As expected, control tumor-bearing animals displayed marked tumor development (32.9 ± 5.1 tumor percentage

area), with histological characteristics of glioma, according to evaluation 21 days after tumor induction (Figure 4A and B). The i.c.v. treatment with the preferential P/Q-type VGCC inhibitors PhTx3-3 (50 pmol/site) or MVIIC (30 pmol/site), at 5, 10 and 15 days after surgery for tumor implantation, caused partial reductions of the tumor areas (20.3 ± 3.1 and 26.0 ± 2.0, respectively), although statistical significance was not observed. The same treatment scheme with N-type VGCC blockers MVIIA (10 pmol/site) or Phα1β (50 pmol/site), administered by i.c.v route, produced marked and significant reductions of gliomas, presenting mean tumor area percentages of 19.0 ± 2.5 and 16.8 ± 3.9, respectively. Of note, the i.t. administration of the recombinant Phα1β (50 pmol/site, at 5, 10 and 15 days) also displayed significant inhibitory effects on mouse glioblastoma development, with a tumor percentage area of 16.0 ± 2.0 (Figure 4B).

To extend the evidence on the *in vivo* effects of the toxins, we also evaluated the immunopositivity for astrocytes (GFAP) and microglia (Iba-1) in the tumor core or the peritumoral region of mouse glioma sections. The number of GFAP-positive astrocytes was not significantly altered in the tumor core of any experimental groups that had been treated with the toxins, when compared to control animals (Figure 5B). A partial, but not significant increase in the number of GFAP-positive astrocytes was observed in the peritumoral region of the groups that received PhTx3-3 from *P. nigriventer*, or MVIIC and MVIIA from *C. magus* by i.c.v. route (Figure 5B). However, the administration of the *P. nigriventer*-derived toxin Phα1β, dosed i.c.v or i.t., led to a marked increase of activated astrocytes in the peritumoral area, displaying thick, dense and darkly stained cell bodies, when compared to the other experimental groups (Figure 5A and B). Concerning the positive immunolabelling for the microglia marker Iba1/AIF1, none of

the evaluated P/Q- and N-type VGCC blockers was able to significantly alter the cell activation in the tumor core (Figure 6B). The i.c.v. treatment with the preferential P/Q-type VGCC inhibitors PhTx3-3 and MVIIC did not significantly change the immunopositiviy for Iba1/AIF1 in the peritumoral region, when compared to control tumor-bearing animals. Noteworthy, there was a significant increase in the number of active microglia cells in the experimental group that had been treated i.c.v with the N-type VGCC inhibitor MVIIA from *C. magus*. Similarly, the animals treated with the N-type VGCC blocker Phα1β from *P. nigriventer*, given by i.c.v. or i.t. routes of administration, displayed a significant increase in the number of Iba1/AIF1 immunopositive microglia cells in the peritumoral areas, when compared to control mice (Figure 6A and B).

Discussion and conclusions

Calcium is a pivotal second messenger that participates in the regulation of several pathophysiological events, such as cell cycle control, survival and apoptosis (Chen *et al.*, 2013; Prevarskaya *et al.*, 2013). VGCC provide one of the pathways to the regulation of intracellular calcium influx. Distinct classes of calcium-permeable channels are abnormally expressed in cancer, and are likely involved in the alterations underlying malignant growth (Monteith *et al.*, 2012; Prevarskaya *et al.*, 2013). GBM are highly lethal and aggressive brain tumors. Despite the recent advances in the current therapies, which includes the combination of surgery and radio/chemotherapy, the treatment of these tumors remains palliative with an average survival rate of one year (Omuro *et al.*, 2013). P/Q- and N-type high-VGCC are widely distributed throughout the central nervous system (CNS), and they have been investigated as possible therapeutic targets for several neurological disorders (Nimmrich *et al.*, 2012). Nevertheless, to the best of our knowledge, there are no available studies on the role of these calcium channels in glioblastomas. Therefore, in this study, we explored the effects of the pharmacological inhibition of P/Q- and N-type VGCC on glioma progression, by means of *in vitro* and *in vivo* approaches.

The *in vitro* effects of the preferential P/Q-type inhibitors MVIIC and PhTx3-3, or the N-type MVIIA and Phα1β were initially evaluated in the human GBM cell lines M059J, U-138MG and U-251MG, in both viability and proliferation protocols. This first set of results revealed significant anti-proliferative effects for the toxins PhTx3-3 and Phα1β obtained from *P. nigriventer*, or MVIIA from *C. magus*, in all the tested cell lines, according to assessment in a pM range. However, *C. magus*-derived MVIIC toxin

displayed only marginal effects on viability and proliferation of human GBM cell lines. From the analysis of *in vitro* experiments, it is feasible to observe that PhTx3-3, Phα1β and MVIIA displayed anti-proliferative effects of the three tested glioma cell lines in a concentration-independent manner. These results are somewhat supported by previous literature data showing the absence of dose-related effects for the same toxins in experimental models of pain and itching (Souza *et al.*, 2008; Maciel *et al.*, 2014).

It has been widely recognized that uncontrolled cell proliferation, invasion and resistance to apoptosis are the major causes of the deadly nature of human gliomas (Watkins *et al.*, 2012). Our *in vitro* data allows suggesting that activation of high-VGCC, including P/Q- and N-type VGCC, are likely relevant for the mechanisms of glioma cell viability and proliferation. Moreover, the present results provide compelling evidence on the ability of the most tested toxins, namely PhTx3-3, Phα1β and MVIIA, to promote the apoptosis of glioblastoma cells and to markedly reduce the cell proliferation, as demonstrated by flow cytometry with Annexin V/PI and Ki67 staining, respectively. The lack of marked antiproliferative effects for MVIIC, in comparison to the other VGCC blockers tested by us might be explained, at least in part, by the different profiles observed in flow cytometry experiments. Whilst PhTx3-3, Phα1β and MVIIA caused cell death via mixed apoptosis/necrosis (M059J cells) or apoptosis (U-138MG and U-251MG), MVIIC induced cell death with predominant necrosis characteristics in U-138MG cells. Furthermore, the flow cytometry analysis for the proliferation marker Ki67 showed less expressive effects for MVIIC, when compared to the other tested VGCC inhibitors. In fact, it was previously demonstrated that PhTx3-3 showed higher efficacy when compared to MVIIC in preventing synaptosomal calcium influx or ischemia-

induced neuronal damage in *in vitro* protocols (Miranda *et al.*, 1998; Pinheiro *et al.*, 2009).

As mentioned before, the exact role of high-VGCC in gliomas had not been explored so far. Nevertheless, a series of previous publications suggested the importance of the T-type low-VGCC in different cancer types, including gliomas (Roger *et al.*, 2006). Remarkably, a recent study demonstrated that pharmacological inhibition of T-type VGCC by mibefradil, or gene knockdown by siRNA, caused anti-proliferative effects and triggered apoptosis of U-251MG human glioma cells (Valerie *et al.*, 2013). Additionally, it was demonstrated that endostatin markedly reduced the proliferation and migration of the human GBM U87 cell line, via inhibition of T-type low-VGCC (Zhang *et al.*, 2012). The results in the present study extend the evidence on the relevance of VGCC in gliomas, providing novel evidence on the role of high-VGCC in this tumor type.

A previous literature report demonstrated the ability of the T-type low-VGCC mibefradil to inhibit the tumor growth and to increase the efficacy of the reference drug temozolamide in an *in vivo* xenograft mouse glioma model (Keir *et al.*, 2013). Herein, we investigated the effects of P/Q- and N-type in an *in vivo* GBM model, induced by orthotopic injection of GL261 cells into the mouse brain. The repeated treatment with the preferential N-type blockers Phα1β and MVIIA, dosed by i.c.v route, was able to markedly reduce the tumor areas at pmol doses, according to the evaluation of histological sections. However, the administration of the P/Q-type VGCC inhibitors PhTx3-3 and MVIIC, at the same schedules of i.c.v treatment, produced only a partial reduction of the tumor areas. Thus, it is reasonable to propose a more significant role for

N-type VGCC, when compared to P/Q-type VGCC, in the mouse glioma model employed by us. To obtain additional evidence on the anti-tumor effects of N-type VGCC, we have also tested the recombinant Phα1β, when dosed by i.t. route. In this case, it was also possible to observe a marked decrease of the mouse glioma areas. Interestingly, a recent study demonstrated beneficial effects for Phα1β and MVIIA, when dosed by i.t. route, to prevent the cancer-related pain in a mouse model of melanoma, at the same range of pmol doses used in our study (Rigo et al., 2013).

Data on immunohistochemistry analysis demonstrated the induction of glial cell activation in the peritumoral region of tumor-bearing mice that had been treated with the N-type VGCC blockers Phα1β (by i.c.v. an i.t. routes), and to a less extent MVIIA (by i.c.v. route), when compared to control tumor-implanted animals. Otherwise, only mild effects were observed after treatment with the preferential P/Q-type VGCC inhibitors MVIIC and PhTx3-3, according to the evaluation of glia activation in the tumor periphery. This is consistent with the superior effects observed for Phα1β and MVIIA regarding the reduction of the tumor areas, and might well explain the marked effectiveness of these toxins on *in vivo* glioma progression. Importantly, Phα1β from *P. nigriventer* led to a marked increase in the number of GFAP-activated astrocytes and Iba-1-positive microglia around the most proliferative and vascularized portion of gliomas, which likely contributes to the invasive behavior of this tumor type (Omuro *et al.*, 2013).

