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**PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR**

# ALVOS MOLECULARES EM MEDULOBLASTOMA:

# UM ESTUDO *IN VITRO*

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## LISTA DE ABREVIATURAS

**BB:** Bombesin (Bombesina)

**BDNF:** Brain-derived Neurotrophic Factor (Fator Neurotrófico Derivado de Cérebro)

**BRS3**: Bombesin-like Receptor 3 (Receptor preferencial para o Subtipo 3 de Bombesina)

**cAMP:** Cyclic Adenosine Monophosphate (Adenosina Monofosfato Cíclica)

**COX2**: Cyclooxygenase 2 (Cicloxigenase 2)

**DAG**: Diacylglycerol (diacilglicerol)

**ERK**: Extracellular Signal-Regulated Kinases (Cinase Reguladora de Sinal Extracelular)

**FAK**: Focal-Adhesion Kinase (Cinase de Adesão Focal)

**GRP**: Gastrin-releasing Peptide (Peptídeo Liberador de Gastrina)

**GRPR**: Gastrin Releasing Peptide Receptor (Receptor preferencial ao Peptídeo Liberador de Gastrina)

**HPSE:** Neurotrophin-Regulated Heparanase (Heparanase Regulada por Neurotrofina),

**IP3**: Inositol 1,4,5-trisphosphate (Inositol 1,4,5-trifosfato)

**MAPK**: Mitogen-Activated Protein (MAP) Kinases (Proteína Cinase Mitógeno-Ativada)

**NFL:** Neurofilament Light (Proteína de Neurofilamento)

**NGF:** Nerve Growth Factor (Fator de Crescimento Neuronal)

**NMB**: Neuromedin B (Neuromedina B)

**NMBR**: Neuromedin B Receptor (Receptor Preferencial à Neuromedina B)

**NMC**: Neuromedin C (Neuromedina C)

**NT-1:** Neurotrofina 1

**NT-3:** Neurotrofina 3

**NT-4:** Neurotrofina 4

**PACAP:** Pituitary Adenylyl Cyclase-Activating Peptide (Peptídeo Ativador da Pituitária Adenilato Ciclase

**PDE4:** Phosphodiesterase 4 (Fosfodiesterase 4)

**PGE2**: Prostaglandin E2 (Prostaglandina E2)

**PI3K**: Phosphoinositide 3-kinases (Fosfatidilinositol 3 cinase)

**PIP2**: Phosphatidylinositol (4,5)-bisphosphate (Fosfatidilinositol (4,5)-bifosfato)

**PKA**: Protein Kinase A (Proteína Cinase A)

**PKC**: Protein Kinase C (Proteína Cinase C)

**PNET:** Primitive Neuroectodermic Tumor (Tumor Neuroectodérmico Primitivo)

**SHH:** Sonic Hedgehog

**TrkA:** Tropomyosin Related Kinase A (Cinase Relacionada a Tropomiosina (A))

**TrkB:** Tropomyosin Related Kinase B (Cinase Relacionada a Tropomiosina (B))

**TrkC:** Tropomyosin Related Kinase C (Cinase Relacionada a Tropomiosina (C))

## RESUMO

Meduloblastoma é o tumor intracranial mais comum em crianças, provavelmente derivado de células precursoras da camada granular externa do cerebelo durante seu desenvolvimento. O tratamento padrão consiste em cirurgia, radioterapia e quimioterapia, que produzem graves sequelas nos pacientes e garantem uma sobrevida baixa, o que demonstra a necessidade de novas alternativas terapêuticas para a doença. Evidências demonstram que o receptor do peptídeo liberador de gastrina (GRPR) está superexpresso em diversos tumores humanos, assim como seu agonista (GRP) pode atuar como um fator de crescimento autócrino em tumores cerebrais. No presente estudo, avaliamos a expressão de GRPR e o efeito de seus agonistas, bombesina (BB) e GRP, além do antagonista RC-3095, sobre a viabilidade celular de linhagens de meduloblastoma humano DAOY, D283 e ONS76. Mostramos que meduloblastomas, apesar de expressarem GRPR, não têm sua viabilidade celular afetada por agonistas e antagonista desse receptor. Uma vez que há evidências de que BDNF (fator neurotrófico derivado de cérebro) esteja relacionado à diferenciação celular em meduloblastomas, também avaliamos o efeito de BDNF sobre a viabilidade celular das linhagens de meduloblastoma humano. As linhagens DAOY e D283 tiveram sua viabilidade celular reduzida pela presença de BDNF. Uma vez que a via da PKA tem sido implicada na iniciação e progressão de vários tumores, também avaliamos o efeito de rolipram, um inibidor de fosfodiesterase tipo IV, sobre a viabilidade celular das linhagens de meduloblastoma humano, sendo que rolipram reduziu a viabilidade celular de todas as linhagens estudadas. Os receptores de BDNF e a via da PKA podem, portanto, ser alvos moleculares promissores para o desenvolvimento de novas terapias para meduloblastomas.



**Palavras-chave**

Meduloblastoma • Bombesina • Peptídeo Liberador de Gastrina • Receptor do Peptídeo Liberador de Gastrina • Sinalização cAMP • Tumores Cerebrais • BDNF

## ABSTRACT

Medulloblastoma is the most common intracranial tumor in children and is believed to arise from the precursor cells of the external granule layer of the developing cerebellum. The standard treatment, consisting of surgery, craniospinal radiotherapy and chemotherapy, produces severe sequelae in patients and provides a poor overall survival, indicating the need for new therapeutic alternatives for treating this disease. Evidences show that the gastrin releasing peptide receptor (GRPR) is overexpressed in various human tumors and its agonist (GRP) can act as an autocrine growth factor in brain tumors. In the present study, we evaluated GRPR expression, as well as the effect of its agonists, bombesin (BB) and GRP, and its antagonist RC-3095, over cell viability of the human medulloblastoma cell lines DAOY, D283 and ONS76. We found that medulloblastomas, in spite of expressing GRPR, do not have its viability affected by the presence of agonists and antagonist of this receptor. Since there are evidences that BDNF (brain-derived neurotrophic factor) is related to cell differentiation in medulloblastomas, we also evaluated the effect of BDNF over the viability of medulloblastoma cell lines. The viability of the cell lines DAOY and D283 was reduced by the presence of BDNF. Since the PKA pathway has been implicated in the initiation and progression of various tumors, we also evaluated the effect of rolipram, a phosphodiesterase IV inhibitor, over the viability of the same medulloblastoma cell lines and we found that rolipram inhibited the viability of all the cell lines studied. BDNF receptors, as well as the PKA pathway, may be therefore promising molecular targets for the development of new therapies for treating medulloblastomas.



**Key words**

Medulloblastoma • Bombesin • Gastrin Releasing Peptide • Gastrin Releasing Peptide Receptor • cAMP Signaling • Brain Tumors • BDNF

**SUMÁRIO**

# 1 INTRODUÇÃO

## 1.1 Meduloblastomas

Os tumores cerebrais são a terceira causa de morte relacionada a cânceres em adultos e a segunda em crianças (PARKER *et al.,* 1997). O meduloblastoma é o tipo mais comum de tumor intracranial em crianças, representando 16% dos tumores cerebrais em pacientes de 0 a 4 anos, sendo que a incidência decai com a idade para cerca de 2% em adultos (CBTRUS, 2007-2008). O meduloblastoma é um tumor altamente metastático, com até 30% dos pacientes apresentando doença disseminada ao diagnóstico (CRAWFORD *et al.*, 2007). A sobrevida após 5 anos dos pacientes tratados com radioterapia ainda é baixa, com taxas de 40% ou menos em pacientes afetados pela doença de alto risco (MACDONALD et al., 2003; TAYLOR et al., 2003; KÜHL et al., 1998).

O meduloblastoma é um tumor embrionário, classificado como um tumor neuroectodérmico primitivo (PNET). O meduloblastoma surge, por definição, da fossa posterior, originando de células não-diferenciadas, precursoras de neurônios da camada granulosa externa do cerebelo em desenvolvimento (OLIVER et al., 2005; UEBA et al., 2008; SCHÜLLER et al., 2008). Estudos recentes têm mostrado que os meduloblastomas também podem ser iniciados em progenitores comprometidos com a linhagem neuronal e também em células-tronco (YANG et al., 2008). Apesar de aparentemente bem delimitado, o tumor é altamente infiltrativo e apresenta forte tendência à disseminação para o neuro-eixo. Os meduloblastomas são divididos pela Organização Mundial de Saúde em meduloblastoma desmoplásico/nodular, meduloblastoma com extensa nodularidade, anaplásico e meduloblastoma de células grandes (LOUIS et al., 2007).

A etiologia dos meduloblastomas na população pediátrica ainda não é clara e permanece difícil de ser elucidada, uma vez que fatores ambientais como fumo, dieta e outras exposições não parecem predispor ao desenvolvimento da doença (WRENSCH et al., 2002). Embora se saiba que síndromes como as de Li-Fraumeni,

Gorlin, Turcot A e Rubenstein-Taybi[1] estejam associadas com a formação de meduloblastomas, apenas uma pequena parcela dos tumores cerebrais são causados por defeitos genéticos hereditários, assim como por irradiação ou supressão imune (DE BONT et al., 2008).

Uma vez que as alterações genéticas mencionadas acima não representam todas as possibilidades de formação de meduloblastomas existentes, outras vias de sinalização celular têm sido investigadas, a fim de compreender a patogênese desses tumores.

### *1.1.1 Vias de Sinalização Celular Envolvidas com Meduloblastomas*

Já está claro que a desregulação de vias de sinalização essenciais para o desenvolvimento cerebral, como as vias Sonic Hedgehog (SHH), Wnt e Notch, desempenham um papel importante na patogênese dos meduloblastomas (CARLOTTI *et al.,* 2008). A via de sinalização SHH atua na promoção de crescimento durante o desenvolvimento cerebelar normal, e 10% a 30% dos pacientes diagnosticados com meduloblastoma têm mutações que resultam em ativação da via SHH (ZURAWEL et al., 2000; DONG et al., 2000; POMEROY et al., 2002; RAFFEL et al., 1997). Pacientes afetados pela síndrome de Gorlin, que possuem mutações que inativam o receptor Patched 1 (PTCH1), que regula negativamente a via SHH, são propensos a desenvolver meduloblastomas (TAYLOR et al., 2002). Além disso, tumores de camundongos mutados em PTCH +/-, que desenvolvem espontaneamente meduloblastomas, mostram uma expressão aumentada de genes envolvidos na ativação de SHH e da via Wnt (DAKOBU et al., 2006). Inibidores de SHH, capazes de inibir Smoothened (SMO), o receptor transmembrana que responde a PTCH1, têm sido desenvolvidos. Esses inibidores de SHH, como ciclopamina e HhAntag-691, regulam negativamente proteínas da via SHH e têm eliminado com sucesso meduloblastomas em modelos de camundongo em estudos pré-clínicos e recentemente entraram em ensaios clínicos de fase I

[1] As síndromes autossômicas dominantes de Li-Fraumeni, Gorlin, Rubenstein-Taybi e a Síndrome Turcot A são causadas por mutações nos genes de P53, PTCH1, CREBBP e APC, respectivamente e estão associadas à formação de diversos tumores nos pacientes afetados.

(ROMER et al., 2004; SASAI et al., 2007; KIMURA et al., 2005; CHEN et al., 2002; BRADBURY et al, 2004).

A ativação da via Wnt, após a união do ligante do receptor Frizzled (FRZ) causa desestabilização do complexo da polipose adenomatosa (APC), resultando em redução da degradação de β-catenina, seguida pela ativação de fatores de transcrição e alvos de Wnt, como c-myc e ciclina D1, que são mediadores da proliferação de células precursoras de neurônios cerebelares da camada granulosa (POLAKIS et al., 2000; NOVAK et al., 1999). A síndrome de Turcot, que é associada com mutação no gene de APC, está associada com uma maior incidência de meduloblastomas. Mutações que ativam a via Wnt, a maioria envolvendo o gene de β-catenina, podem ocorrer em 15% dos casos de meduloblastoma (CLIFFORD et al., 2006; THOMSON et al., 2006).

A via Notch está envolvida na determinação do destino celular e diferenciação de várias células e tecidos. Notch também é crítico para o crescimento e sobrevivência de meduloblastomas induzidos por SHH, uma vez que Notch2, normalmente expresso em células em proliferação precursoras de células da camada granulosa do cerebelo, está superexpressa em alguns meduloblastomas (BARON et al., 2003).

Embora estratégias terapêuticas tenham sido desenvolvidas tendo as vias SHH, Wnt e Notch como alvos, outras vias de sinalização também são consideradas relevantes em meduloblastomas. Em especial, vias de sinalização que desempenham papel importante durante o desenvolvimento, como as dos receptores de neurotrofinas.

Além dessas, foi demonstrado que o fator de crescimento de hepatócitos (HGF), o fator de crescimento epidermal (EGF), o receptor 1 do fator de crescimento semelhante à insulina (IGFR1) e o receptor do fator de crescimento derivado de plaqueta (PDGFR) estão envolvidos na sinalização de meduloblastomas.

HGF é neuroprotetor para células da camada granulosa do cerebelo e promove o crescimento de meduloblastomas humanos em cultura e em camundongos xenotransplantados (BINNING et al., 2008).

O receptor de EGF, ErbB2, tem impacto prognóstico em meduloblastomas, sendo que sua alta expressão está relacionada com desfecho negativo e é encontrada em até 84% dos casos de meduloblastoma (GILBERTSON et al., 1995; BAL et al., 2006; HERNAN et al., 2003). O receptor de IGF também representa um

alvo promissor para terapias anti-câncer (SELL et al., 1993; CARBONI et al., 2005; LOPEZ et al., 2002; YAKAR et al., 2005), pelo seu papel na promoção de crescimento celular e inibição de apoptose. Além disso, o IGFR1 foi descrito como superexpresso em linhagens e amostras de tumors de meduloblastomas (DEL VALLE et al., 2002). IGF1 e IGF2 estão envolvidos no controle da proliferação de meduloblastomas e de células precursoras cerebelares (PATTI et al., 2000), sendo que IGF2 é um alvo da via SHH (HAHN et al., 2000). Similarmente, a superexpressão de PDGFRβ está associada com metástase em meduloblastomas e PDGFRα é altamente expresso em meduloblastomas (GILBERTSON et al., 2003).

A interação entre diferentes vias de sinalização tem sido descrita em diversos tipos de câncer, desempenhando um papel importante no comportamento de tumores. A relação entre vias de sinalização que afetam o desenvolvimento cerebral e a proliferação celular e diferenciação não podem ser analisados como um único fenômeno. O resultado da ativação de diversos receptores de fatores de crescimento pode ocorrer através de interações entre diferentes vias de sinalização, que podem ativar múltiplas respostas em células.

### *1.1.2 Tratamento de Meduloblastomas*

O tratamento de meduloblastomas é baseado na ressecção cirúrgica máxima, quimioterapia baseada em cisplatina intravenosa e radioterapia. Um desafio clínco em relação aos meduloblastomas são as respostas altamente variadas dos pacientes ao tratamento: alguns respondem bem, com remissão do tumor, enquanto outros apresentam doença progressiva (PACKER et al., 1999). Os meduloblastomas têm alta tendência a recorrência (RIFFAUD et al., 2009) que na maioria das vezes ocorre nos 2 primeiros anos após o tratamento inicial, sendo que a sobrevida mediana dos pacientes após os sintomas de recorrência do tumor é de 4 a 5 meses (SAUNDERS et al., 2003; TORRES et al., 1994).

Uma preocupação a longo prazo em relação aos pacientes tratados é também o risco de desenvolvimento de tumores secundários, como gliomas e meningiomas. O tratamento padrão, com radioterapia e quimioterapia, também causa fortes efeitos negativos sobre a qualidade de vida dos pacientes a longo prazo. Dificuldades

neurocognitivas são os efeitos mais comuns em pacientes de todas as idades (PALMER; REDDICK; GAJJAR, 2007), uma vez que as doses de radioterapia necessárias para o controle da doença causam um dano cerebral significativo, o que é especialmente danoso em crianças mais jovens (RIS et al., 2001). Os quocientes de inteligência decaem de 20 a 30 pontos cerca de 2 a 3 anos depois de o tratamento com radioterapia. O desenvolvimento de sequelas endocrinológicas, como insuficiência de hormônio do crescimento, com um consequente retardo no crescimento, é observado na maioria dos pacientes (RIS et al., 2001; RADCLIFFE et al., 1992; PACKER, et al., 2001), que são então tratados com terapia de reposição de hormônio do crescimento (PACKER et al., 2001). A toxicidade relacionada ao tratamento também pode causar tumores secundários (GESSI et al., 2008), ototoxicidade, toxicidade ginecológica e consequentes complicações neonatais, toxicidade cardíaca, toxicidade pulmonar e até mesmo a morte dos pacientes (PEREZ-MARTINEZ et al., 2005).

Enquanto a sobrevivência das crianças com meduloblastoma não-metastático pode atingir uma taxa de 50 a 70% após 5 anos, os resultados para lactentes, crianças mais jovens e crianças com meduloblastomas metastático são ainda muito baixas (MACDONALD et al., 2003). Em pacientes de alto risco tratados com cirurgia e radioterapia, as taxas de sobrevida sem progressão da doença podem atingir quase 50% com a adição de quimioterapia (TAYLOR et al., 2005).

Embora vários avanços tenham sido alcançados na clínica para o tratamento de medublastomas (HOFF et al., 2009; (GRODMAN; KRETSCHMAR, 2009; SKOWRONSKA-GARDAS, 2009), a maior parte dos tratamentos convecionais fracassam e portanto novas alternativas terapêuticas são necessárias. Além disso, as formas de melhorar a qualidade de vida a longo prazo dos pacientes reduzindo as seqüelas sem comprometer o controle da doença ainda são controversas.

Nos últimos anos, muitos avanços têm permitido um melhor entendimento da biologia tumoral, viabilizando assim a identificação de novos alvos terapêuticos e consecutivamente o desenvolvimento de novas drogas antitumorais com mecanismos de ação específicos (FERNANDO *et al*., 2003; SCHALLY *et al.*, 2004).

A administração sistêmica de anticorpos monoclonais contra HGF prolongou a sobrevivência de camundongos com meduloblastomas induzidos por SHH e HGF, estimulando apoptose (BINNING et al., 2008). Erlotinib ou Gefitinib inibe a sinalização por ErbB2 em células de meduloblastoma humano e podem ter um

potencial terapêutico (HERNAN et al., 2003). Ensaios clínicos usando Erlotinib e Lapatinib, um inibidor reversível potente de ErbB1 e ErbB2, estão em curso. Estratégias para inibir a função de IGFR1 também poderiam servir como ações suplementares a terapias convencionais. A inibição de IGFR1 oligonucleotídeos anti-senso para o mRNA desse receptor reduz o crescimento de meduloblastomas (WANG et al., 2001). Vários ensaios clínicos têm investigado novas terapias que têm como alvo os domínios extracelulares de IGFR1 e o uso de anticorpos monoclonais contra IGFR1 tem mostrado benefícios em diversos tipos de câncer quando usado sozinho ou combinado com quimioterapia (HALUSKA et al., 2007; KARP et al., 2008). Foi demonstrado que o tratamento com Imatinib, um inibidor de tirosina cinase que afeta PDGFR, inibe migração e invasão e induz apoptose e inibição de proliferação celular em meduloblastomas, indicando que esse pode ser um alvo importante em meduloblastomas (ABOUANTOUN et al., 2009).

Há evidências crescentes de que o prognóstico e possivelmente a resposta aos tratamentos dependem da variante histológica do tumor e também das vias de sinalização envolvidas no desenvolvimento e crescimento do tumor (GARRÉ et al., 2009). Um maior conhecimento a respeito das vias oncogências afetadas nos meduloblastomas pode, portanto, elucidar as características do desenvolvimento e proliferação desses tumores e contribuir para o desenvolvimento de novos alvos terapêuticos para essa doença.

## 1.2 Peptídeo Liberador de Gastrina

O peptídeo bombesina (BB) foi originalmente isolado da pele do anfíbio *Bombina bombina* (ANASTASI *et al*., 1971). Outros três peptídeos homólogos à bombesina foram posteriormente descobertos: o peptídeo liberador de gastrina (GRP), a neuromedina C (NMC) e a neuromedina B (NMB) (McDONALD *et al*., 1979). Estes peptídeos foram classificados de acordo com a região C-terminal (KROOG *et al*., 1995; PRESTON *et al*., 1996), sendo que o GRP e a NMC têm uma leucina como penúltimo resíduo da região C-terminal, enquanto a NMB tem uma fenilalanina como penúltimo resíduo (SHIN *et a*l., 2006).

O peptídeo liberador de gastrina é um importante fator de crescimento autócrino e parácrino (CUTTITA *et al*., 1985; KIM *et al*., 2002), pois afeta a proliferação e diferenciação celular pela ligação ao seu receptor (SCOTT *et al*., 2004; PATEL *et al*., 2004; MOODY & MERALI, 2004; OHKI-HAMAZAKI *et a*l., 2005; PATEL *et al*., 2006; ROESLER *et al*., 2006). Além disso, o GRP possui uma potente atividade mitogênica, promovendo o crescimento de tecido normal e tumoral (FRUCHT *et al*., 1991; CASANUEVA *et al*., 1996; CASSANO *et al*., 2001; SCOTT *et al*., 2004). O peptídeo liberador de gastrina é o homólogo humano de bombesina (ver figura 1.1) e foi assim denominado por estimular a liberação da gastrina *in vivo*, com atividade similar à da bombesina (PRESTON *et al*., 1996). O GRP possui 27 aminoácidos e é codificado no cromossomo 18 (SCOTT *et al.,* 2004).


**Figura 1.1** (A) Sequências de aminoácidos de bombesina e GRP. As porções C-terminais de bombesina e GRP, marcadas em negrito, são idênticas (SUNDAY *et al*., 1988). (B) Alinhamento das sequências de aminoácidos de GRP e bombesina, indicando homologia. (C) Predição de folding de GRP.

Em humanos, três subtipos de receptores que se ligam aos peptídeos da família da bombesina foram identificados: o receptor preferencial ao peptídeo liberador de gastrina (GRPR), receptor preferencial à neuromedina B (NMBR) e receptor para o subtipo 3 de bombesina (BRS3) (CASSANO *et al*., 2001), todos pertencentes à família dos receptores acoplados à proteína G e com grau de homologia de 45 a 54% entre os três receptores em humanos (Figura 1.2).

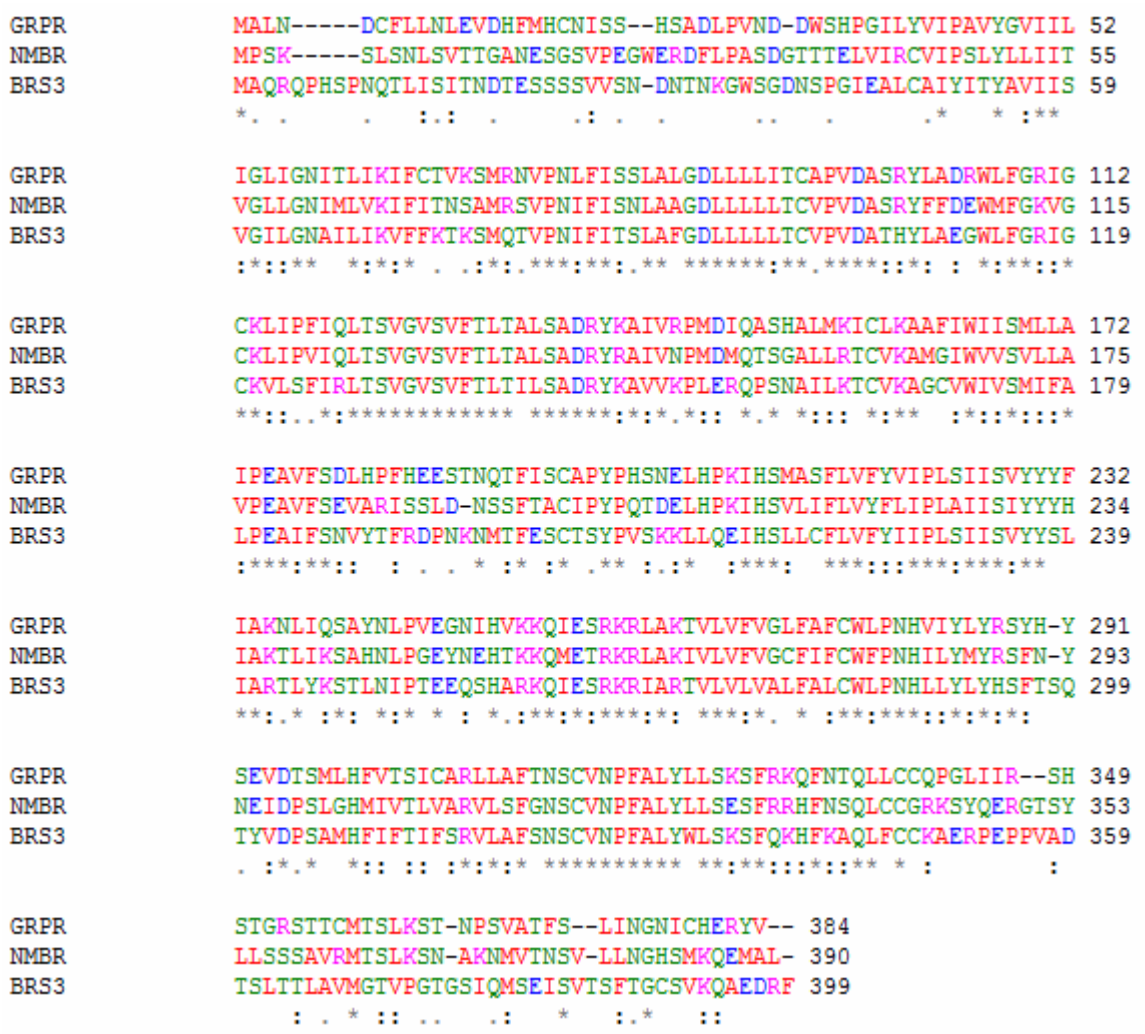


**Figura 1.2** Alinhamento de sequências dos receptores do peptídeo liberador de gastrina (GRPR), do receptor preferencial à neuromedina B (NMBR) e do receptor para o subtipo 3 de bombesina (BRS3) de humanos, indicando sua homologia. As cores idicam as caracteríscias dos resíduos de aminoácidos: vermelho (hidrofóbico), azul (ácido), magenta (básico) e verde (hidroxila ou amina ou básico).

As sequências de GRPR e NMBR são bem conservadas entre espécies, com homologia acima de 90% entre mamíferos. A similaridade de BRS3 entre mamíferos

é mais baixa, mas ainda está acima de 84% (OHKI-HAMAZAKI *et al*., 2005). De acordo com Patel e colaboradores (2004), o GRP se liga ao GRPR com alta afinidade e ao BRS3 com baixa afinidade.

O GRPR é um receptor transmembrana com 384 aminoácidos, codificado pelo cromossomo X (Xp22) e pertencente à família dos receptores acoplados à proteína G heptahelical (BENYA *et al*., 2000; XIAO *et al*., 2001). (Figura 1.3).

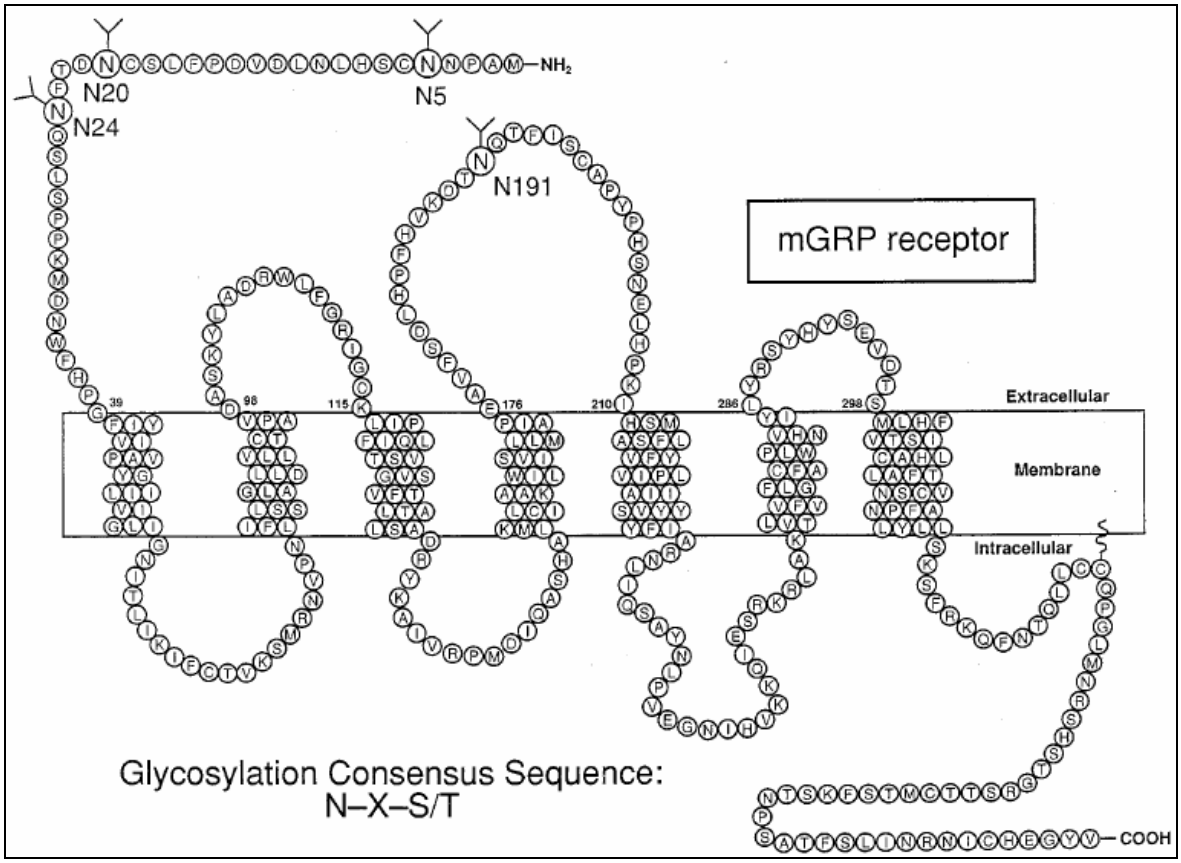


**Figura 1.3** Estrutura molecular do GRPR, com aminoácidos em código de uma letra, possíveis sequências transmembrana e porções glicosiladas (BENYA *et al*., 2000).