It is well established that brain resident cells can influence the tumor growth (Puduvalli, 2001). Astrocytes maintain homeostasis of the brain microenvironment and under pathological conditions, such as trauma, ischemia or neurodegenerative alterations, they become activated (Sofroniew, 2005). These reactive astrocytes have been shown to

protect neurons from injury-induced apoptosis (Chen *et al.*, 2005; Sofroniew, 2005; Mahesh *et al.*, 2006). An inverse correlation between the levels of astrocyte activation and glioma malignancy grade has been demonstrated before, clearly suggesting that astrocytes have a crucial role in the regulation of gliomas (Deck *et al.*, 1978; Duffy *et al.*, 1980). Additional *in vitro* evidence demonstrated a marked reduction of the proliferative ability of GFAP-expressing glioma cells (Rutka *et al.*, 1993), whereas GFAP-knockdown led to increased invasiveness and proliferation of GFAP-positive U-251MG glioma cells (Rutka *et al.*, 1994). Furthermore, a recent *in vivo* study conducted by Valiente et al.(2014) found that plasmin expression by astrocytes triggered apoptotic cell death in cancer cells and also prevented cell spreading along brain capillaries (Valiente *et al.*, 2014). Thus, it is tempting to propose that Phα1β is able to modulate some of the mechanisms underlying the cancer immunosurveillance by astrocytes, finally resulting in the inhibition of glioma development.

Microglia is a major brain immune cell type, which is activated during inflammation and under development of primary brain tumors (Hoelzinger *et al.*, 2007). Some studies demonstrated that microglia can be modulated by tumor cells, contributing for tumor progression. However, emerging studies have used microglia cells as therapeutic vectors, demonstrating potential cytotoxicity effects in glioma cells, opening new possibilities in GBM gene therapy (Ribot *et al.*, 2007; Lima *et al.*, 2012). In agreement, our results demonstrated a marked increase of Iba-1-positive microglia cells in the tumor periphery of animals that had been treated with either Phα1β or MVIIA, what might further explain the anti-tumor effects of these toxins in gliomas.

In summary, our data provide novel evidence indicating the potential role of P/Q-, and especially N-type high-VGCC in growth and proliferation of gliomas. It is worth noting that Phα1β from *P. nigriventer* demonstrated marked anti-tumor effects, when dosed by both i.c.v. and i.t. routes, making it an attractive therapeutic approach for the treatment of glioblastomas in the clinical practice. The anti-tumor effects of the FDA-approved N-type VGCC blocker MVIIA from *C. magus* by i.c.v route can also be of great interest as a novel alterative for treatment of advanced gliomas.

Acknowledgments

The authors thank Mrs Luciana Adolfo Ferreira for her excellent technical assistance in histological and immunohistochemical analysis. We are grateful to Dr Ana Maria Battastini, from Federal University of Rio Grande do Sul (Brazil), for donating both tested cell lines.

Financial support

This study was Supported by the FINEP research grant "Implantação, Modernização e Qualificação de Estrutura de Pesquisa da PUCRS" (PUCRSINFRA) # 01.11.0014-00, CNPq, CAPES and FAPERGS. N.F.N. is a PhD student in Cellular and Molecular Biology receiving a grant from CAPES AUX-PE/Toxinologia and PROBOLSAS/PUCRS; T.C.E. is an undergraduate student in Pharmacy supported by PIBITI/CNPq and BPA/PUCRS; R.F.Z. is a post-doc fellow supported by PNPD/CAPES.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

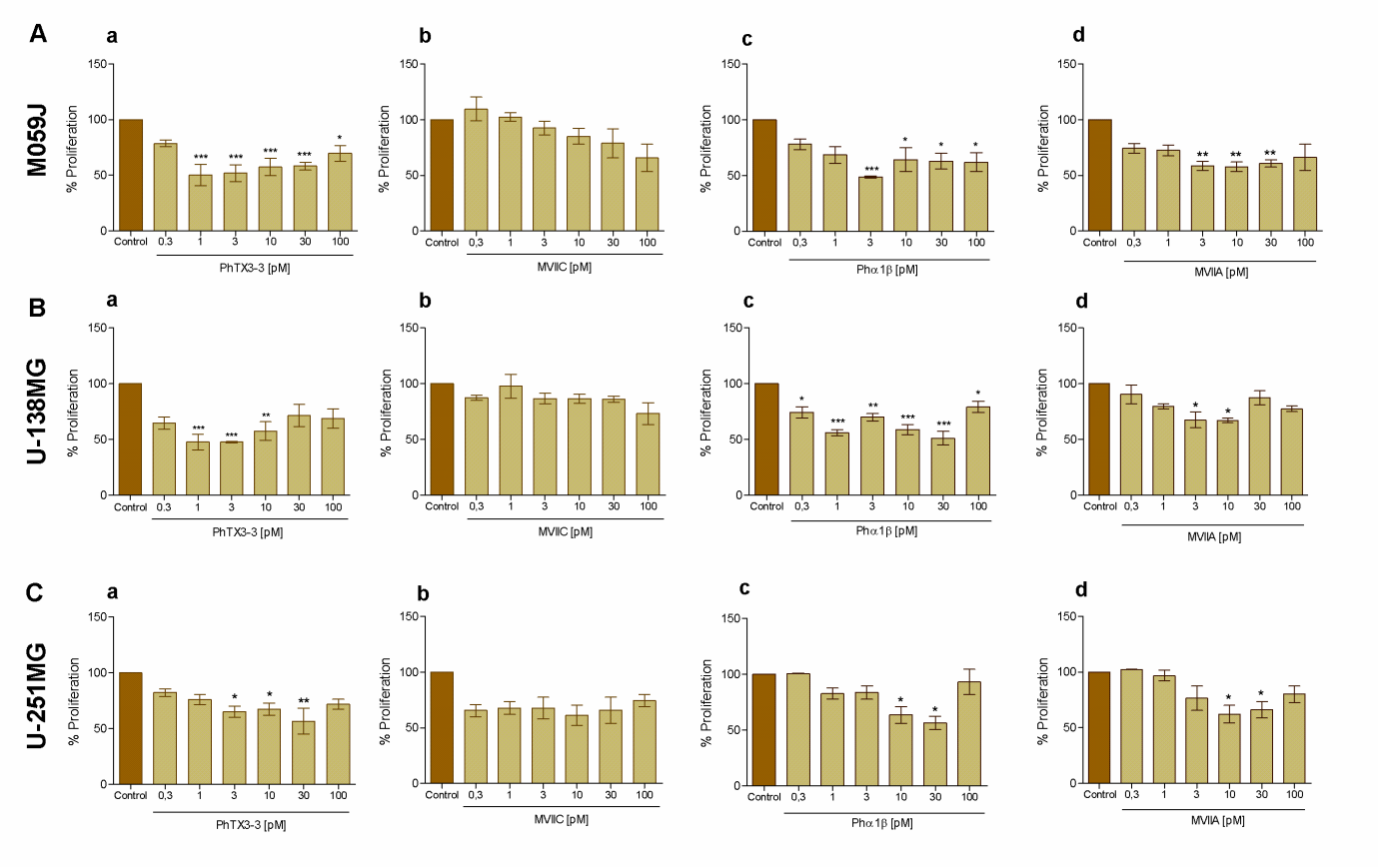


Figure 1. Effect of treatment with PhTx3-3 and Phα1β from *Phoneutria nigriventer*, or MVIIC and MVIIA from *Conus magus* (0.3 to 100 pM) on cell counting of **(A)** M059J, **(B)** U-138MG and **(C)** U-251MG human glioma cells, after 18 h. The experiments were carried out at least three times in triplicate. Each column represents the mean±SEM. *p<0.05; **p<0.01; ***p<0.001 for comparison *versus* the control 0.5% FBs, as determined by ANOVA with Tukey's post-hoc test.