No tecido normal, a distribuição de GRP é restrita ao sistema nervoso central (BATTEY & WADA, 1991), células neuroendócrinas do pulmão de fetos (SCOTT *et al*., 2004), fibras nervosas no plexo mientérico do trato gastro-intestinal (MORAN *et al.,* 1988), mucosa intestinal (CHU *et al*., 1995) e glândulas mamárias (SZEPESHAZI *et al.,* 1991; PRESTON *et al*., 1996).

O GRP participa da regulação de diversos processos fisiológicos no sistema nervoso central, sistema imune, pulmonar e gastro-intestinal, (CARROLL *et al*., 1999). Este peptídeo age como um hormônio gastro-intestinal, estimulando o crescimento epitelial do intestino (DEMBIŃSK *et al.,* 1991), a liberação de gastrina através de células G antrais, a secreção exócrina pancreática e modulando a motilidade gastro-intestinal (SCOTT *et al.*, 2004).

Bombesina e GRP estão envolvidos na indução da secreção de hormônios e ácidos gástricos, secreção de muco, regulação da contração do músculo esquelético, promoção de quimiotaxia, modulação neuronal, controle da temperatura corporal (MARKI *et al*., 1981), regulação do ritmo circadiano (ALBERS *et al*., 1991), saciedade (McCOY & AVERY, 1990) e alguns aspectos do comportamento e formação da memória (LIEBOW *et al.,* 1994; CARROLL *et al.,* 2000, CASSANO et al., 2001; CASANUEVA *et al*., 1996; ROESLER *et al*., 2006).

Sabe-se que bombesina, GRP e NMB atuam como um fator de crescimento tumoral (KROOG *et al*., 1995; PRESTON *et al*., 1996), promovendo a proliferação celular e aderência das células tumorais na matriz extracelular (GLOVER *et al*., 2004). Análises imuno-histoquímicas usando anticorpos contra BB, GRP e NMB revelaram a existência de peptídeos similares altamente conservados no cérebro e em tecidos gástricos de várias espécies (OHKI-HAMAZAKI *et al*., 2005) (Figura 1.4).

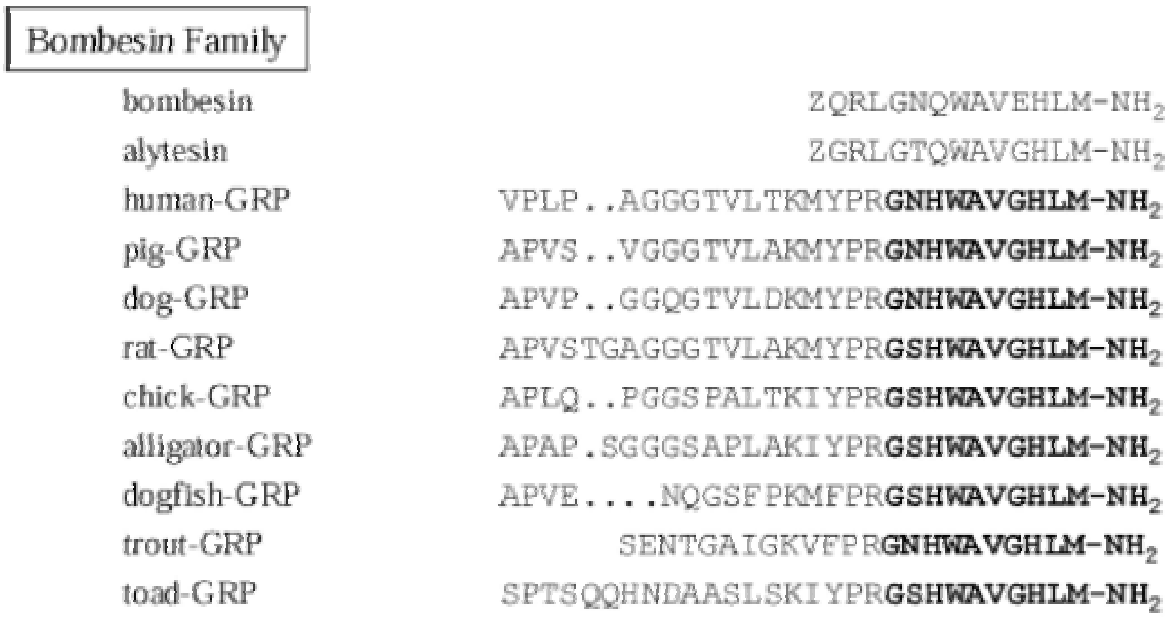


**Figura 1.4** Estrutura da bombesina e seus peptídeos relacionados (Adaptado de OHKI-HAMAZAKI *et al*., 2005).

Recentes evidências indicam que os receptores de GRP estão relacionados a doenças neurodegenerativas e neuropsiquiátricas, incluindo Doença de Alzheimer, autismo e ansiedade (ITO *et al*., 1994; ISHIKAWA-BRUSH *et al.*, 1997; MELLER *et al*., 2004; ROESLER *et al*., 2004a; GIBSON & HUANG, 2005; ROESLER *et al*., 2006). Alterações na densidade de GRPR e disfunções em BB têm sido mostradas em fibroblastos e leucócitos de pacientes com Doença de Alzheimer (ITO *et al*., 1994; GIBSON AND HUANG, 2005). Adicionalmente, o GRP é também relatado em processos neoplásicos e parece estar relacionado à promoção de tumores (PRESTON *et al*., 1996).

Alguns estudos têm mostrado que tanto o GRP quanto o GRPR são expressos de forma aberrante em muitos tipos de cânceres, como glioblastomas (WADA *et al*., 1991; WANG *et al*., 1992), câncer de próstata (SCHULZ *et al*., 2006; SUN *et al*., 2000a), gastro-intestinal (CARROLL *et al*., 1999; 2000; KIM *et al*., 2000; REUBI *et al*., 2002; PATEL *et al*., 2006), de pulmão, (CORJAY *et al*., 1991; MOODY *et al*., 1992; SIEGFRIED *et al*., 1997; MOODY *et al.*, 2006), de mama (XIAO *et al.*, 2001), de ovário (SUN *et al*., 2000b), neuroblastoma (SEBESTA *et al.,* 2001) e câncer de cabeça e pescoço (PRESTON *et al*., 1996; JENSEN *et al*., 2001). Essa superexpressão parece estar vinculada a características invasivas dos tumores, bem como com o desenvolvimento e progressão da doença (CARROLL *et al.,* 2000; PATEL *et al*., 2004). Neste contexto, Scott e colaboradores (2004) sugerem que a expressão aberrante do receptor de GRP pode ser suficiente para estimulação autócrina e parácrina de alguns carcinóides e para dirigir a proliferação das células neoplásicas. Em células que expressam alto número de GRPR, há uma dessensibilização crônica do receptor quando comparado às células que expressam pouco GRPR (MILLAR & ROZENGURT, 1990).

A interação entre GRP e outros mitógenos potenciais pode ocorrer em nível de membrana celular pela modulação de receptores GRP (PRESTON *et. al*, 1996). Além disso, estudos dirigidos por Patel e colaboradores (2006) sugerem que múltiplos fatores de crescimento devem atuar em conjunto para a progressão das neoplasias.

### *1.2.1 RC-3095: Um Antagonista de GRPR*

Radulovic e colaboradores (1991) desenvolveram um antagonista sintético para o receptor de bombesina/GRP, o RC-3095: [D-Tpi$^{6}$,Leu$^{13}\Psi$ ($CH_2$-NH)Leu$^{14}$]bombesina-(6-14). Este pseudononapeptídeo tem sido testado contra diferentes tipos de cânceres, demonstrando ser um potente agente antitumoral (RADULOVIC *et al*., 1991; LIEBOW *et al*., 1994; CASANUEVA *et al*., 1996; SZEPESHAZI *et al*., 1997; CHATZISTAMOU *et a*l., 2001; SCHWARTSMANN *et al*., 2004; 2005; CORNÉLIO *et al*., 2007).

RC-3095 inibe o crescimento celular das linhagens tumorais de glioblastoma U-87MG e U373MG, após a estimulação com GRP (PINSKI *et al*., 1994b). Além disso, em modelos de enxerto não-autólogo, a proliferação celular também é inibida com o uso de RC-3095 (KIARIS *et al.,* 1999). Glover e colaboradores (2004) mostraram que GRP e GRPR atuam na diferenciação de células tumorais em camundongos *knockout.*

RC-3095 tem se mostrado eficaz na diminuição da progressão de lesões pré-malignas induzidas, bem como o crescimento e diferenciação de muitas neoplasias *in vitro* e *in vivo*, incluindo cânceres gástricos, colorretais, pancreáticos, de mama, de próstata e de pulmão (SZEPESHAZI *et al.,* 1991; 1992; PINSKI *et al.*, 1994a; 1994b; LIEBOW *et al.*, 1994; MAHMOUD *et al.,* 1991; HALMOS & SCHALLY, 1997; KOOPAN *et al.*, 1998; KIARIS *et al.,* 1999; DORSAM & GUTKIND, 2007).

## 1.3 Vias de Sinalização Celular Envolvidas com GRPR

Os receptores de GRP (representados na figura 1.5 como BBS, bombesina) se ligam a uma proteína Gαq (Gq), ativando fosfolipase C-β (PLC-β) que cliva o fosfatidilinositol bifosfato (PIP2), resultando na produção de diacilglicerol (DAG) e inositol 1,4,5-trifosfato (IP3), elevando os níveis de cálcio intracelular e, deste modo, ativando cascatas de proteínas cinases (MEK, ERK), que podem levar à liberação de cromogranina-A (CGA), um marcador tumoral, para o meio extracelular (GILADI *et al*., 1993; SHUMYATSKY *et al*., 2002; ROESLER *et al.*, 2003; MOODY & MERALI,

2004; ROESLER *et al*., 2004a; 2004b; 2005; 2006; PATEL *et al*., 2004; OHKI-HAMAZAKI *et al*., 2005).

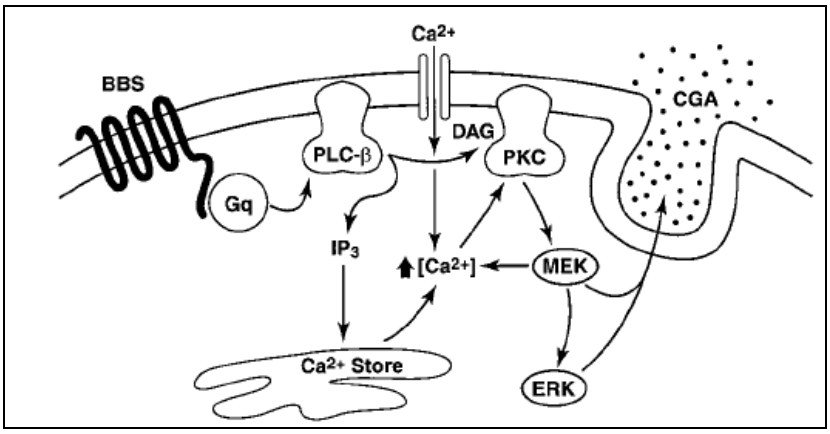


**Figura 1.5** Modelo de sinalização de receptores de GRPR (HELLMICH, 1999).

Entre as vias de sinalização celular que são ativadas pelos receptores de GRP, já foram caracterizadas as da proteína cinase mitógeno-ativada (mitogen-activated protein kinase, MAPK), proteína cinase C (protein kinase C, PKC), cinase de adesão focal (focal adhesion kinase, FAK), e proteína cinase regulada por sinais extracelulares (extracellular signal-regulated protein kinase, ERK) (APRIKIAN *et al*., 1997; HELLMICH *et al.*, 1999; KIM *et al.,* 2000; QU *et al.*, 2002; XIAO *et al.,* 2003; CHEN & KROOG, 2004; SCHWARTSMANN *et al.,* 2005; STANGELBERGER *et al*., 2005; THOMAS *et al*., 2005).

Os receptores de GRP, além de promoverem crescimento e proliferação, também estão envolvidos na migração celular e angiogênese, pois estimulam a pequena GTPase Rho (LEFRANC *et al.,* 2002), que tem um papel central na migração celular através do estímulo da proteína ROCK (MARINISSEN & GUTKIND, 2001), e ativam a fosfolipase A2 (PLA2) e cicloxigenase 2 (COX2), aumentando a produção de prostaglandina E2 (PGE2) (ROZENGURT *et al*., 2002) (Figura 1.6).

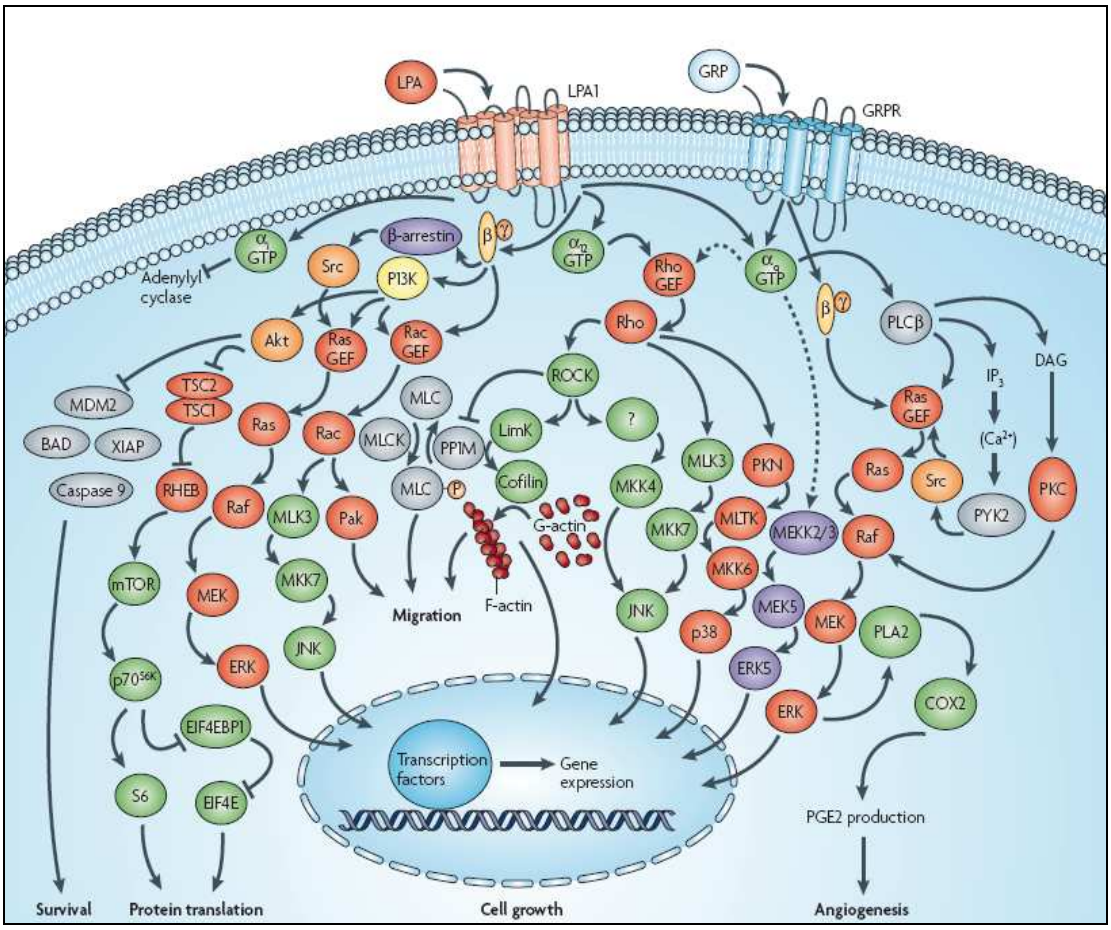


**Figura 1.6** Vias de sinalização ativadas pelo GRPR (DORSAM & GUTKIND, 2007).

## 1.4 Via do cAMP/PKA

A adenosina monofosfato cíclica (cAMP) é um segundo mensageiro que participa na transdução de sinal intracelular de vários estímulos. A enzima adenilato ciclase catalisa a conversão de ATP a cAMP, que por sua vez ativa a cinase dependente de cAMP (proteína cinase A ou PKA). Um dos substratos ativados por PKA é a fosfodiesterase, que converte cAMP em AMP, reduzindo a quantidade de cAMP que pode ativar PKA (WALSH *et al.,* 1948). A fosfodiesterase tipo 4 (PDE4) é uma hidrolase seletiva de cAMP e é amplamente expressa em tumores cerebrais e superexpressa na linhagem de meduloblastoma humano DAOY, promovendo sua proliferação celular *in vitro* (GOLDHOFF, 2008).

A PKA está envolvida no controle de vários processos celulares como motilidade, adesão, interação célula-célula, transdução de sinais externos

(KONDRASHIN, 1999). Sabe-se que o aumento de cAMP intracelular inibe proliferação (KATO, 1994) e pode estimular apoptose (HARADA, 1999) em diferentes tipos celulares.

Alguns estudos demonstraram que os níveis intracelulares de adenosina monofosfato cíclica (cAMP) são importantes na regulação do crescimento e diferenciação celulares (CHO-CHUNG & CLAIR, 1993) e em 1977, Furman e Shulman compararam os níveis de cAMP e avaliaram a atividade de adenilil ciclase no cérebro normal e em gliomas, mostrando existir uma correlação inversa entre o grau de malignidade e os níveis de cAMP em gliomas malignos. Entreanto, os mecanismos de sinalização envolvidos nas ações geradas por GRP e GRPRs, principalmente em relação à participação da via de adenosina monofosfato cíclica (cAMP) e da proteína cinase A (PKA), na proliferação celular de meduloblastoas, ainda não estão totalmente esclarecidos.

Algumas evidências sugerem que a via da PKA poderia interagir com a proliferação de células tumorais, pois ativadores de cAMP/PKA, como forskolin, 8-Br-cAMP e rolipram, diminuem a proliferação celular, aumentam a diferenciação e induzem apoptose na linhagem de glioblastoma humano, A-172 (CHEN *et al*., 1998; 2002). Neste contexto, Walter e colaboradores descreveram em 1977 a existência de PKA na superfície externa em células C6 de gliomas de ratos. Mais tarde, inibidores dessa via foram usados, resultando no aumento da proliferação celular desta mesma linhagem (HELMBRECH & RENSING, 1999). PERRY e colaboradores (2004) indicam que a PKA parece estar relacionada à proliferação na linhagem celular U-87MG.

Na interação entre GRPR e a via do D1R/cAMP/PKA em células de linhagem COS-7, a ativação de GRPR e D1R inibiu a atividade de proteínas cinases (CHAN & WONG, 2005). Recentemente foi demonstrado que a ativação de GRPR e a cascata de sinalização de cAMP/PKA podem atuar em sinergismo para a promoção da proliferação celular em glioblastomas humanos *in vitro* (FARIAS *et al,* 2008).

A via da PKA foi implicada na iniciação e progressão de vários tumores e é considerada um alvo molecular com relevância crescente e alto potencial terapêutico em câncer. Quando não controlada, a via da PKA pode levar a uma proliferação celular exacerbada.

Em meduloblastomas, a atividade da PKA é influenciada pelo Pepetídeo Ativador da Pituitária Adenilato Ciclase (PACAP), que foi demonstrada como

reguladora da proliferação de células precursoras da camada granulosa do cerebelo *in vitro* e identificada como um fator fisiológico que regula a patogênese da via SHH, associada com meduloblastomas (LELIEVRE et al., 2008). A regulação dos níveis de cAMP pode estar também envolvido nos efeitos antiapoptóticos do receptor de citocina CXCR4, que é importante para o cerebelo em desenvolvimento (YANG et al., 2007).

### *1.4.1. Rolipram: ativador da via da PKA por inibição de PDE4*

O rolipram é um inibidor específico da PDE4 (Figura 1.7), levando a um aumento da concentração de cAMP intracelular. Em astrócitos em cultura tratados com isoproterenol, o rolipram a partir da concentação de 100nM é capaz de aumentar significativamente as concentrações de cAMP intracelular, e em neurônios, esse efeito é observado com rolipram a partir de 10nM (YAMASHITA et al., 1997).

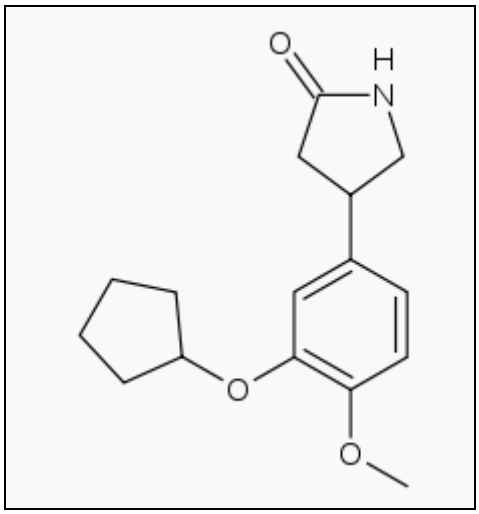


**Figura 1.7** Estrutura molecular do rolipram (4-(3-ciclopentilóxi-4-metóxi-fenil)pirolidina-2-ona).

O rolipram inibe o crescimento, aumenta a diferenciação e induz apoptose em células A-172 de glioma humano com tratamento de 48h em concentrações de até

1mM (CHEN, 2002). O tratamento com rolipram também induz a liberação de citocromo C mitoncondrial, ativação de caspase 9 e 3 e clivagem de PARP em células de leucemia linfocítica crônica. (MOON et al., 2003). Por outro lado, rolipram a 10µM combinado com GRP foi capaz de promover o crescimento de células de glioma humano (FARIAS et al., 2008), indicando que a ativação da cascata de sinalização de cAMP/PKA pode promover proliferação celular sob certas condições.

Além de seu efeito antiinflamatório, o rolipram já é proposto para o tratamento de depressão e esclerose múltipla (SCOTT et al., 1991; SOMMER et al, 1995) e já foi utilizado em ensaios clínicos que demonstraram vantagens de seu uso, por ser um agente de utilização oral que atravessa a barreira hemato-encefálica que inibe a atividade da fosfodiesterase IV no cérebro (MCLAUGHLIN et al., 1993; KRAUSE et al., 1989). Dados sobre farmacocinética e farmacodinâmica foram estudados por Krause e colaboradores (1993). Rolipram (até 3mg/dia) se mostrou bem tolerado em humanos, sendo que os efeitos colaterais incluem náusea, sudorese e constipação (HEBENSTREIT et al., 1989).

Em um estudo recente, Goldhoff e colaboradores (2008) demonstraram que a inibição de PDE4 por rolipram suprimiu o crescimento de xenotransplantes de glioblastoma. No entanto, nenhum estudo avaliou o efeito de rolipram sobre linhagens celulares de meduloblastoma humano.

## 1.5 Neurotrofinas

As neurotrofinas, também chamadas de fatores neurotróficos, são uma família de proteínas que favorecem a sobrevivência de neurônios. Essas proteínas pertencem à família dos fatores de crescimento, que são proteínas que estão na corrente sangüínea e são capazes de se unir a receptores de determinadas células para estimular sua sobrevivência, crescimento ou diferenciação.

A família de neurotrofinas é formada pelo Fator de Crescimento Neuronal (NGF), pelo Fator Neurotrófico Derivado de Cérebro (BDNF), pela Neurotrofina 1 (NT-1), NT-3, NT-4 e NT-6 (Figura 1.8). Os receptores de neurotrofinas são do tipo tirosina cinase, capazes de adicionar um grupamento fosfato a certos resíduos de tirosina em proteínas alvo. O receptor cinase relacionado a tropomiosina (A) (TrkA) é

o principal receptor de alta afinidade para NGF. As neurotrofinas que ativam o receptor TrkB são BDNF, NT-4 e NT-3. O receptor TrkC se une e é ativado somente por NT-3 (HUANG & REICHARDT, 2003). O BDNF se liga a no mínimo dois receptores: TrkB e LNGFR (p75) e age em neurônios do sistema nervoso central e periférico, dando suporte à sobrevivência dos neurônios existentes e estimulando o crescimento e diferenciação de novos neurônios e sinapses. As quatro neurotrofinas também se ligam ao receptor LNGFR, também conhecido como p75, que ainda não possui um papel biológico claro. Foi demonstrado, no entanto, que p75 pode sinalizar morte celular via apoptose (CHEN *et al.,* 2009.)

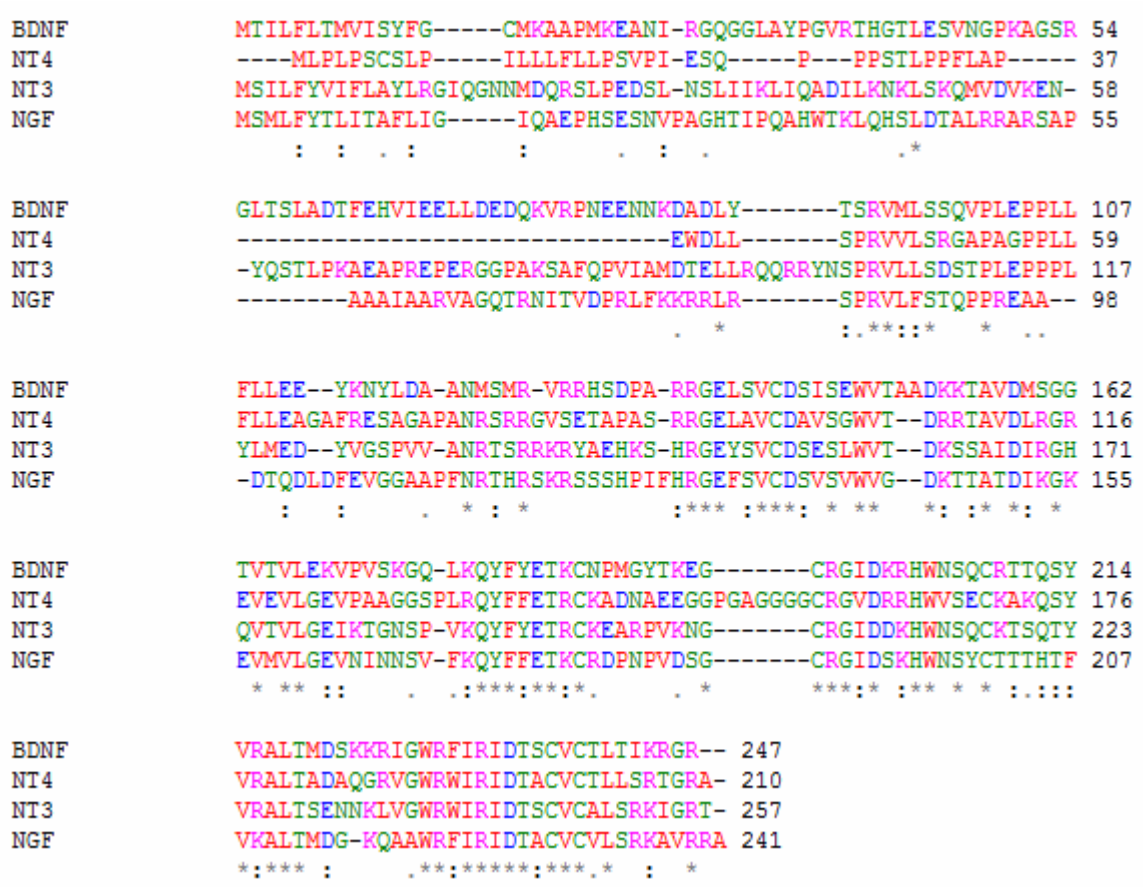


**Figura 1.8** Alinhamento das sequências de NGF, BDNF, NT-3 e NT-4.

As neurotrofinas estão entre os sinais extracelulares, além de outros neuropeptídos, que afetam funções celulares também em tumores. Em neurônios e células gliais do sistema nervoso central normal adulto e em desenvolvimento, essas neurotrofinas desempenham um papel fisiológico relacionado à regulação de

transmissão neuronal, plasticidade sináptica, sobrevivência e proliferação celular. Células neoplásicas de meduloblastomas e outros tumores neuroectodérmicos primitivos apresentam características de células progenitoras do sistema nervoso central em desenvolvimento e podem apresentar fenótipos moleculates característicos de neurônios imaturos. Neurotrofinas regulam proliferação, diferenciação e apoptose em células neuroectodérmicas que estão comprometidas com a linhagem neuronal. A expressão de seus receptores pode ser fator um prognóstico para tumores neuroectodérmicos. As neurotrofinas são importantes na regulação de proliferação celular, diferenciação e sobrevivência no cérebro normal e poderiam também influenciar a progressão de meduloblastomas (CHOU et al. 1997; NAKAGAWARA 2001; TAJIMA et al. 1998; WASHIYAMA et al. 1996).