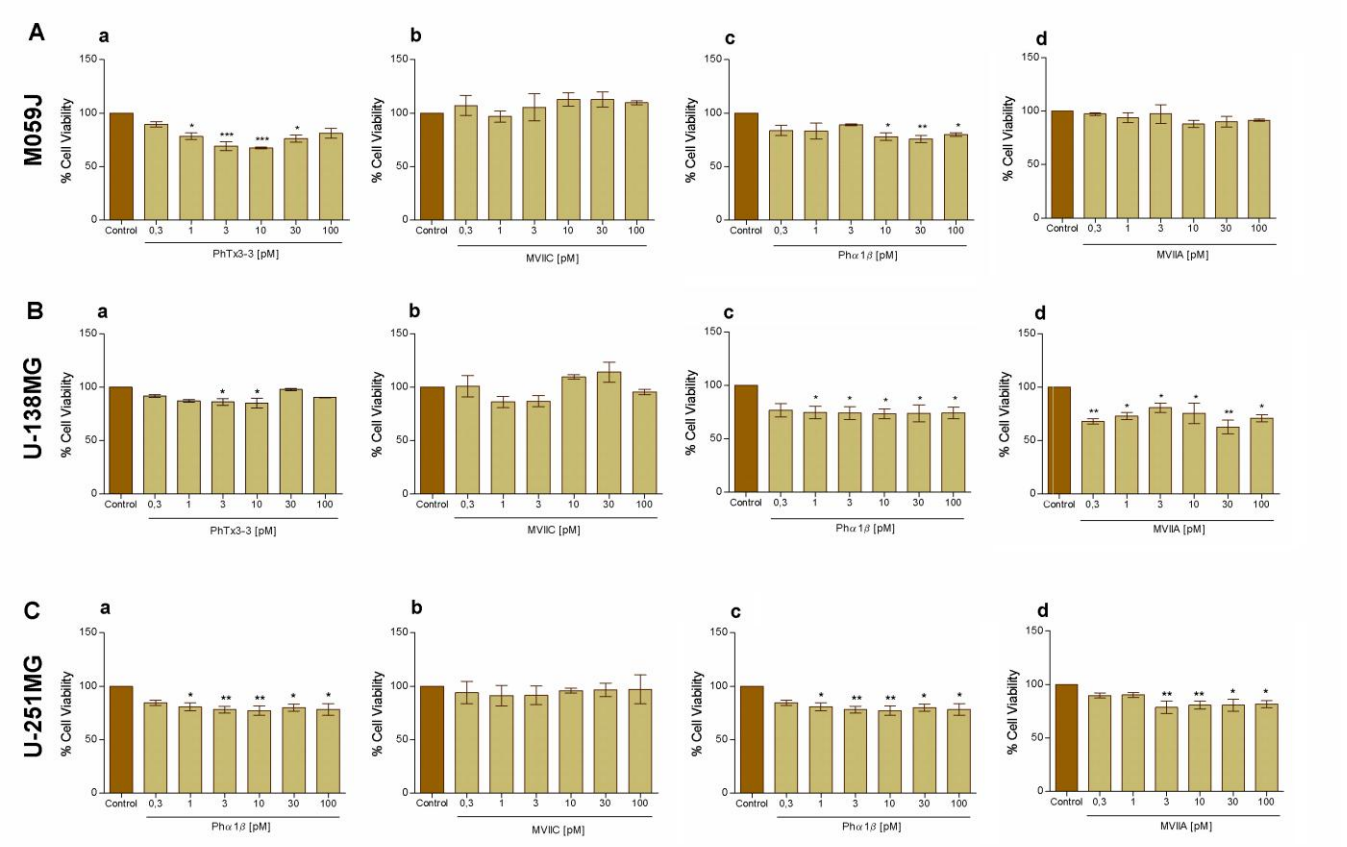


Figure 2. Effect of treatment with PhTx3-3 and Phα1β from *Phoneutria nigriventer*, or MVIIC and MVIIA from *Conus magus* (0.3 to 100 pM) on cell viability of **(A)** M059J, **(B)** U-138MG and **(C)** U-251MG human glioma cells, after 18 h. The experiments were carried out at least three times in triplicate. Each column represents the mean±SEM. *p<0.05; **p<0.01; ***p<0.001 for comparison *versus* the control 0.5% FBs, as determined by ANOVA with Tukey's post-hoc test.

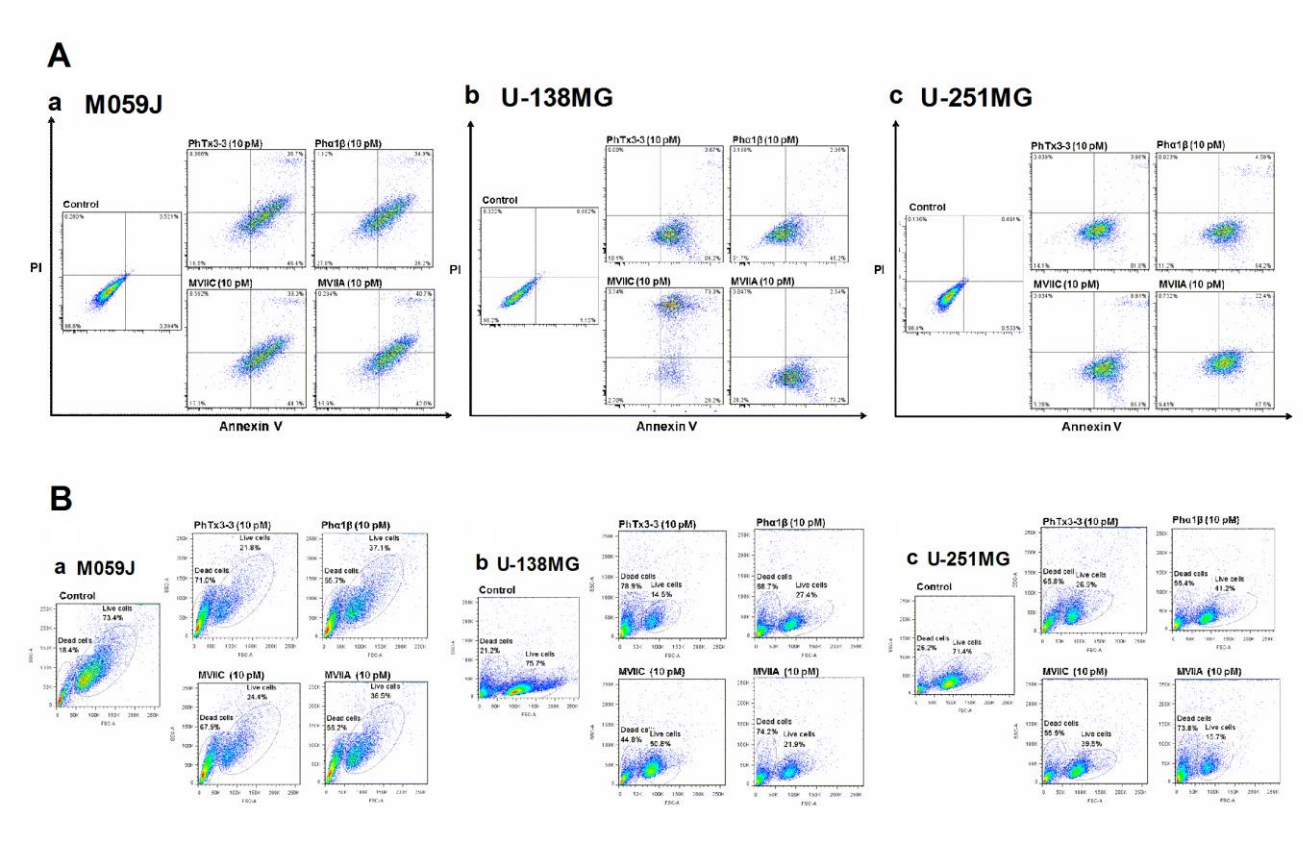


Figure 3. Dot plot with percentage of **(A)** Annexin V/PI positive and **(B)** ki67 marker in **(a)** M059J, **(b)** U-138MG and **(c)** U-251MG cells, 18 h after treatment with PhTx3-3 and Phα1β from *Phoneutria nigriventer*, or MVIIC and MVIIA from *Conus magus* (10 pM). Each sample has 50,000 cells. Data shown is representative of at least two independent experiments in duplicate.