### *1.5.1 Neurotrofinas e Meduloblastomas*

Neurotrofinas e seus receptores são expressos em amostra de biópsia e linhagens de meduloblastoma (CHIAPPA et al. 1999; TAJIMA et al. 1998; WASHIYAMA et al. 1996). Sabe-se que a superexpressão de TrkA e TrkC são fatores prognósticos positivos em meduloblastomas, sendo que o receptor de NT-3 (TrkC) foi o primeiro receptor de fator de crescimento do tipo tirosina cinase a ser associado com aspectos clínicos de meduloblastomas. Existem evidências de que esse receptor é um marcador prognóstico positivo confiável (SEGAL et al., 1994; GROTZER et al., 2000) e também desempenha um papel importante em meduloblastomas, uma vez que a ativação de TrkC pelo seu ligante NT-3 afeta o desfecho da doença, inibindo o crescimento tumoral através da promoção de apoptose (KIM et al., 1999). Uma baixa expessão de TrkC está relacionada a um prognóstico menos favorável para os pacientes afetados e alterações nesse receptor são encontradas em até 48% dos casos de meduloblastoma (MACDONALD et al., 2003). Existe também uma evidência recente de que a heparanase regulada por neurotrofina (HPSE), uma enzima singular que degrada matriz extracelular – associada com a progressão tumoral em uma ampla variedade de cânceres e expressa em até 76% dos casos de meduloblastoma – pode desempenhar um papel

crítico na invasão tumoral e progressão em um contexto ligado à ativação dos receptores TrkC e p75 (MARCHETTI et al., 2007).

A expressão do receptor de NGF (NGFR ou TrkA) também está associada com níveis aumentados de apoptose em células de meduloblastoma (OHTA et al., 2006). NGF induz apoptose em linhagens de meduloblastomas que expressam TrkA (MURAGAKI *et al.,* 1997). A exposição a NGF bloqueia a proliferação celular, indicando que NGF poderia talvez induzir diferenciação através de TrkA pela interferência em proliferação e sobrevivência (ANTONELLI et al., 2007). Além disso, o fator de transcrição Zhangfei, expresso em neurônios diferenciados, também leva a diferenciação e apoptose pela indução da expressão de TrkA em células de meduloblastoma (VALDERRAMA et al., 2009). O cotratamento com NGF e cisplatina também pode reduzir os efeitos colaterais citotóxicos da cisplatina. (ANTONELLI et al., 2007)

### *1.5.2 TrkB e BDNF*

Evidências crescentes sugerem que alterações na sinalização por TrkB pode causar a formação de tumores e mestástase. O receptor TrkB está superexpresso em vários tipos de câncer, desde de neuroblastomas até adenocarcinomas, onde pode provocar expansão tumoral e pode contribuir para a resistência a drogas anti-tumorais. Em geral, o TrkB age *in vitro* como um supressor de apoptose, no entanto os resultados da ativação desse receptor podem variar (DESMET et al., 2006).

Evidências sugerem que a adição de BDNF na concentração de 50ng/mL a culturas de células tronco mesequimais com fenótipo dopaminérgico durante 9 dias é capaz de promover maturação funcional dessas células (TRZASCA et al., 2009).

Tajima e colaboradores (1998) encontraram co-expressão de BDNF e TrkB em diversos casos de meduloblastoma em pacientes, que também expressavam NFL (proteína de neurofilamento), que é um marcador neuronal, sugerindo que BDNF pode agir de maneira parácrina através do receptor TrkB para induzir diferenciação neuronal em meduloblastomas. No entanto, não há estudos avaliando o efeito de BDNF sobre a viabilidade celular de linhagens de meduloblastoma humano.

# 2 OBJETIVOS

## *2.1 Objetivo Geral*

O tratamento padrão para os pacientes afetados por meduloblastomas produz sequelas e garante uma sobrevida baixa, o que demonstra a necessidade de novas terapias para tratar essa doença. Este trabalho objetivou avaliar o efeito de agonistas e antagonista do GRPR, do ativador da via de AMPc/PKA rolipram e de BDNF sobre a viabilidade celular de linhagens de meduloblastoma humano *in vitro*, uma vez que esses podem ser alvos moleculares promissores para o desenvolvimento de alternativas terapêuticas para meduloblastomas.

## *2.2 Objetivos Específicos*

- Analisar a expressão de mRNA para GRPR por reação em cadeia de polimerase por transcriptase reversa (RT-PCR) em diferentes linhagens de células de meduloblastoma humano (DAOY, D283 e ONS-76);
- analisar a expressão de GRPR por imuno-histoquímica em diferentes linhagens de células de meduloblastoma humano (DAOY, D283 e ONS-76);
- analisar os efeitos dos agonistas GRPR, BB e GRP, e do antagonista GRPR, RC-3095, sobre a viabilidade celular em diferentes linhagens de células de meduloblastoma humano (DAOY, D283 e ONS-76);
- analisar os efeitos do inibidor de fosfodiesterase IV, rolipram, sozinho ou combinado com GRP, sobre a viabilidade celular em diferentes linhagens de células de meduloblastoma humano (DAOY, D283 e ONS-76);
- analisar os efeitos de BDNF sobre a viabilidade celular em diferentes linhagens de células de meduloblastoma humano (DAOY, D283 e ONS-76).

## 3 RESULTADOS

Visando ordenar os assuntos abordados, o trabalho está apresentado na forma de três capítulos contendo artigos científicos.

O Capítulo I contém o artigo científico que apresenta os experimentos relacionados com o efeito de agonistas e antagonista do GRPR, do ativador da via de AMPc/PKA rolipram e de BDNF sobre a viabilidade celular de linhagens de meduloblastoma humano *in vitro*. Esse trabalho mostra que o efeito inibitório de rolipram sobre a viabilidade celular de meduloblastomas é observado nas linhages humanas DAOY, D283 e ONS76. Também foi demonstrado que o efeito inibitório de rolipram não foi modificado pelo co-tratamento com GRP em DAOY. Nesse trabalho também foi demonstrado que BDNF inibe a viabilidade das linhagens de meduloblastoma humano DAOY e D283, enquanto agonistas e o antagonista GRPR RC-3095 não tiveram efeito sobre a viabilidade celular dessas linhagens, que expressam os receptores GRPR e TrkB. Esses resultados sugerem que BDNF/TrkB e PDE4, mas não o GRPR, regulam a viabilidade de células de meduloblastoma.

O Capítulo II abrange uma carta ao Editor da revista Clinical Cancer Research. O artigo trata da evidência de que o inibidor de PDE4, rolipram, inibe a viabilidade celular da linhagem de meduloblastoma humano DAOY *in vitro*, representando a primeira evidência na literatura de que rolipram é capaz de inibir a proliferação de células de meduloblastoma. O artigo também aponta que rolipram pode, sob certas condições, aumentar ao invés de diminuir a proliferação de células de câncer, como no co-tratamento com GRP, de acordo com um trabalho anterior.

Para concluir, o Capítulo III contém um artigo de revisão a respeito de recentes avanços em relação ao tratamento de meduloblastomas, focando em novos alvos moleculares e em avanços nos estudos translacionais para o tratamento dessa doença.

## 3.1 CAPÍTULO I: BDNF and PDE4, but not the GRPR, regulate the viability of human medulloblastoma cells

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# BDNF and PDE4, but not the GRPR, Regulate Viability of Human Medulloblastoma Cells

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**Abstract** Medulloblastoma is the most common brain tumor of childhood. Emerging molecular targets in medulloblastoma include neurotrophin and neuropeptide receptors. In the present study, we have examined the influence of brain-derived neurotrophic factor (BDNF)/TrkB receptor- and gastrin-releasing peptide receptor (GRPR)-mediated signaling on the viability of human medulloblastoma cells. The expression of TrkB and GRPR was confirmed by immunohistochemistry and mRNA for both BDNF and GRPR was detected by reverse transcriptase polymerase chain reaction in Daoy, D283, and ONS76 cells. Treatment with BDNF significantly inhibited the viability of Daoy and D283, but not ONS76 cells, measured with the MTT assay. Neither the GRPR agonists GRP and bombesin nor the GRPR antagonist RC-3095 affected cell viability. Because previous findings have indicated that the viability of glioma cells might be enhanced by GRP when combined with the cAMP phosphodiesterase-4 (PDE4) inhibitor rolipram, we also examined the effects of rolipram alone or combined with GRP on cell viability. Rolipram significantly reduced the viability of all three cell lines, and the inhibitory effect of rolipram in Daoy cells was not modified by cotreatment with GRP. The results suggest that BDNF/TrkB and PDE4, but not the GRPR, regulate the viability of medulloblastoma cells.

**Keywords** Brain-derived neurotrophic factor · cAMP phosphodiesterase-4 · Gastrin-releasing peptide receptor · Cell signaling · Medulloblastoma · Brain tumors

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## Introduction

Medulloblastoma, a primitive neuroectodermal tumor (PNET) of the central nervous system (CNS), is the most common brain tumor of childhood. In spite of significant advances in therapy, about 30% of patients still have a low chance of being cured and survivors experience long-term neurocognitive and/or neuroendocrine sequelae. Thus, the development of novel therapies is urgently needed (Crawford et al. 2007; Packer and Vezina 2008; Rossi et al. 2008). A better understanding of the molecular pathways involved in medulloblastoma formation and progression may allow the discovery and

development of innovative targeted therapies. Multiple cell signaling pathways have been implicated in the pathogenesis of medulloblastoma, including Hedgehog, Notch, Wnt, receptor tyrosine kinases, and the oncoprotein Myc (Carlotti et al. 2008; de Bont et al. 2008; Guessous et al. 2008; Gulino et al. 2008; Rossi et al. 2008).

External signals affecting the behavior of brain tumor cells include neurotrophins and neuropeptides, which affect cellular functions through the activation of cell surface receptor-mediated signaling pathways. In neurons and glial cells of the normal developing and adult CNS, such neurotrophin and neuropeptide systems play physiological roles related to the regulation of neural transmission, synaptic plasticity, and cell survival and proliferation. The neurotrophin family consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4, and NT-6, which act by activating specific receptor tyrosine kinases of the Trk family. Thus, NGF, BDNF and NT-3 activate, respectively, TrkA, TrkB, and TrkC receptors (Huang and Reichardt 2003). Neoplastic cells in medulloblastoma and other PNETs display features of progenitor cells of the developing CNS and may present a molecular phenotype characteristic of immature neurons. Neurotrophins may influence medulloblastoma cell function (Chou et al. 1997; Nakagawara 2001; Tajima et al. 1998; Washiyama et al. 1996), and expression of neurotrophins and their receptors has been shown in both medulloblastoma biopsy samples and medulloblastoma cell lines (Chiappa et al. 1999; Tajima et al. 1998, Washiyama et al. 1996). Importantly, TrkC receptor expression has been associated with a better clinical outcome in patients with medulloblastoma (Grotzer et al. 2000; Segal et al. 1994). Although these previous studies have detected the expression of BDNF and its receptor, TrkB, in medulloblastoma biopsies and cell lines, to our knowledge, previous studies have not examined the effects of BDNF on the growth of medulloblastoma.

Neuropeptides involved in both neurotransmission in the normal brain and progression of brain tumors include gastrin-releasing peptide (GRP). GRP, a mammalian bombesin-like peptide that acts by binding to the GRP-preferring type of bombesin receptor (gastrin-releasing peptide receptor [GRPR], BB2 receptor), has been put forward as an important growth factor in many types of human cancer (Cornelio et al. 2007; Patel et al. 2006). GRPR overexpression has been detected in glioma cells lines, and drugs that modulate GRPR activation influence the growth of experimental glioma in vitro and in vivo (de Oliveira et al. 2009; Flores et al. 2008; Kiaris et al. 1999; Pinski et al. 1994). In spite of the increasing importance of GRPR as a target in brain tumors, previous studies have not examined whether GRPR is expressed in medulloblastoma and influences the proliferation and viability of medulloblastoma cells.

In this study, we have examined the expression of BDNF, TrkB, and GRPR, as well as the effects on cell viability of BDNF and GRPR ligands, in human medulloblastoma cell lines. We also examined the effects of the cAMP phosphodiesterase-4 (PDE4) inhibitor rolipram, alone or combined with GRP, on cell viability because of previous findings indicating that the growth of glioma cells might be promoted by GRP combined with rolipram (Farias et al. 2008).

## Materials and Methods

### Cell Culture and Treatments

All experimental procedures were approved by the institutional research ethics committee. Daoy, D283, and ONS76 human medulloblastoma cells originally obtained from the American Type Culture Collection (Rockville, MD, USA) were kindly donated by Dr. Michael D. Taylor (The Hospital for Sick Children, Toronto, Canada). The cells were plated into 96 multiwell plates (TPP) at a density of $5\times10^3$ cell per well in sextuple and grown and maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Carlsbad, USA) containing 2% (*w*/*v*) H-glutamine and 15% (*v*/*v*) fetal bovine serum (Sorali, Campo Grande, Brazil). After 24 h, the cultures were starved for 24 h in 0.5% serum medium then treated with human recombinant BDNF (0.074, 0.74, or 7.4 nM), human recombinant GRP (0.00.1, 0.01, 0.1, or 1 μM; Sigma-Aldrich, St. Louis, USA), the GRPR agonist bombesin (0.00.1, 0.01, 0.1, or 1 μM; Sigma-Aldrich, St. Louis, USA), the GRPR antagonist [D-Tpi$^6$, Leu$^{13}$ psi ($CH_2NH$)-Leu$^{14}$] bombesin (RC-3095; 0.01, 0.1, 1.0, or 10 μM; Zentaris GmbH, Frankfurt, Germany), rolipram (1, 10, or 100 μM; Sigma-Aldrich), or GRP (0.1 μM) combined with rolipram (10 nM). Drug doses were chosen on the basis of previous studies using glioma and neuroblastoma cells (Abujamra et al. 2009; Farias et al. 2008; Flores et al. 2008). Cells were kept at a temperature of 37 C, a minimum relative humidity of 95%, and an atmosphere of 5% $CO_2$ in air.

### MTT Assay

Cell viability was measured by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) 48 h after the treatment. The cells were washed with Hank's balanced salt solution (Invitrogen, São Paulo, Brazil) and 11 μl of MTT 5 mg/mL solution was added to each well then were incubated for 4 h at 37 C. The plate was left at room temperature until completely dry. Dimethyl sulfoxide was added and the absorbance was read at 492 nm in a multiplate reader (Farias et al. 2008).

### RT-PCR Analysis of BDNF and GRPR mRNA Expression

Total RNA was extracted from Daoy, D283, and ONS76 cells using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions and reverse transcribed with SuperScript™ III First-Strand Synthesis SuperMix® (Invitrogen, USA). The human BDNF and GRPR primers were designed according to the corresponding GenBank sequence: BDNF primers 5′-GCGTGAATGGGCCCAAGGCAGG-3′ (forward) and 5′-TGTGACCGTCCCGCCCGACATG-3′ (reverse); GRPR primers 5′-CAAGATCTTCTGCAC GGTCA-3′ (forward) and 5′-TCAGTTTGCAGCCAAT TCTG-3′ (reverse). The polymerase chain reaction (PCR) experiments were carried out with 1.5 mM $MgCl_2$, 0.1 μM for each primer, 0.2 mM deoxynucleotide triphosphates (dNTPs), 0.5 M betaine (only to BDNF primers), 1 U *Taq* Platinum® (Invitrogen, USA), and 2 μl cDNA template. The expression of β-actin was measured as an internal control using the primers 5′-AAACTGGAACGGTGAAGGTG-3′ (forward) and 5′-AGAGAAGTGGGGTGGCTTTT-3′ (reverse). The PCR reaction was performed in a total volume of 20 μL using a concentration of 0.04 mM dNTPs, 0.2 U *Taq* polymerase in the supplied reaction buffer, 0.3 mM $MgCl_2$, and 10 pmol of each primer. The amplification consisted of 1 min at 95 C followed by 35 cycles of denaturation at 94 C for 30 s, annealing at 59 C for 30 s, extension of primers at 72 C for 45 s, and a final extension at 72 C for 10 min. The products of BDNF (362 bp), GRPR (190 bp), and β-actin (190 bp) were electrophoresed through 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet illumination (Farias et al. 2008; Flores et al. 2008). Each experiment was performed in replicate using RNA isolated from independent cell cultures.