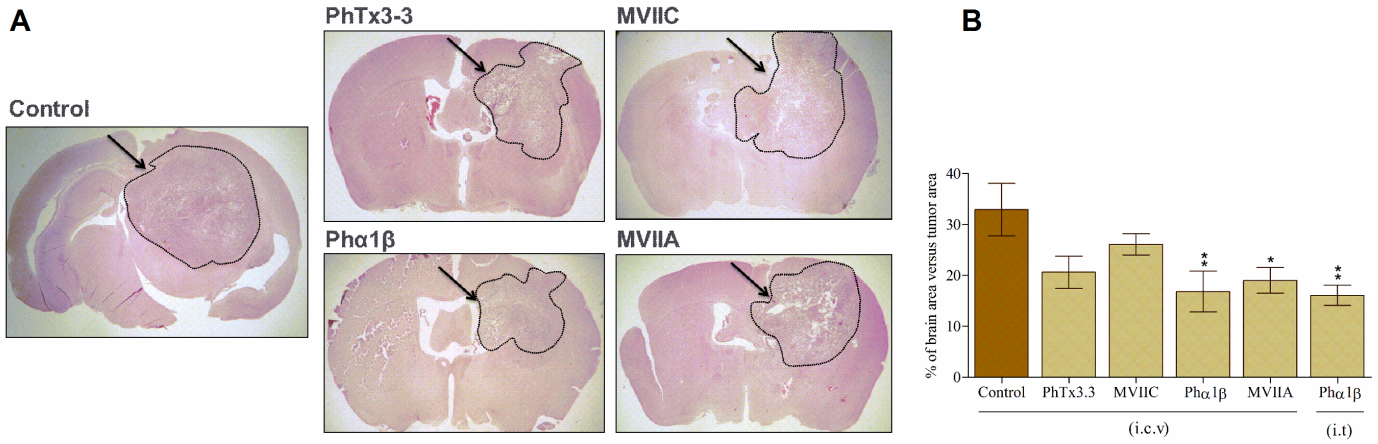
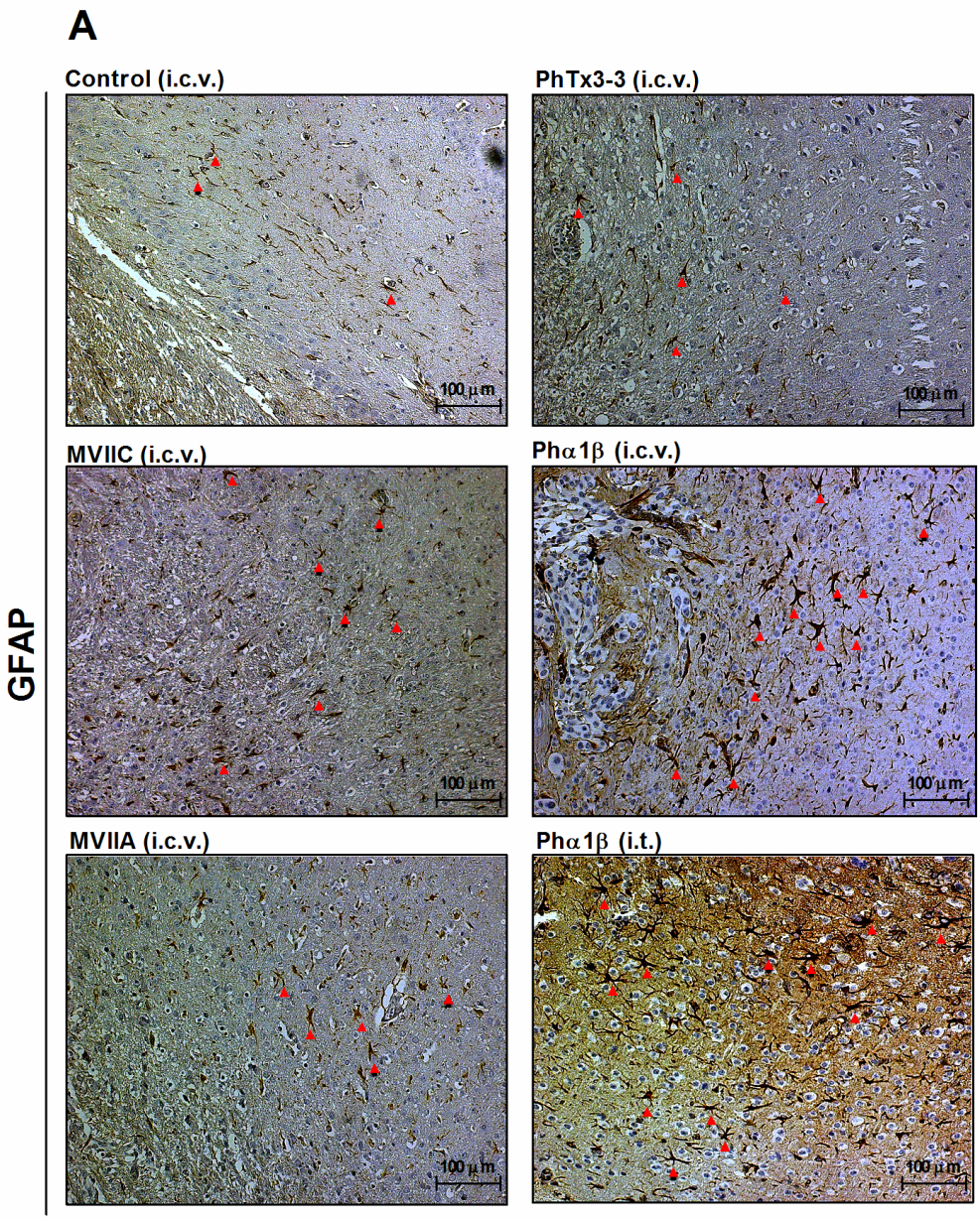


Figure 4. (**A**) Representative H&E-stained brain coronal sections and (**B**) tumor area quantification in mouse brain after glioma GL261-induced tumors of five animals per group divided as follows: control (vehicle; i.c.v.); PhTx3-3 (50 pmol/site; i.c.v.);; MVIIC (30 pmol/site; i.c.v.); Phα1β (50 pmol/site; i.c.v.); MVIIA (10 pmol/site; i.c.v.) and Phα1β (50 pmol/site; i.t.). Each column represents the mean±SEM. *p<0.05; **p<0.01 for comparison versus control group, as determined by ANOVA with Tukey's post-hoc test.


A
Control (i.c.v.)
PhTx3-3 (i.c.v.)
MVIIC (i.c.v.)
Phα1β (i.c.v.)
MVIIA (i.c.v.)
Phα1β (i.t.)
GFAP
100 µm

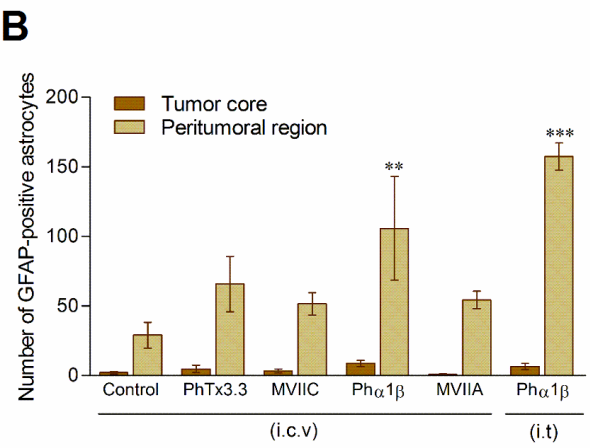
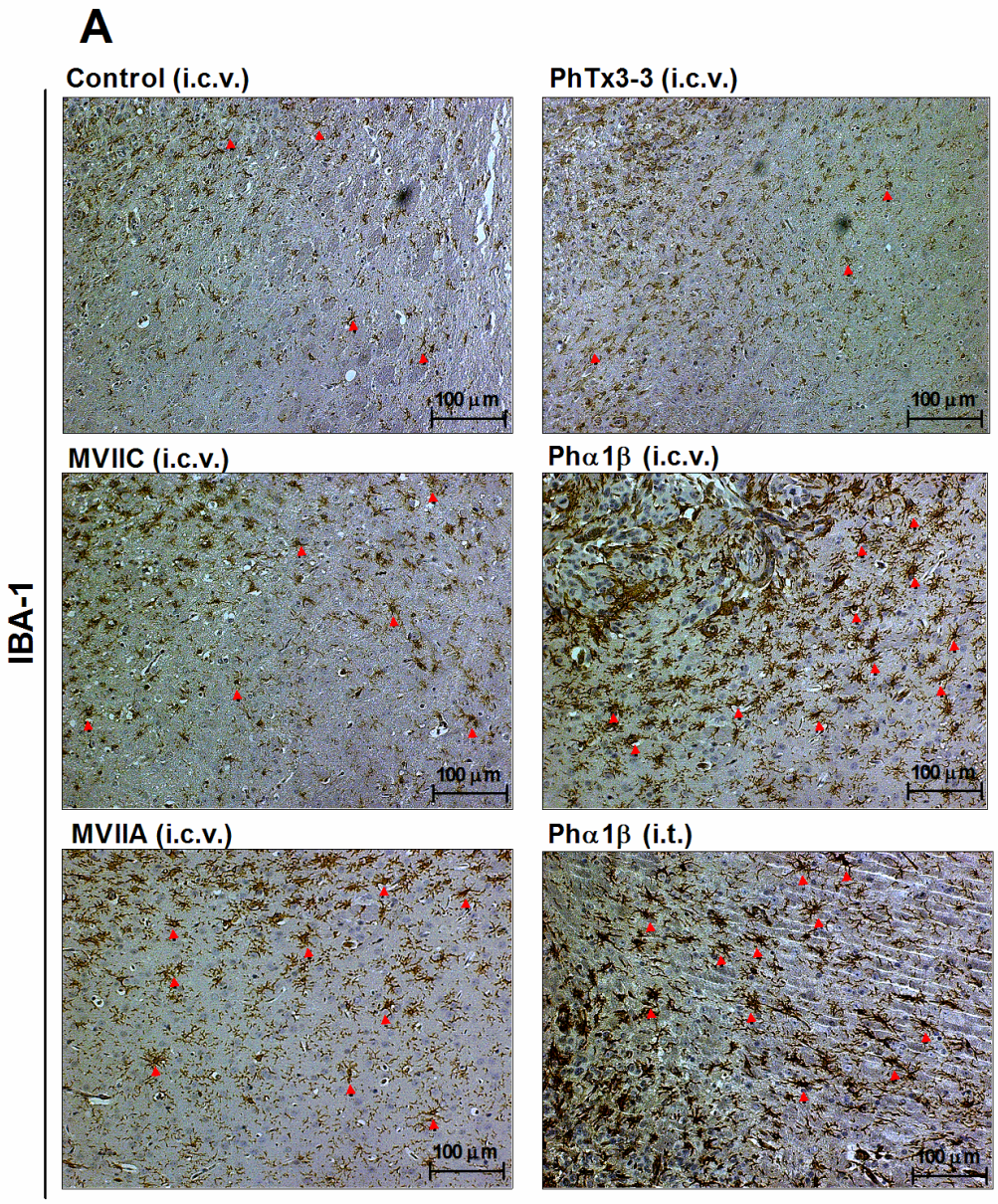

B
Number of GFAP-positive astrocytes
200
150
100
50
0
Tumor core
Peritumoral region
**
***
Control
PhTx3.3
MVIIC
Phα1β
MVIIA
Phα1β
(i.c.v)
(i.t)

Figure 5. (A) Representative immunohistochemistry analysis on the number of GFAP-positive astrocytes in the peritumoral region of mouse gliomas treated with PhTx3-3 (50 pmol/site; i.c.v.); MVIIC (30 pmol/site; i.c.v.); Phα1β (50 pmol/site; i.c.v.); MVIIA (10 pmol/site; i.c.v.) and Phα1β (50 pmol/site; i.t.). **(B)** GFAP-positive astrocytes quantification in the core and the peritumoral regions of mouse gliomas. Each column represents the mean±SEM. ***$p<0.01$; * $p<0.05$ for comparison versus C57/BL6 control group.


A
Control (i.c.v.)
PhTx3-3 (i.c.v.)
MVIIC (i.c.v.)
Phα1β (i.c.v.)
MVIIA (i.c.v.)
Phα1β (i.t.)
IBA-1
100 µm

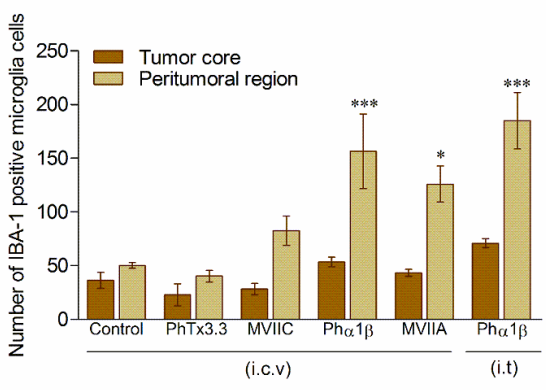

B
Number of IBA-1 positive microglia cells
250
200
150
100
50
0
Tumor core
Peritumoral region
***
*
***
Control
PhTx3.3
MVIIC
Phα1β
MVIIA
Phα1β
(i.c.v)
(i.t)

Figure 6. (A) Representative immunohistochemistry analysis on the number of Iba-1-positive microglia in the peritumoral region of mouse gliomas treated with PhTx3-3 (50 pmol/site; i.c.v.); MVIIC (30 pmol/site; i.c.v.); Phα1β (50 pmol/site; i.c.v.); MVIIA (10 pmol/site; i.c.v.) and Phα1β (50 pmol/site; i.t.). **(B)** Iba-1-positive microglia quantification in the core and the peritumoral regions of mouse gliomas. Each column represents the mean±SEM. ***$p<0.01$; * $p<0.05$ for comparison versus C57/BL6 control group.

Capítulo V

4. CONSIDERAÇÕES FINAIS

5. PERSPECTIVAS

4. CONSIDERAÇÕES FINAIS

A terapia dos gliomas é baseada em dois aspectos essenciais: o controle local do tumor primário e o bloqueio da invasão de células tumorais no tecido cerebral normal (40). Apesar dos recentes avanços acerca da patogênese molecular do GBM, as opções atuais para a terapia tumoral ainda são muito limitadas. A ressecção cirúrgica é adotada como tratamento de escolha para os diferentes graus de malignidade dos gliomas (I-IV), seguida de radioterapia e quimioterapia, que contribuem pouco para prolongar a sobrevida média dos pacientes, que varia de nove a doze meses (26, 29). A eficiência da cirurgia para a remoção do GBM é dificultada pela natureza invasiva do tumor, sendo a infiltração do parênquima cerebral um fator limitante para o sucesso da ressecção cirúrgica que invariavelmente permite a permanência de populações de células neoplásicas no tecido cerebral adjacente (7). Frente às limitações apresentadas pela ressecção cirúrgica, a radioterapia e quimioterapia são de extrema relevância, a fim de eliminar as populações celulares remanescentes de caráter neoplásico, responsáveis pela rápida recidiva tumoral e baixa sobrevida.

Apesar de as vias de sinalização envolvidas no desenvolvimento de gliomas malignos já estarem relativamente bem caracterizadas, a origem celular destes tumores é desconhecida. Alterações na expressão gênica e anormalidades em vias de sinalização relacionadas à proliferação, sobrevivência e apoptose, são comumente encontradas em gliomas e parecem estar diretamente correlacionadas com o grau de malignidade do tumor. Como descrito anteriormente, o GBM pode ser dividido em dois subtipos, de acordo com as características clínicas e histológicas apresentadas e recentemente sugeriu-se associar uma subclassificação molecular a fim de aprimorar a caracterização do GBM e direcionar o tratamento. Neste sentido, o GBM também pode ser caracterizado como proneural, neural, clássico e mesenquimal (28). O GBM primário é caracterizado por excesso de ativação, amplificações e mutações em EGFR, perda da heterozigosidade do cromossomo 10q22-25, com consequente deleção de PTEN e deleção do gene supressor tumoral p16 (4). A perda da expressão de PTEN resulta em ativação de uma das vias cruciais que regulam a sobrevivência celular, a via AKT. O GBM secundário ou *de novo*, manifestado em pacientes jovens e originário de um astrocitoma de baixo grau, é caracterizado por mutações no gene p53, super-expressão de PDGF e anormalidade nas vias que envolvem p16 e Rb, além de perda da heterozigosidade do cromossomo 10q (26, 142). É possível ainda, que a sinalização da

ERK e de PKC module a transcrição gênica necessária para a proliferação das células gliais, enquanto que a sinalização da PI3K promove um aumento da síntese proteica, necessária para a célula progredir no ciclo celular ou, ainda, para a pró-sobrevivência destas células, o que pode justificar as características de radiorresistência apresentadas por este tipo de tumor (143). Apesar das diferenças genéticas apresentadas, os GBM primários e secundários não são distintos morfologicamente e apresentam resposta semelhante à terapia convencional, mas provavelmente respondam de forma diferente a terapias focadas em alvos moleculares (4).

Estudos prévios em nosso grupo de pesquisa demonstraram que diferentes sistemas medeiam importantes ações em modelos de glioma *in vivo* e *in vitro* (144-147). Substâncias endógenas como a BK e o Ca^{2+} medeiam respostas que podem interagir através de múltiplos mecanismos de sinalização, que desencadeiam importantes ações nas células tumorais, como proliferação, migração e invasão. Cabe salientar a elevada expressão dos receptores de BK encontrados nos gliomas, o que evidencia que os receptores de cininas podem apresentar funções importantes no crescimento do tumor e na migração das células tumorais. Neste sentido, nosso grupo de pesquisa demonstrou o aumento da proliferação e viabilidade em linhagens celulares de câncer de bexiga, além da elevada expressão dos B_1R em biópsias de pacientes com este tipo de tumor (148).

Diante do exposto, nossa hipótese baseou-se no efeito da sinalização desencadeada pela BK e da sinalização Ca^{2+}-dependente na regulação do crescimento e desenvolvimento dos gliomas. Neste sentido, este trabalho explorou o efeito dos receptores B_1R e B_2R de cininas e da sinalização de Ca^{2+} via CCVD tipo-P/Q e -N em modelo de glioma *in vitro* e *in vivo*.

Deste modo, os Capitulos II e III do presente estudo evidenciaram a importância dos receptores B_1 e B_2 de cininas no desenvolvimento dos gliomas *in vitro* e *in vivo*. Primeiramente, avaliamos o comportamento das linhagens celulares de GBM humano (grau IV) frente à exposição a diferentes ligantes dos B_1R e B_2R. Foi possível observar que a ativação destes receptores através do uso dos agonistas des-arg^9-BK e BK, ocasionou um aumento importante na proliferação de linhagens celulares de GBM, através da ativação das vias ERK1/2 e PI3K/Akt, envolvidas na proliferação e migração das células tumorais (69, 149, 150). Ao contrário, quando realizado o antagonismo seletivo de B_1 (SSR240612) e B_2 (HOE-140), ocorreu um notável decréscimo na proliferação e viabilidade destas células, além de intensa morte celular desencadeada

pelo bloqueio destes receptores. De interesse, a ativação de componentes da família MAPK e a presença de citocinas inflamatórias parecem estar envolvidas na regulação e superexpressão do gene para B_1R. Com relação ao B_2R, a BK promove a ativação de eNOS através da fosforilação pela AKT e elevação de Ca^{2+} intracelular, promovendo um aumento transitório de NO (151). Em um ambiente inflamatório, os metabólitos des-Arg agem em B_1R, ocasionando a fosforilação e ativação de iNOS, através da via da ERK/MAPK, o que leva a um aumento prolongado de NO (52, 152). Além disto, a ativação da via PI3K/Akt também pode ser estimulada pela ação da BK, contribuindo para a proliferação e migração em diferentes tipos de celulares (69, 149, 150).