### Immunohistochemical Analysis of TrkB and GRPR Expression

Expression of the GRPR and TrkB receptor in Daoy, D283, and ONS76 cells was accessed by immunohistochemistry. The primary antibodies used was a rabbit polyclonal antibody against GRPR (OPA1-15619, Affinity Bioreagents, Golden, CO, USA), corresponding to the second extracellular loop of human GRPR, and a mouse monoclonal antibody raised against an extracellular domain of the human TrkB receptor (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

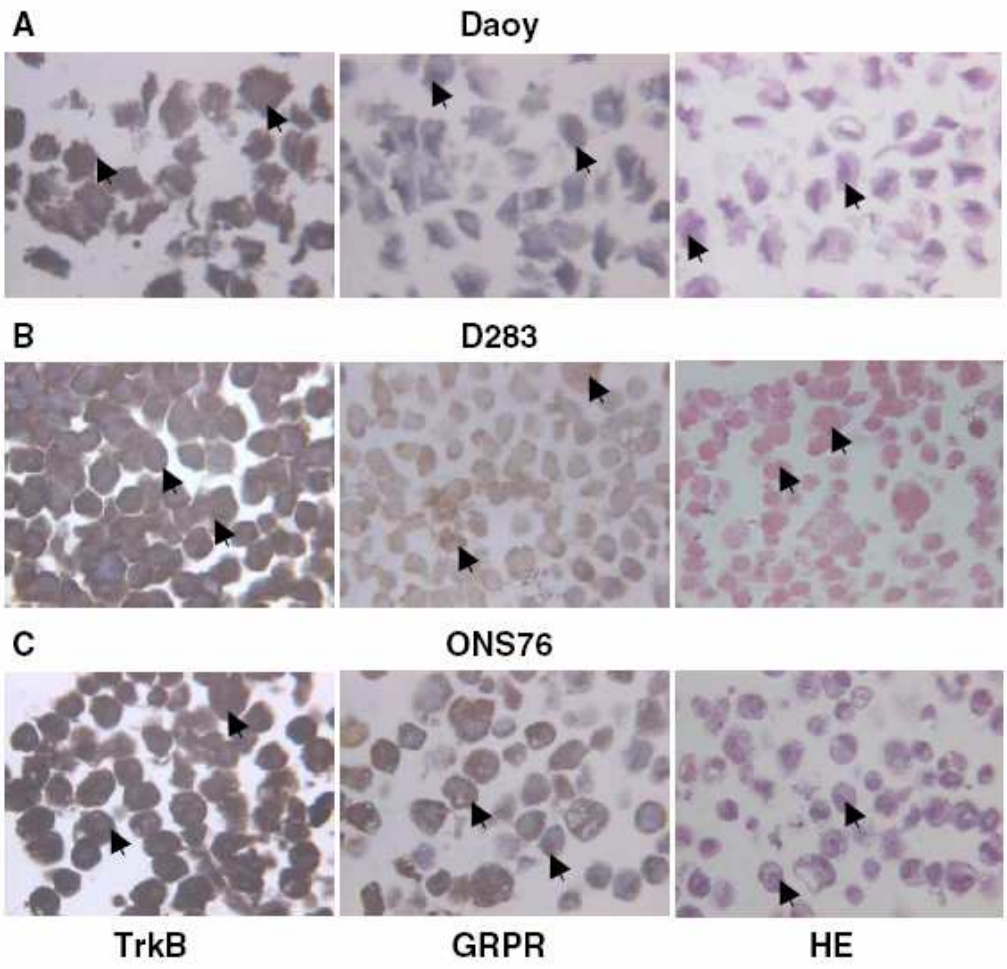


**Figure 1** Immunohistochemical analysis of TrkB receptor and GRPR expression in a Daoy, b D283, and c ONS76 human medulloblastoma cells. Sections were incubated with anti-TrkB or anti-GRPR antibody, sequentially treated with biotinylated anti-rabbit IgG and streptavidin–biotin–peroxidase solution, and then developed with diaminobenzidine as chromogen (*brown/left*, TrkB receptor expression on the membranes of two cells is indicated by *arrowheads*, ×1,000; *center/left*, GRPR expression on the membranes of two cells in indicated by *arrowheads*, ×1,000). Cell nuclei were lightly counterstained with hematoxylin–eosin as a control (*blue/right*, two cells indicated by *arrowheads*, ×1,000)

Cells were seeded in flasks of 25 $cm^3$. The cells grown were at least confluent. The cells were detached with a trypsin/ethylenediaminetetraacetic acid solution. After centrifugation, the cell pellet was resuspended in 3 mL of formol and embedded into paraffin wax. Four-micrometer-thick sections were mounted on organosilane-coated slides and dried overnight at 37 C. Sections were deparaffinized in stove, rehydrated in graded alcohols, and washed with distilled water. The procedure to antigenic recuperation was performed in the microwave, the inactivation of the endogenous peroxidase through immersion in hydrogen peroxide, and blocking cross-reaction with normal serum. The primary antibody diluted in solution (1:50) was incubated for 12 h at 4 C, followed by an application of the streptavidin–biotin–peroxidase complex (LSAB, Dako) and the revelation with diaminobenzidine tetrahidroclore (Kit DAB, Dako). Cell nuclei were lightly counterstained with hematoxylin–eosin as a control (Farias et al. 2008; Flores et al. 2008).

### Statistics

Data are shown as the mean ± standard error of the mean (SEM) number of cells. Differences between mean values were evaluated by one-way analysis of variance followed by Tukey post hoc tests when appropriate. In all comparisons, $P<0.05$ was considered to indicate statistical significance.

## Results

### Immunohistochemical Detection of TrkB Receptors and GRPRs in Medulloblastoma Cells

We first verified whether TrkB and GRPR were expressed in Daoy, D283, and ONS76 cells. Immunohistochemical analysis using a mouse monoclonal antibody raised against an extracellular domain of the human TrkB receptor or a synthetic rabbit polyclonal antibody against GRPR corresponding to the second extracellular loop of human GRPR confirmed that all three cell lines express TrkB receptors and GRPRs (Fig. 1).

### RT-PCR Analysis of BDNF Expression in Medulloblastoma Cells

Reverse transcriptase polymerase chain reaction (RT-PCR) analyses demonstrated that Daoy, D283, and ONS76 cells express mRNA for BDNF. A transcript size of 362 bp, representing a fragment of BDNF, was identified in all cell lines (Fig. 2a).

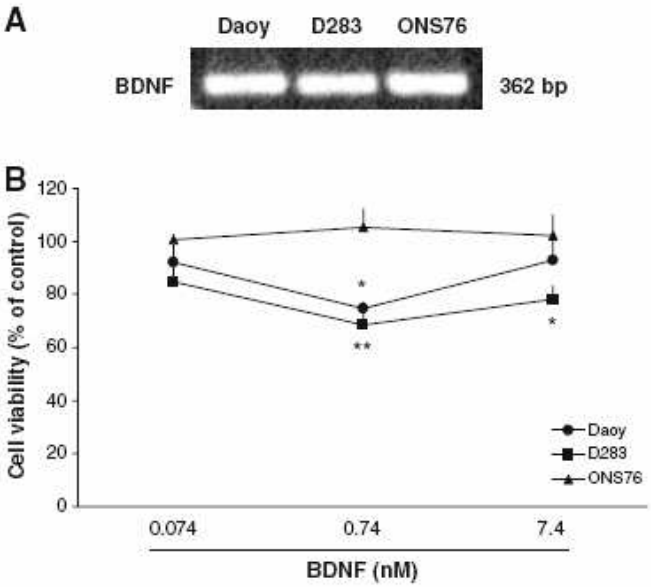


**Figure 2** BDNF inhibits the viability of human medulloblastoma cells in vitro. Daoy, D283, and ONS76 cells were treated with BDNF (1, 10, or 100 ng/ml). a RT-PCR analysis of BDNF mRNA expression in Daoy, D283, and ONS76 human medulloblastoma cells. RNA was extracted from the cells and RT-PCR analysis was performed as described in the "Materials and Methods" section. A transcript size of 362 bp representing a fragment of BDNF was identified. b Cell viability was measured using MTT assay 48 h after treatment with BDNF (0.074, 0.74, or 7.4 nM) as described in the "Materials and Methods" section. Data represent the mean ± SEM of three different experiments performed in sextuple wells each. The mean value for control cells was taken as 100%; *$P<0.05$ and **$P<0.01$ compared to control cells

### BDNF-Induced Inhibition of Medulloblastoma Cell Viability

Treatment with human recombinant BDNF at 0.74 nM significantly inhibited viability of Daoy cells assessed by the MTT assay ($P<0.017$ compared to control cells), whereas BDNF at either 0.74 or 7.4 nM inhibited viability in the D283 cell line ($P$s=0.002 and 0.02, respectively, compared to control cells). The viability of ONS76 cells was not significantly affected by BDNF (Fig. 2b).

### RT-PCR Analysis of GRPR mRNA Expression in Medulloblastoma Cells

RT-PCR analyses demonstrated that Daoy, D283, and ONS76 cells express mRNA for GRPR. A transcript size of 190 bp, representing a fragment of the GRPR, was identified in the cells (Fig. 3a).

### Lack of Effect of GRPR Stimulation or Inhibition on Medulloblastoma Cell Viability

Treatment with human recombinant GRP, the amphibian peptide and GRPR agonist bombesin, or the synthetic GRPR

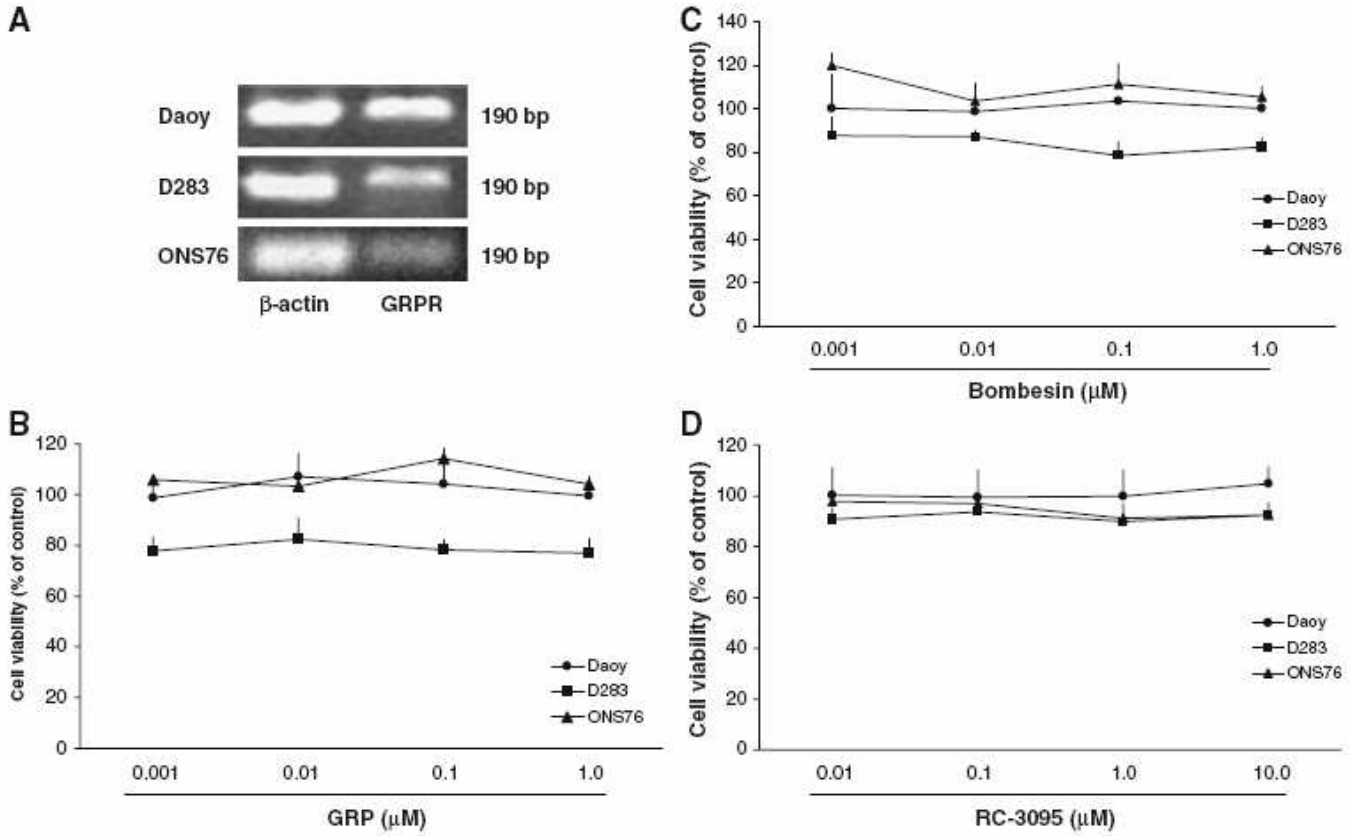


**Figure 3** Drugs acting on the GRPR do not affect the viability of human medulloblastoma cells in vitro. a RT-PCR analysis of GRPR mRNA expression in Daoy, D283, and ONS76 human medulloblastoma cells. RNA was extracted from the cells and RT-PCR analysis was performed as described in the "Materials and Methods" section. A transcript size of 190 bp representing a fragment of the GRPR was identified. Daoy, D283, and ONS76 cells were treated with b GRP (0.001, 0.01, 0.1, or 1 μM), c bombesin (0.00.1, 0.01, 0.1, or 1 μM), or d RC-3095 (0.01, 0.1, 1.0, or 10 μM). Cell viability was measured using MTT assay 48 h after treatment as described in the "Materials and Methods" section. Data represent the mean ± SEM of three to four different experiments performed in sextuple wells each. The mean value for control cells was taken as 100%. There were no significant differences among groups

antagonist RC-3095 did not significantly affect cell viability in the Daoy, D283, or ONS76 cells (Fig. 3b–d). Although the different doses of bombesin and GRP induced a reduction of about 13–23% in viability of D283 cells, these effects fell short of statistical significance (bombesin, $F_{4,10}=2.41$, $P=0.12$; GRP, $F_{4,10}=3.23$, $P=0.06$). The results indicate that modulation of GRPR activity does not significantly affect cell viability in human medulloblastoma cells.