Para explorar e compreender melhor o papel dos receptores de cininas nos tumores primários do SNC utilizou-se a técnica de implante das células GL261 de glioma (grau IV) *in vivo.* Nossos principais resultando destacaram de forma interessante que tanto o *knockout* genético como o antagonismo farmacológico combinado dos receptores B_1 e B_2 (SSR240612 + HOE-140) diminuiu o volume e a progressão tumoral dos gliomas analisados. Além disso, a diminuição do índice mitótico nos tumores onde os B_1R e B_2R foram bloqueados ou deletados reforça a nossa hipótese e é um indicativo da menor taxa de proliferação e agressividade do GBM, o que representa um bom prognóstico na prática clínica (4). Alguns estudos clínicos têm focado na utilização de ligantes peptídicos e não peptídicos dos B_1R e B_2R para o tratamento de diferentes tipos de tumor. Uma revisão recente publicada por Costa e colaboradores (86) enfatiza o perfil favorável dos antagonistas de cininas em alguns tipos de câncer, como o câncer de pulmão, próstata e o câncer de mama. Com relação aos tumores primários do SNC, um estudo de fase II utilizando o agonista B_2R RMP-7 associado à carboplatina não apresentou o sucesso clínico esperado em crianças com glioma (87, 88). Cabe ressaltar, que o antagonista do receptor B_2, HOE-140, utilizado neste estudo foi recentemente aprovado para o tratamento do angioedema, o que o torna um composto interessante para aplicação em outras patologias, entre elas o câncer (153, 154).

Notavelmente, a deleção do receptor B_1R desencadeou um incontrolável crescimento tumoral nos animais *knockout* para este receptor, como observado neste estudo. Este evento pode ser justificado pelo mecanismo de compensação que aumenta a expressão de B_2R nestes animais (89, 155, 156). Por outro lado, os animais com deleção genética de B_2R não tiveram o desenvolvimento tumoral alterado, destacando que apenas a presença de B_1R não é suficiente para estimular a progressão tumoral.

Frente a este achado, podemos inferir a maior relevância do receptor B_2R no crescimento e progressão do GBM.

Através do uso de ferramentas genéticas e farmacológicas, nossos resultados demonstram o claro envolvimento dos receptores de cininas nos gliomas malignos. Desta maneira, podemos propor que o bloqueio farmacológico combinado de antagonistas seletivos para os receptores B_1 e B_2 pode representar uma alternativa viável no tratamento do GBM.

No Capítulo IV, buscamos compreender o envolvimento dos CCVD tipo-P/Q e -N na fisiopatologia dos gliomas. Para a realização do estudo foram utilizadas frações da toxina proveniente da aranha *P. nigriventer* (PhTx3-3 bloqueadora de canais do tipo-P/Q; Phα1β bloqueadora de canais do tipo-N) e ω-conotoxinas obtidas do *C. magus* (MVIIC bloqueadora de canais do tipo-P/Q; MVIIA bloqueadora de canais do tipo-N).

Os experimentos *in vitro* demonstraram que o bloqueio dos canais de Ca^{2+} subtipo P/Q pela toxina PhTx3-3 diminui a proliferação e a viabilidade das linhagens celulares de GBM humano, enquanto o bloqueio destes canais pela ω-conotoxina MVIIC não alterou estes parâmetros nas linhagens celulares analisadas. Além disso, o bloqueio dos canais tipo-N também foi capaz de reduzir a proliferação e viabilidade das células de GBM tanto pelo uso da fração Phα1β provenientes da *P. nigriventer* como pelo uso da ω-conotoxina MVIIA, extraída do *C. magus*. Ademais, o bloqueio dos CCVD tipo-P/Q e -N ocasionou a morte celular nas linhagens testadas, com intensa característica de morte celular por apoptose. Desta maneira, as análises realizadas *in vitro,* demonstraram o potencial envolvimento dos CCVD tipo-P/Q e -N no desenvolvimento dos tumores cerebrais de origem glial. Dentre as patologias relacionadas com a regulação anormal da homeostase de Ca^{2+}, podem-se destacar os diferentes tipos de câncer (108, 109). Para os canais neurais da classe *Cav2*, particularmente os do tipo-P/Q e -N, o principal mecanismo de modulação ocorre via ativação de GPCRs. A sinalização de Ca^{2+} regula os mecanismos de proliferação celular através da modulação da atividade de diversos fatores de transcrição reguladores do ciclo celular, como as ciclinas, as quinases dependentes de ciclina e proteínas associadas (157, 158). Além disso, um número considerável de evidências indica que mudanças em vias de sinalização que envolvem o Ca^{2+} podem contribuir em importantes eventos durante a progressão tumoral, tais como proliferação, migração, invasão e metástase (109, 112, 159). De interesse, recentemente foi descrito que a inibição dos CCVD do

subtipo T também foi capaz de inibir a proliferação e migração celular em modelo utilizando células de glioma humano (118).

A segunda etapa do Capítulo IV envolveu os ensaios utilizando a técnica de implante das células GL261 de glioma *in vivo*, a fim de aprimorar a compreensão acerca do envolvimento dos CCVD tipo-P/Q e -N no crescimento e progressão dos gliomas malignos. Nossos resultados demonstraram que ambas as toxinas Phα1β e MVIIA, potentes bloqueadoras dos canais do tipo-N, foram efetivas em diminuir o crescimento e a progressão tumoral nos animais tratados. Com relação a Phα1β, duas vias de administração, intracerebroventricular e intratecal, foram utilizadas para o tratamento do glioma e ambas as vias foram eficazes na redução do tumor cerebral. Cabe ressaltar que a toxina Phα1β já foi patenteada e sua forma recombinante, além da possível administração pela via intratecal, a torna um composto atrativo na prática clínica. Ainda, a versão sintética do peptídeo MVIIA foi aprovado pelo FDA para o tratamento da dor em pacientes que necessitam de analgesia intratecal e é atualmente comercializada como Prialt®.

Além disso, nossos resultados destacaram o envolvimento de células gliais, astrócitos e micróglia, na progressão tumoral. Foi possível observar o aumento da presença e da ativação destes dois tipos celulares na região peritumoral dos tumores tratados com Phα1β (ativação de astrócitos e micróglia) e com MVIIA (ativação somente da micróglia), que tiveram menor crescimento tumoral. Cabe ressaltar a intensa atividade, tanto dos astrócitos como da micróglia na região peritumoral, mais vascularizada e proliferativa, e responsável pelo comportamento agressivo e infiltrativo do tumor. Neste sentido, podemos destacar o possível envolvimento do sistema imune no combate ao tumor cerebral.

Com relação aos diferentes tipos de câncer, o CCVD do tipo-T tem sido apontado como o principal alvo para conter a proliferação e migração das células tumorais (115, 116, 160). No que se refere aos gliomas, alguns grupos têm associado o bloqueio dos canais do tipo-T com um declínio da proliferação e migração de diferentes linhagens de GBM humano (118, 161). Por fim, de forma interessante, o bloqueio destes canais foi relacionado ao aumento da potência e da citotoxicidade da TMZ em modelo de glioma *in vitro* e *in vivo* (162). Embora o interesse pelos diferentes tipos de CCVD seja crescente, ainda não existem evidencias consistentes sobre o envolvimento dos canais de Ca^{2+} tipo-P/Q e -N em tumores cerebrais. Desta forma, os nossos

resultados podem ser considerados pioneiros no que diz respeito a modulação destes canais no desenvolvimento e progressão dos gliomas malignos.

Através dos dados obtidos neste estudo, podemos concluir que (i) o estímulo dos receptores B_1 e B_2 das cininas promove a proliferação em linhagens celulares de glioblastoma humano, através da ativação das vias ERK1/2 e PI3K/Akt; (ii) em modelo de glioma *in vivo* o bloqueio farmacológico combinado dos antagonistas seletivos para os receptores B_1 e B_2 de cininas inibiu de forma importante o crescimento tumoral do glioblastoma; (iii) o bloqueio dos CCVD do tipo-P/Q e -N modulou a proliferação e promoveu a morte celular por apoptose em ensaios utilizando células de glioblastoma humano *in vitro*; (iv) enquanto somente o bloqueio dos canais do tipo-N foram capazes de inibir o crescimento e a progressão tumoral em modelo de glioma *in vivo*.

Levando-se em consideração que o tratamento dos gliomas malignos permaneceu praticamente inalterado nas últimas décadas, a elucidação de novos mecanismos moleculares em torno da patologia dos gliomas representa um passo interessante na descoberta de novos alvos farmacológicos, a fim de melhorar o prognóstico e aumentar a sobrevida destes pacientes. Frente ao exposto neste estudo, tanto os B_1R e B_2R de cininas, como os CCVD tipo-P/Q e –N, surgem como potenciais alvos terapêuticos no manejo dos tumores cerebrais e podem ser apontados como novas perspectivas no tratamento e na progressão dos gliomas malignos.