### Inhibition of Medulloblastoma Cell Viability by Rolipram Alone or Combined with GRP

We examined the effects of rolipram alone or combined with GRP on the viability of medulloblastoma cells because we have previously observed that GRP promotes the viability of human U138-MG glioblastoma cells only when combined with the PDE4 rolipram or other agents that enhance cellular levels of cAMP (Farias et al. 2008). In Daoy cells, rolipram inhibited cell viability at all concentrations tested (1 μM, $P=0.03$; 10 μM, $P<0.001$; 100 μM, $P<0.001$ compared to control cells), confirming and extending previous findings (Schmidt et al. 2009). Cell viability was dose-dependently inhibited in D283 and ONS76 cells by rolipram at 100 μM ($P$s<0.001 compared to control cells; Fig. 4a). Rolipram inhibited the viability of Daoy cells also when combined with GRP ($P$s<0.001, comparisons between cells treated with rolipram alone or rolipram plus GRP compared to control cells; Fig. 4b).

## Discussion

Although the expression of both BDNF and its receptor TrkB has been detected in a subset of biopsy specimens of medulloblastoma (Tajima et al. 1998; Washiyama et al. 1996), previous studies have not verified whether BDNF influences the proliferation and viability of medulloblastoma cells. We found that treatment with human recombinant BDNF significantly inhibited the viability of two human medulloblastoma cell lines expressing both BDNF mRNA

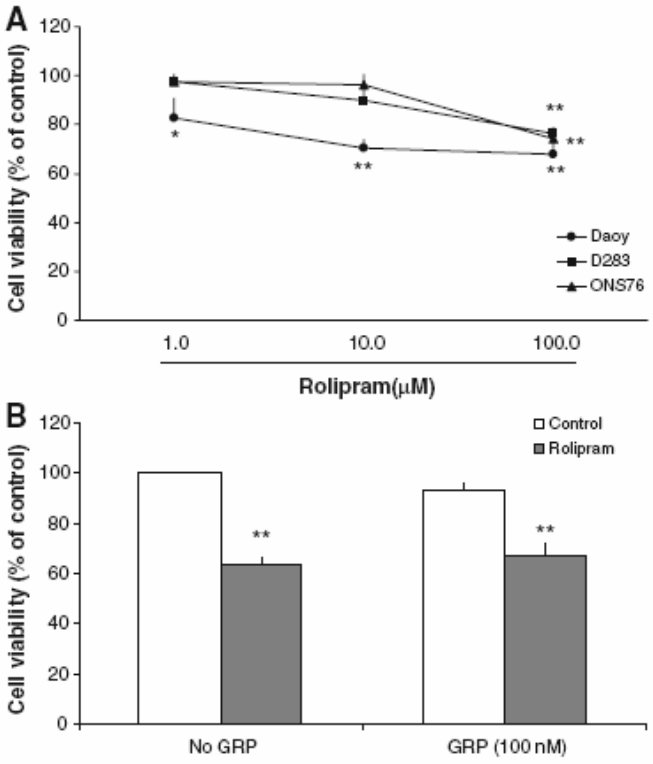


**Figure 4** The cAMP PDE4 inhibitor rolipram alone or combined with GRP inhibits the viability of human medulloblastoma cells in vitro. **a** Daoy, D283, and ONS76 cells were treated with rolipram (1, 10, or 100 μM); **b** Daoy cells were treated with rolipram (10 μM), GRP (0.1 μM), or rolipram combined with GRP. Cell viability was measured using MTT assay 48 h after treatment as described in the "Materials and Methods" section. Data represent the mean ± SEM of three to seven different experiments performed in sextuple wells each. The mean value for control cells was taken as 100%; $*P<0.05$ and $**P<0.01$ compared to control cells

and TrkB receptors. These findings suggest that BDNF might hinder rather than stimulate survival and proliferation of medulloblastoma cells. Although in neuronal cells BDNF is known to promote cell survival and neurogenesis (Bath and Lee 2006; Huang and Reichardt 2003), a few previous studies have indicated that BDNF can also reduce cell proliferation in the CNS. Thus, BDNF inhibits the proliferation of neural progenitor cells through a mechanism involving neuronal nitric oxide synthase in the mouse brain (Cheng et al. 2003). In addition, BDNF has been shown to reduce the proliferation of retinal cells, and a Trk receptor antagonist blocked this effect (dos Santos et al. 2003). It remains to be determined why BDNF affected cell viability at a lower, but not a higher, dose in Daoy cells. Previous studies have found biphasic dose–response patterns for the effects of BDNF. For instance, a low dose of BDNF promoted, while a higher dose inhibited, axonal regeneration in rat motoneurons (Boyd and Gordon 2002).

BDNF/TrkB signaling has been proposed as a target in several types of cancer (reviewed in Desmet and Peeper 2006). BDNF promotes survival and proliferation of ovarian and breast cancer cells (Descamps et al. 2001; Qiu et al. 2006) and activates antiapoptotic pathways in lung adenocarcinomas (Perez-Pinera et al. 2007). In neuroblastoma, BDNF and TrkB expression is associated with poor prognosis, increased invasiveness and metastatic potential, and enhanced therapy resistance (Cimmino et al. 2009). Thus, TrkB inhibitors have been put forward as a potential anticancer therapy (Desmet and Peeper 2006; Ruggeri et al. 1999). In contrast, we found that BDNF decreased the viability of medulloblastoma cells. In studies investigating TrkC receptor expression in medulloblastoma, the better outcome of patients showing higher levels of TrkC receptors has been associated with a possible differentiation-promoting activity of NT-3. The significance, if any, of the inhibitory effect of BDNF on cell viability observed in the present study for tumor progression in medulloblastoma remains to be determined. A further understanding of the role of BDNF and the TrkB receptor in influencing medulloblastoma growth may lead to the validation of the TrkB receptor as a novel therapeutic target in medulloblastoma.

Previous studies on the role of the GRPR in the proliferation and viability of cancer cells have shown that GRPR agonists enhance, whereas antagonists inhibit, the proliferation of a variety of in vitro and in vivo experimental cancers (reviewed in Cornelio et al. 2007). We and others have shown that bombesin stimulates and GRPR antagonists, such as RC-3095, reduce the proliferation of glioma cells in vitro when used at doses and treatment schedules similar to the ones used in the present study (de Oliveira et al. 2009; Flores et al. 2008; Kiaris et al. 1999; Pinski et al. 1994). In Neuro2A neuroblastoma cells, we recently found that RC-3095 either inhibits or promotes cell proliferation depending on the dose (Abujamra et al. 2009). The mechanisms involved in the inhibition of cancer cell proliferation by GRPR antagonism might include altered expression and/or release of neurotrophins (Farias et al. 2009). Surprisingly, although we found that all three medulloblastoma cells used express both the mRNA and protein for GRPR, cell viability was not affected by GRP, bombesin, or RC-3095. Although we did not examine the expression of GRP (the endogenous GRPR ligand) in medulloblastoma cells, previous studies using human glioma cell lines have shown that cells that do not express the mRNA for GRP can still express GRPR and respond to GRP and RC-3095 treatments, suggesting that GRP may stimulate brain tumor cells as a paracrine rather than an autocrine mechanism (Kiaris et al. 1999). One possibility to explain the lack of effect of GRPR ligands in medulloblastoma cells would be that, in medulloblastoma, GRP acts by regulating differentiation rather than as a growth factor promoting mitogenesis. Such a role for GRP as a morphogen contributing to tumor differentiation has been proposed in colon cancer (Glover et al. 2005). Given that

the GRPR is now established as a therapeutic target in cancer and GRPR antagonists have been developed as potential anticancer agents, it will be important to determine whether GRPR signaling is involved or not in medulloblastoma formation and progression.

We have recently found that GRP failed to affect the viability of cultured human glioblastoma cells when given alone, but significantly promoted cell viability when combined with rolipram or other stimulators of cAMP signaling (Farias et al. 2008). We thus aimed to examine whether GRP would affect the viability of medulloblastoma cells when combined with rolipram. Rolipram inhibits PDE4 leading to an increase in cellular levels of cAMP and stimulation of the cAMP/protein kinase A pathway. Goldhoff et al. (2008) have recently shown that rolipram promoted tumor regression and enhanced survival in mice bearing U87-MG glioblastoma xenografts. In addition, these authors showed that PDE4A1 overexpression in medulloblastoma cells stimulated tumor growth. Consistent with these findings and our own previous results using Daoy cells (Schmidt et al. 2009), rolipram induced a significant inhibition of the viability of all three medulloblastoma cell lines used in this study. The inhibitory effect of rolipram in Daoy cells was not modified by cotreatment with GRP, providing further evidence that, in medulloblastoma cells, GRP does not promote cell survival or viability.

Together, our results indicate that (1) BDNF can inhibit the viability of human medulloblastoma cells in vitro; (2) the GRPR is expressed in medulloblastoma cells, but might not be involved in regulating cell viability in medulloblastoma; and (3) the PDE4 inhibitor rolipram inhibits the viability of medulloblastoma cells regardless of concomitant GRPR stimulation. The possible involvement of GRPR in the differentiation of medulloblastoma cells, as well as the involvement of the TrkB receptor and PDE4 in regulating medulloblastoma growth and their potential as therapeutic targets in medulloblastoma, should be further explored in future studies.

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## 3.2 CAPÍTULO II: Phosphodiesterase-4 Inhibition and Brain Tumor Growth

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# Phosphodiesterase-4 Inhibition and Brain Tumor Growth

**To the Editor:** We read with great interest the December 1, 2008, article by Goldhoff et al. (1) on the role of cyclic AMP phosphodiesterase-4 (PDE4) in brain tumor growth. The authors found that PDE4 is expressed in human brain tumors of glial and neuronal lineage (glioblastoma, medulloblastoma, ependymoma, and meningioma). In addition, overexpression of PDE4A1, a brain-specific isoform of PDE4, in Daoy medulloblastoma and U87 glioblastoma cells was associated with increased tumor growth in intracranial xenografts. Conversely, the PDE4 inhibitor rolipram promoted tumor regression and enhanced survival in mice bearing U87 xenografts. Based on these findings, the authors conclude that PDE4 is a novel molecular target for brain tumor therapy and PDE4 inhibitors such as rolipram should be evaluated in dinical trials for malignant brain tumors. We would like to address two issues concerning the study by Goldhoff et al. (1).

First, we too have recently examined the effects of rolipram on the growth of brain tumor cells and have found results complementary to those of Goldhoff et al. (1). Although Goldhoff et al. (1) have shown that PDE4A1 overexpression in medulloblastoma cells stimulated tumor growth and rolipram suppressed the growth of glioblastoma xenografts, they did not address whether rolipram can also inhibit the growth of experimental medulloblastoma. We have recently observed that rolipram significantly inhibits proliferation of Daoy medulloblastoma cells *in vitro*. In our experiments, treatment with 10 μmol/L rolipram reduced cell viability measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay by 36.12% compared with controls at 48 hours after treatment ($P < 0.001$, $n = 3$ experiments in sextuples). This finding supports and extends those reported by Goldhoff et al. (1) and to our knowledge provides the first evidence that rolipram inhibits proliferation of medulloblastoma cells.

Second, although we agree that PDE4 is a promising target for the development of novel therapeutic strategies for glioblastoma and medulloblastoma, we would like to point out that rolipram might, under certain conditions, stimulate rather than inhibit brain tumor cell growth. We have recently reported (2) that rolipram as well as other agents that enhance cellular cyclic AMP levels induced a significant increase in proliferation of U138-MG human glioblastoma cells when the cells were stimulated by gastrin-releasing peptide, a neuropeptide that acts as a growth factor in several types of cancer. These findings suggest that additional studies are required to further characterize the effects of PDE4 inhibitors in preclinical models before rolipram is evaluated in clinical trials for malignant brain tumors.

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## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## 3.3 CAPÍTULO III: Recent Therapeutic Advances for Treating Medulloblastomas: a Focus on New Molecular Targets

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RECENT THERAPEUTIC ADVANCES FOR TREATING MEDULLOBLASTOMA: A FOCUS ON NEW MOLECULAR TARGETS

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**Abstract:** Medulloblastoma is the most common malignant brain tumor in children. This malignant tumor of the cerebellum commonly affects children and is believed to arise from the precursor cells of the external granule layer or neuroepithelial cells from the cerebellar ventricular zone of the developing cerebellum. The standard treatment, consisting of surgery, craniospinal radiotherapy and chemotherapy, still provides a poor overall survival for infants and young children. Furthermore, the dose of radiation that can be safely given without causing extensive neurocognitive and endocrinologic sequelae is limited. Therefore, the understanding of the oncogenic pathways that lead to medulloblastoma, as well as the identification of specific molecular targets with significant therapeutic implications in order to develop new strategies for therapy, is crucial to improve patient survival without substantially increasing toxicity. In this review, we discuss recent therapeutics for treating medulloblastoma, focusing on new molecular targets, as well as advances in translational studies for the treatment of this malignancy.

**Keywords:** medulloblastoma, CNS tumor, cancer therapies, molecular targets, translational studies.

## INTRODUCTION

Medulloblastoma is the most common malignant brain tumor in children. This tumor of the cerebellum commonly affects children; less than 30% of medulloblastoma cases occur in individuals older than 16 years. Medulloblastoma is considered an embryonic tumor since it originates from precursor cells present during cerebellar development, and may derive from cerebellar granule cell precursors, which form a layer of committed proliferating cells in the subpial surface of the cerebellum (external granule layer) during the fetal and the postnatal period. Neuroepithelial cells of the cerebellar ventricular zone, displaced around the midline of the neuroectodermal tube, may constitute the other cells that may originate medulloblastomas. The standard treatment consists of surgery, craniospinal radiation therapy and chemotherapy, however these strategies still provide poor overall progression-free survival rates in infants and young children, and a disappointingly high incidence of sequelae in patients. Most children, as well as adults, who survive the various treatment-related toxicities have significant neurological and cognitive impairment. An understanding of the signaling pathways that play a role in the development of medulloblastoma is therefore necessary in order to discover promising targets and develop more effective and less toxic therapeutic strategies to manage this malignancy. It is clear that the sonic hedgehog (SHH), wingless (Wnt) and Notch pathways play a crucial role in medulloblastoma signaling, and that growth factor receptor pathways, specially those which activate Myc, represent promising targets for treating these tumors. This review focuses on the new molecular targets for treating medulloblastoma by providing an overview of the molecular biology of this malignancy and how its understanding may improve therapies and outcomes for patients. Indeed, the increasing number of studies focusing on the molecular signals involved in the development and exacerbation of medulloblastoma, as well as the advances in translational studies, may change the management of this disease in the near future.

## MEDULLOBLASTOMA

Medulloblastoma is an embryonic tumor that accounts for 16% of all brain tumors in children from 0 to 4 years of age; its incidence decreases with age, and therefore it accounts for approximately 2% of all adult brain tumors. With a higher incidence in males than females, the median age of diagnosis is well under 20 years of age [1]. Children under the age of 3 diagnosed with medulloblastoma have an inferior survival in comparison to older children. This tumor is also highly metastatic, with up to 30% of children having evidence of disseminated disease at diagnosis. [2]

Medulloblastoma exemplifies one of the most common individual histologies found in CNS tumors in patients up to 19 years of age. Although not conclusively established, it has been postulated to arise from the two germinal zones of the cerebellum: the ventricular zone,

which contains stem progenitor cells, for classic and midline tumors [3], and the external granular layer for the less common, laterally placed and often desmoplastic tumors that possibly arise from more restricted neuronal progenitor cells [4,5]. Recent studies have shown that medulloblastoma can be initiated in neuronal lineage-restricted progenitors, and also in stem cells [6].