5. PERSPECTIVAS

1) Explorar a possível interação entre os receptores B_1R e B_2R das cininas e os CCVD Tipo-P/Q e -N sobre o desenvolvimento e a progressão dos gliomas malignos.

2) Analisar a presença dos receptores B_1R e B_2R de cininas e dos CCVD tipo-P/Q e -N em tecidos tumorais de diferentes graus de glioma (I-IV). Este projeto já possui aprovação pelo Comitê de Ética em Pesquisa da Irmandade da Santa Casa de Misericórdia de Porto Alegre (CEP/163.360) e as amostras de glioma já foram coletadas e armazenadas para as análises de imunohistoquímica e RT-qPCR.

3) Avaliar a interação dos receptores B_1R e B_2R de cininas e do receptor purinérgico P2X7 no desenvolvimento de gliomas *in vitro* e *in vivo*.

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ANEXOS

ANEXO A - Carta de Aprovação da Comissão de Ética para o Uso de Animais

ANEXO B - Parecer do Orientador no exterior pelo período do Doutorado Sanduíche (Université de Montreal, Canadá)

ANEXO C - Graus obtidos no período de Doutorado Sanduiche

ANEXO D - Artigo publicado "Polymethylmethacrylate dermal fillers: evaluation of the systemic toxicity in rats", Int. J. Oral Maxillofac. Surg., 2013.

ANEXO E - Artigo publicado "Efficacy and gastrointestinal tolerability of ML3403, a selective inhibitor of p38 MAP kinase and CBS-3595, a dual inhibitor of p38 MAP kinase and phosphodiesterase 4 in CFA-induced arthritis in rats", Rheumatology, 2013.

ANEXO F - Artigo publicado "Effects of the compounds resveratrol, rutin, quercetin, and quercetin nanoemulsion on oxaliplatin-induced hepatotoxicity and neurotoxicity in mice", Naunyn-Schmiedeberg's Arch Pharmacol, 2014.

ANEXO G - Artigo publicado "Protective Effects of Resveratrol on Hepatotoxicity Induced by Isoniazid and Rifampicin via SIRT1 Modulation", J. Nat. Prod, 2014.

Anexo A

Carta de Aprovação da Comissão de Ética para o Uso de Animais

CEUS/PUCRS/2011

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Ofício 154/11 – CEUA

Porto Alegre, 01 de dezembro de 2011.

Senhora Pesquisadora:

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou seu protocolo de pesquisa, registro CEUA 11/00258 intitulado: **"Interação entre os receptores para cininas e o receptor purinérgico P2X7 no desenvolvimento de gliomas".**

Sua investigação está autorizada a partir da presente data.

Atenciosamente,

Profa. Dra. Anamaria Gonçalves Feijó
Coordenadora da CEUA/PUCRS

Ilma. Sra.
Fernanda Morrone
FFARM
Nesta Universidade

Campus Central
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Anexo B

Parecer do Orientador no exterior pelo período do Doutorado Sanduíche

Université de Montreal, QC, Canadá

Université de Montréal

Faculté de médecine
Département de physiologie

7 November 2013

Ref: Evaluation of Ms Natália Nicoletti

Sir/Madam,

This is to confirm that Ms Natália Fontana Nicoletti from Pontifícia Universidade do Rio Grande do Sul spent a period of 6 months (6 April to 4 October 2013) in my laboratory at the Université de Montréal in the frame of a Sandwich-PhD program (3-995-1-9) between Canada and Brazil. During that period, she was registered in a Research Training course of 9 credits in Physiology (PSL6003). She was also compelled to take three one-day courses: *Health and Security at work, training in animal experimentation and training with radioactive elements.*

During her stay at UdeM, Natália worked on a project entitled: *"Involvement of kinin B1 and B2 receptors in tumor proliferation using a murine glioma model in vivo"*. She performed experiments using Western blotting and receptor autoradiography to assess the expression and distribution of both kinin receptors in the brain of mice implanted with tumor cells. She studied the impact of *in vivo* treatments with selective agonists and antagonists of B1 and B2 receptors on tumor cell proliferation and the expression of both receptors. Thanks to her hard work she obtained compelling data, yet other analyses are still in progress.

Natália was highly dedicated to her project and was a gift student. She has demonstrated a high level of autonomy and ability to work with other students in my team. She learned new techniques that she could use in the frame of her project in Brazil.

Natália had a great facility to integrate our culture and learned quickly basic French although all scientific exchanges were in English. This will certainly help her in her future research endeavour.

I remain available to pursue this successful collaboration with her host laboratory and to welcome her again in our laboratory to complete some experiments if necessary.

With kind regards,

Réjean Couture, PhD
Professor
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Faculty of Medicine
Université de Montréal
Montréal, Qc, Canada
H3C 3J7
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Anexo C

Graus obtidos no período de Doutorado Sanduiche

Université de Montreal, QC, Canadá

RELEVÉ DE NOTES

Université de Montréal

Natalia Fontana Nicoletti

408, La Rue Pedro Chaves
Caxias Do Sul 95090-230
Brésil

Date d'émission: 2014-03-06
Matricule: 1051372
CPER QC: FONN03628407

Dossier Cycles supérieurs

Été 2013

Programme d'études: 399519 - Programmes d'échanges - 3e c. (Échange)

Cours	Description	Crédits:	Suivis	Obtenus	Note	Points	Moy.gr.
PSL 6003	Stage de rech. en physio 3 3-995-1-9 Cours au choix		9.000	9.000	A+	38.700	

			Crédits:	Suivis	Obtenus	Moy.	Points
Moyenne générale trimestrielle	4.300	Total du trimestre		9.000	9.000	9.000	38.700

Automne 2013

Programme d'études: 399519 - Programmes d'échanges - 3e c. (Échange)

Cours	Description	Crédits:	Suivis	Obtenus	Note	Points	Moy.gr.
PSL 6001	Stage de rech. en physio 1 3-995-1-9 Cours au choix		3.000	0.000	REM	0.000	

			Crédits:	Suivis	Obtenus	Moy.	Points
Moyenne générale trimestrielle	0.000	Total du trimestre		3.000	0.000	0.000	0.000

Fin des Cycles supérieurs

MARIE-CLAUDE BINETTE
REGISTRAIRE

Pour être officiel, le relevé de notes doit porter le sceau de l'Université et la signature du Registraire. VOIR NOTES AU VERSO

Anexo D

Polymethylmethacrylate dermal fillers: evaluation of the systemic toxicity in rats.

Medeiros CC, Borghetti RL, Nicoletti NF, da Silva VD, Cherubini K, Salum FG, de Figueiredo, M.A.

Artigo publicado no *International Journal of Oral and Maxillofacial Surgery*, 2013.

Int. J. Oral Maxillofac. Surg. 2014; 43: 62–67
http://dx.doi.org/10.1016/j.ijom.2013.06.009, available online at http://www.sciencedirect.com

International Journal of Oral & Maxillofacial Surgery

Research Paper
Cosmetic Surgery

Polymethylmethacrylate dermal fillers: evaluation of the systemic toxicity in rats

C. C. G. Medeiros[1], R. L. Borghetti[1], N. Nicoletti[2], V. D. da Silva[3], K. Cherubini[1], F. G. Salum[1], M. A. Z. de Figueiredo[1]

[1]Division of Oral Medicine, PUCRS, Pontifical Catholic University of Rio Grande do Sul, Porto Alegre, RS, Brazil; [2]Postgraduate Program of Cellular and Molecular Biology, PUCRS, Pontifical Catholic University of Rio Grande do Sul, Porto Alegre, RS, Brazil; [3]Division of Pathology, PUCRS, Pontifical Catholic University of Rio Grande do Sul, Porto Alegre, RS, Brazil

C. C. G. Medeiros, R. L. Borghetti, N. Nicoletti, V. D. da Silva, K. Cherubini, F. G. Salum, M. A. Z. de Figueiredo: Polymethylmethacrylate dermal fillers: evaluation of the systemic toxicity in rats. Int. J. Oral Maxillofac. Surg. 2014; 43: 62–67.

Abstract. This study evaluated local and systemic reactions after an intravascular injection of polymethylmethacrylate (PMMA) at two concentrations in a murine model. Thirty rats were divided equally into three groups: 2% PMMA, 30% PMMA, and a control group (normal saline only injection). The filler was injected into the ranine vein. The rats were sedated at 7 and 90 days and a clinical evaluation performed. After euthanasia, the right lung, liver, and right kidney were removed, weighed, and microscopically analyzed. The submandibular lymph nodes and tongue were removed and examined microscopically. Serum was subjected to liver and kidney function tests. No groups showed clinical alterations. Microspheres were not observed at any distant organ. Two samples from the 2% PMMA group showed a local inflammatory response at day 7 and another two samples from the 30% PMMA group at day 90. The group injected with 30% PMMA presented higher levels of alanine aminotransferase ($P = 0.047$) after 90 days when compared with the other groups. The data obtained in this study demonstrate that intravascular injections of PMMA fillers show potential health risks such as chronic inflammation at the implantation site.

Key words: oral medicine; polymethylmethacrylate; adverse effects; toxicology.

Accepted for publication 17 June 2013
Available online 18 July 2013

Anexo E

Efficacy and gastrointestinal tolerability of ML3403, a selective inhibitor of p38 MAP kinase and CBS-3595, a dual inhibitor of p38 MAP kinase and phosphodiesterase 4 in CFA-induced arthritis in rats.

Koch DA, Silva RB, de Souza AH, Leite CE, Nicoletti NF, Campos MM, Laufer S, Morrone FB.

Artigo publicado no periódico *Rheumatology*, 2013.

RHEUMATOLOGY

Rheumatology 2014;53:425–432
doi:10.1093/rheumatology/ket369
Advance Access publication 15 November 2013

Original article

Efficacy and gastrointestinal tolerability of ML3403, a selective inhibitor of p38 MAP kinase and CBS-3595, a dual inhibitor of p38 MAP kinase and phosphodiesterase 4 in CFA-induced arthritis in rats

Diana A. Koch[1,*], Rodrigo B. M. Silva[2,*], Alessandra H. de Souza[3], Carlos E. Leite[2,4], Natália F. Nicoletti[5], Maria M. Campos[2,6], Stefan Laufer[1] and Fernanda B. Morrone[2,7]

Abstract

Objective. Mitogen-activated protein kinase (MAPK) p38 inhibitors have entered the clinical phase, although many of them have failed due to high toxicity and lack of efficacy. In the present study we compared the effects of the selective p38 inhibitor ML3403 and the dual p38–PDE4 inhibitor CBS-3595, on inflammatory and nociceptive parameters in a model of polyarthritis in rats.

Methods. Male Wistar rats (180–200 g) were used for the complete Freund's adjuvant (CFA)-induced arthritis model and they were evaluated at 14–21 days. We also analysed the effects of these pharmacological tools on liver and gastrointestinal toxicity and on cytokine levels.

Results. Repeated CBS-3595 (3 mg/kg) or ML3403 (10 mg/kg) administration produced significant anti-inflammatory actions in the chronic arthritis model induced by CFA. CBS-3595 and ML3403 treatment also markedly reduced the production of the proinflammatory cytokine IL-6 in the paw tissue, whereas it widely increased the levels of the anti-inflammatory cytokine IL-10. Moreover, CBS-3595 produced partial anti-allodynic effects in the CFA model at 4 and 8 days after treatment. Notably, ML3403 and CBS-3595 did not show marked signs of hepatoxicity, as supported by unaltered histological observations in the liver sections. Finally, both compounds were safe in the gastrointestinal tract, according to evaluation of intestinal biopsies.

Conclusion. CBS-3595 displayed a superior profile regarding its anti-inflammatory effects. Thus p38 MAPK/PDE4 blocking might well constitute a relevant strategy for the treatment of RA.

Key words: p38 inhibitors, PDE4 modulation, ML3403, CBS-3595, inflammation, arthritis model.

Anexo F

Effects of the compounds resveratrol, rutin, quercetin, and quercetin nanoemulsion on oxaliplatin-induced hepatotoxicity and neurotoxicity in mice.

Schwingel TE, Klein CP, Nicoletti NF, Dora CL, Hadrich G, Bica CG, Lopes TG, da Silva VD, Morrone FB.

Artigo publicado no periódico *Naunyn-Schmiedeberg's Archives of Pharmacology,* 2014.

Naunyn-Schmiedeberg's Arch Pharmacol
DOI 10.1007/s00210-014-0994-0

ORIGINAL ARTICLE

Effects of the compounds resveratrol, rutin, quercetin, and quercetin nanoemulsion on oxaliplatin-induced hepatotoxicity and neurotoxicity in mice

Tania E. Schwingel · Caroline P. Klein · Natalia F. Nicoletti · Cristiana L. Dora · Gabriela Hadrich · Cláudia Bicca · Tiago G. Lopes · Vinicius Duval da Silva · Fernanda B. Morrone

Received: 18 September 2013 / Accepted: 16 May 2014

Abstract Oxaliplatin (OXA) is a platinum compound widely used in the treatment of some solid tumors, especially colorectal cancer. Despite its usefulness, oxaliplatin-associated neurotoxicity represents the main dose-limiting factor of this drug, and until now, there is no suitable treatment. Chemotherapy with oxaliplatin also increases the rate of developing hepatic damages with inflammatory activity, termed chemotherapy-associated steatohepatitis (CASH). In the present study, we aimed to compare the effects of a series of antioxidant compounds on simultaneous development of oxaliplatin-induced hepato- and neurotoxicity in mice. Mice BALB/c were treated with oxaliplatin for 6 weeks, 10 mg/kg, intraperitoneally, resulting in mechanical allodynia and hepatic steatosis. We administered the following antioxidant compounds—rutin (RT) (20 mg/kg), resveratrol (RVS) (100 mg/kg), quercetin (QT) (20 mg/kg), and quercetin nanoemulsion (NQT) (20 mg/kg)—daily by gavage to BALB/c, and *N*-acetylcysteine (NAC) was used as positive control. Treatments with RSV, RUT, or NQT were able to prevent mechanical allodynia when compared to the OXA group, and this effect was associated with decreased c-Fos immunopositivity in the lumbar spinal cord. Regarding the effects on steatohepatitis, RVS, QT, and NQT almost completely reversed the mean liver weight increase induced by OXA. In accordance with these previous data, histological evaluation indicated attenuation of all features of hepatic steatosis evaluated in RSV, RUT, QT, and NQT groups. These compounds were able to reduce the immunopositivity for the apoptosis marker caspase-3. On the other hand, only QT and NQT treatments were able to reduce neutrophil migration measured by myeloperoxidase (MPO) activity. These results suggest that the compounds tested, RSV, RU, QT, and NQT, would be useful for the clinical treatment of neuro- and hepatoxicity induced by oxaliplatin.

Tania E. Schwingel and Caroline P. Klein have contributed equally to this paper.

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Anexo G

Protective Effects of Resveratrol on Hepatotoxicity Induced by Isoniazid and Rifampicin via SIRT1 Modulation.

Nicoletti NF, Rodrigues-Junior V, Santos AA Jr, Leite CE, Dias AC, Batista EL Jr, Basso LA, Campos MM, Santos DS, Souto AA.

Artigo publicado no *Journal of Natural Products*, 2014.

JOURNAL OF NATURAL PRODUCTS

Article

pubs.acs.org/jnp

Protective Effects of Resveratrol on Hepatotoxicity Induced by Isoniazid and Rifampicin via SIRT1 Modulation

Natália F. Nicoletti,[†,‡] Valnês Rodrigues-Junior,[†,§] André A. Santos, Jr.,[†,‡] Carlos E. Leite,[∥] Ana C. O. Dias,[†] Eraldo L. Batista, Jr.,[⊥] Luiz A. Basso,[†,‡,§] Maria M. Campos,[†,§,∥,⊥] Diógenes S. Santos,[†,‡] and André A. Souto*[,‡,#]

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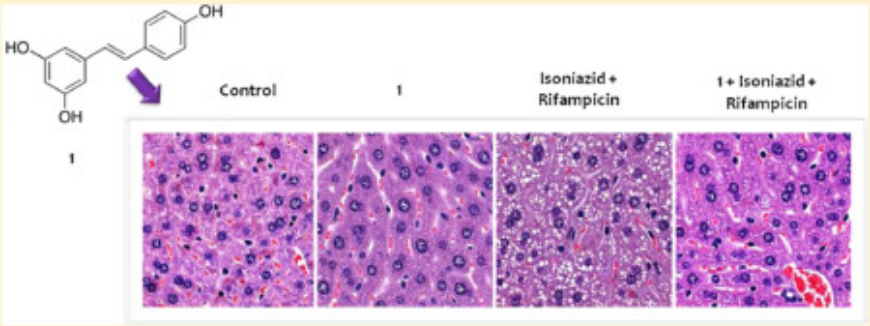

ABSTRACT: Acute liver injury was induced in male BALB/c mice by coadministering isoniazid and rifampicin. In this work, the effects of resveratrol (**1**) were investigated in the hepatotoxicity caused by isoniazid−rifampicin in mice. Compound **1** was administered 30 min prior to isoniazid−rifampicin. Serum biochemical tests, liver histopathological examination, oxidative stress, myeloperoxidase activity, cytokine production (TNF-α, IL-12p70, and IL-10), and mRNA expression of SIRT1−7 and PPAR-γ/PGC1-α were evaluated. The administration of **1** significantly decreased aspartate transaminase and alanine aminotransferase levels, myeloperoxidase activity, and cytokine levels. Furthermore, **1** reverted the decrease of catalase and glutathione activities and ameliorated the histopathological alterations associated with antituberculosis drugs. Modulation of SIRT1 and PPAR-γ/PGC1-α expression is likely involved in the protective effects of **1**. The results presented herein show that **1** was able to largely prevent the hepatotoxicity induced by isoniazid and rifampicin in mice, mainly by modulating SIRT1 mRNA expression.