These tumors are divided by the World Health Organization in subsets that include desmoplastic/nodular medulloblastoma, medulloblastoma with extensive nodularity, anaplastic, and large cell medulloblastoma [7]. Although it has become clear that the deregulation of signaling pathways that are essential for brain development, such as sonic hedgehog (SHH), Wnt, and Notch pathways, plays an important role in the pathogenesis and biological behavior of medulloblastoma [8-10], the etiology of this tumor in the pediatric population remains unknown and is more onerous to elucidate, since environmental factors, such as smoking, diet, and other exposures, do not likely predispose to its development [11]. Although it is known that syndromes such as Li-Fraumeni, Gorlin's, Turcot's A and Rubenstein-Taybi are associated with medulloblastoma formation, only a small proportion of brain tumors are caused by hereditary gene defects, as well as by irradiation or immune suppression [8]. As the genetic alterations mentioned above do not account for all instances of medulloblastoma formation, other pathways have been investigated to better understand the pathogenesis of these tumors.

**TREATMENT**

The standard treatment for medulloblastoma consists of maximal surgical resection, craniospinal radiotherapy and intravenous cisplatin-based chemotherapy and other drugs which have a role in combined chemotherapy. There are also efforts to substitute cisplatin with other drugs due to its serious side effect of hearing loss [12].

With current means of therapy, children with non-disseminated medulloblastoma have a high likelihood of long-term survival, considering that 80% or more will be alive 5 years after diagnosis and treatment, many free of the disease [13]. In the other hand, approximately 30% of patients with medulloblastoma will have evidence of disseminated disease at diagnosis, and in younger children, especially infants, the likelihood of disseminated disease is higher. Even in those without evidence of frank dissemination, because treatment with primary- site irradiation alone results only in disease control in less than 20% of patients, the standard therapy is craniospinal radiotherapy supplemented with local boost radiotherapy. Treatment with radiotherapy to the entire neuroaxis is associated with 5-year disease-free survival rates of 50% to 60% in patients with average-risk disease and 40% or less in patients with high-risk disease [14].

Average risk medulloblastoma have 5-year event-free survival of 81% and 5-year overall survival of 86% in patients treated with reduced-dose craniospinal radiotherapy and one of two postradiotherapy chemotherapies: lomustine, cisplatin, and vincristine, or cyclophosphamide, cisplatin, and vincristine, according to the phase III randomized clinical trial published by Packer and colleagues in 2006 [15]. New therapies are needed for infants and young children, for children with high-risk disease at diagnosis and for all patients with recurrent disease who present a two-year survival rate after relapse in medulloblastoma of 5 to 20% [16].

Although there have been advances in survival achieved by conventional therapy, there is a strong need for novel, less toxic treatment for medulloblastoma. Standard treatment with radiation and chemotherapy has significant negative effects on the quality of life of long-term survivors. Neurocognitive difficulties are one of the most pervasive of all long-term effects across all age groups [17], since the doses of radiotherapy required for disease control cause significant brain injury, which is especially damaging in younger children [18]. Full-scale intelligence quotients drop 20 to 30 points about 2 to 3 years after radiotherapy, and the development of significant endocrinologic sequelae, such as growth hormone insufficiency with resultant linear growth retardation, are observed in most patients [18-19]. Growth hormone replacement therapy has been introduced in those cases [20]. Treatment-related toxicities may also cause secondary tumors [21], ototoxicity, gynecological toxicity and subsequent neonatal complications, cardiac toxicity, pulmonary toxicity and, at times, complications that lead to the patient´s death [22]. While the survival of older children with non-metastatic medulloblastoma can reach a 5-year survival rate of 50% to 70%, the outcomes for infants, younger children and those with metastatic medulloblastoma are highly disappointing [23]. In high-risk patients treated with surgery and radiation therapy, progression-free survival rates of up to 40% can be achieved, approaching 50% with the addition of chemotherapy, which is still considered subpar by the medical community [24]. Even though many advances for treating medulloblastoma in the clinic have been made, (for review see [25-27]) most conventional treatments fail, and therefore new alternative treatments are still warranted. Moreover, the means to improve long-term quality of life by reducing sequelae without compromising disease control remain controversial.

There is increasing evidence that prognosis, and possibly the response to therapy, depend on the tumor's histological variant and on the signaling pathways involved in tumor development and growth [28]. New strategies of risk stratification based on genetic and molecular approaches have been proposed, but have not yet found their way into clinical practice [29]. Additional knowledge about the activated oncogenic pathways in medulloblastoma may therefore elucidate the characteristics of development and proliferation of this tumor and contribute to the development of new molecular therapeutic targets.

## MOLECULAR TARGETS

### Sonic Hedgehog (SHH), Wingless (Wnt) and Notch pathways

The known mutations found in syndromes that predispose patients to the development of medulloblastoma are frequently found in genes related to cellular signaling pathways that regulate brain development, such as sonic hedgehog (SHH), wingless (Wnt), and Notch.

The SHH pathway mediates the signal transduction that promotes growth during normal cerebellar development, and approximately 10% to 30% of patients diagnosed with medulloblastoma have mutations that invariably result in SHH-pathway activation, such as increased expression of the SHH downstream transcription factor GLI1, which leads to exacerbated cellular proliferation [30-33]. Gorlin syndrome-affected patients, who have inactivating mutations on the gene that encodes the SHH receptor, Patched 1 (PTCH1), which negatively regulates SHH signaling, are prone to medulloblastoma [34]. Furthermore, tumors of $PTCH^{+/-}$ mutated mice, which spontaneously develop medulloblastoma, show increased expression of genes involved in the activation of both SHH and Wnt signaling pathways [35]. Inhibitors of SHH, able to block smoothened (SMO), the transmembrane receptor that responds to PTCH1, have been developed. These SHH inhibitors, such as cyclopamine (GDC-0449) and HhAntag-691, downregulate the downstream proteins in the SHH pathway and have successfully eliminated medulloblastoma in mouse models for pre-clinical studies [36-42].

Activation of the Wnt pathway, after ligand binding to its receptor, Frizzled (FRZ), causes destabilization of the adenomatous polyposis coli (APC) complex, resulting in decreased β-catenin degradation, translocation of beta-catenin into the nucleus, and activation of downstream transcription factors and Wnt targets, such as c-myc and cyclin D1, which are mediators of the proliferation of cerebellar granule cell precursors [44, 45]. Turcot's syndrome, which is associated with a mutation in the APC gene, is associated with a higher incidence of medulloblastoma. Activating mutations in the Wnt pathway, most of them concerning the β-catenin gene, may occur in 15% of medulloblastoma cases [46, 47]. Abnormalities in the Wnt signaling pathway have been associated with a molecularly distinct subset of tumors with a more favorable prognosis [47, 48]. Another marker associated with activation of the Wnt signaling pathway is survivin. This apoptosis inhibitor, which is highly expressed during neurogenesis, is upregulated in medulloblastoma and is an unfavorable outcome predictor of recurrent medulloblastoma and basal cell carcinoma, [49, 50] independent of clinical staging or tumor histology [51, 52]. Furthermore, the Wnt pathway can be regulated by the SOX gene family members [53], and there is a trend towards better survival with increasing SOX4 expression [54], which may be overexpressed in medulloblastoma [55, 56].

The Notch pathway is involved in cell fate determination and differentiation in various cells and tissues. Notch is also critical for the growth and survival of SHH-induced medulloblastoma, since Notch2, which is normally expressed in proliferating cerebellar granule cell precursors, is overexpressed in a subset of medulloblastoma. Activation of the Notch signaling pathway results in the activation of HES1 transcription factor [57], which is associated with decreased survival rates in medulloblastoma patients [58]. Treatment of medulloblastoma xenografts with soluble Delta ligand or gamma secretase inhibitors of the Notch signaling pathway results in decreased proliferation and increased apoptosis [59].

Although the knowledge and therapeutic strategies involving SHH, Wnt and Notch signaling pathways (Fig. 1) have evolved and brought valuable therapeutic strategies, there are still improvements to be undertaken. Other pathways involved in brain development and cellular differentiation are also being investigated as promising targets for treating medulloblastoma, considering their embryonic background.

----------Figure 1----------

**Other Targets From Developmental Pathways**

Although data from clinical studies and animal models suggest an important interaction between SHH, Wnt and Notch pathways in medulloblastoma pathogenesis [60], other factors are involved in the biological background of medulloblastoma that cannot be elucidated by the activation of these three embryonic pathways. Therapies targeting other molecular or genetic abnormalities that are likely a contributing factor to the development and progression of medulloblastoma are recently under study [61, 62] and two of them are exemplified below.

*Neurotrophins*

Neurotrophins are growth factors required by discrete neuronal cell types for survival and maintenance, with a broad range of activities in the central and peripheral nervous system in the developing and adult mammal. Neurotrophic factors regulate proliferation, differentiation and apoptosis of neuroectodermic cells. Their role is so well established that the expression of neurotrophin receptors has been used as a prognostic factor for neuroectodermic tumors.

Neurotrophin-3 receptor (also known as TrkC) was the first of the tyrosine-kinase growth factor receptors to be associated with clinical aspects in medulloblastoma. There is increasing evidence that it plays a broader role than being just a passive marker of positive prognosis [63, 64], since TrkC activation by its ligand, neurotrophin-3 (NT-3), affects disease outcome by inhibiting tumor growth through the promotion of apoptosis [65]. Low TrkC expression is related to an unfavorable outcome, and alterations in this receptor are found in up to 48% of medulloblastoma cases [61]. There is also recent evidence that neurotrophin-

regulated heparanase (HPSE), a unique extracellular matrix-degrading enzyme associated with tumor progression in a wide variety of cancers and expressed in up to 76% of medulloblastoma cases, may be involved in tumor invasion and progression in a context linked to TrkC and p75 receptor activation [66].

Nerve growth factor receptor (TrkA, also known as NGF-R) expression is also associated with increased levels of apoptosis in medulloblastoma cells [67]. NGF exposure blocks cellular proliferation and promotes the expression of TrkA in these malignant cells [68], suggesting that NGF, by interfering with mechanisms associated with proliferation and survival, might induce differentiation through TrkA-mediated pathways. It has been shown that NGF induces apoptosis in medulloblastoma cell lines that express TrkA [69]. Furthermore, Zhangfei, a transcription factor expressed in differentiated neurons, also leads to differentiation and apoptosis by inducing the expression of TrkA in medulloblastoma cells [70]. Zhangfei was detected in mature neurons but not in neuronal tumor cells, suggesting that ectopic expression of this protein in medulloblastoma cells may induce their differentiation [70]. Neurotrophins may also be beneficial to patients receiving overly toxic treatments. For example, co-treatment with NGF and cisplatin can also reduce cisplatin-induced cytotoxic side-effects [71].

*Hepatocyte Growth Factor (HGF)*

The hepatocyte growth factor (HGF) and its tyrosine kinase receptor, Met, are known to contribute to both normal cerebellar development as well as to the development and progression of human brain tumors, and therefore have emerged as a new pathway involved in medulloblastoma growth [72, 73]. HGF and c-Met are commonly expressed in malignant gliomas and embryonic neuroectodermal tumors, including medulloblastoma. It has been suggested that HGF is functionally linked to c-Myc by inducing its expression [74], which involves both PI3K signaling and the Wnt pathway. However, the role of HGF and c-Met signaling in medulloblastoma is not fully understood, since it can either promote or inhibit medulloblastoma cell death via pathway- and context- specific mechanisms.

Interestingly, the HGF-mediated induction of c-myc promoted proapoptotic effects in part via downregulation of the Bcl-XL anti-apoptotic protein [74]. The c-myc-induced apoptosis regulated by Bcl-XL has been reported in other cell types. This c-myc deregulation is proposed to be involved in disease progression rather than its induction [75], as c-myc appears to play a causal role in inducing anaplasia in recurrent medulloblastoma. HGF-induced activation of the Met receptor also results in Tissue Factor expression by medulloblastoma cells. Since Tissue Factor up-regulation is often observed on the surfaces of cancer cells and high Tissue Factor levels may be associated with poor prognosis in cancers, it is suggested that Tissue Factor expression stimulated by HGF may contribute to

tumor proliferation by enabling the formation of a provisional fibrin matrix [76]. HGF also promotes medulloblastoma cell death induced by the death receptor ligand TRAIL (tumor necrosis factor-related apoptosis-inducing ligand). Treating cells with a specific c-Met receptor tyrosine kinase inhibitor, PHA-665752, abrogated the enhancement of TRAIL-induced cell death by HGF, indicating that this effect requires Met activation [77].

On the other hand, HGF is neuroprotective for cerebellar granule cells and promotes growth of human medulloblastoma cells in culture and in murine xenografts. Furthermore, there is a high frequency of medulloblastoma formation in mice after postnatal expression of HGF in cooperation with SHH, and systemic administration of a monoclonal antibody against HGF prolonged survival of these mice by stimulating apoptosis. This finding indicates a role for HGF in medulloblastoma initiation and growth and shows the efficacy of HGF-targeted therapy in a mouse model of endogenously arising tumors [78].

**Crosstalk Pathways**

Crosstalk pathways have been proven to play a role in various types of cancers. The SHH and Wnt signaling pathways not only interact with each other and with Notch, but also with other signaling pathways, such as those activated through epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor (IGFR) signaling. Four tyrosine-kinase growth factor receptors are now associated with medulloblastoma: TrkC, TrkA, ErbB2, and platelet-derived growth factor receptor (PDGF-R), all of which have been a research focus into small-molecule target therapy.

*ErbB2*

Epidermal growth factor (EGF) receptor tyrosine kinases regulate cell behavior during normal development and include ErbB1, ErbB2, ErbB3 and ErbB4 receptors [79-81]. These are activated by a variety of ligands, including various neuroregulins which are important in regulating the development of neuronal tissue. The ErbB2 receptor (also known as Her2/neu), found in up to 84% of medulloblastoma cases, also has a prognostic impact in this malignancy and is regarded as a potential medulloblastoma oncogene, since its high expression is related to an unfavorable outcome [82-84]. There is also evidence suggesting that ErbB signaling is regulated by SHH signaling [85]. ErbB2 and ErbB4 co-expression is related to an increased risk of metastases and is associated with poor prognosis in medulloblastoma [86]. Interestingly, CYT1 is the only isoform of ErbB4 that is able to activate the antiapoptotic phosphatidyl inositol 3-kinase (PI3K)/protein kinase B (PKB)/AKT signaling, [87] which is important in medulloblastoma development. Overexpression of the specific CYT1 ErbB4 isoform correlates with ErbB2 expression levels and the anaplastic medulloblastoma subtype. ErbB4, especially the CYT1 isoform, is overexpressed in tumors

with low Gli1 levels, which is a SHH pathway effector, suggesting that ErbB signaling is regulated by SHH signaling [85].

Considering the ErbB pathway as a target, the OSI-774 compound (erlotinib) inhibits ErbB2 signaling in human medulloblastoma cells and may have a therapeutic potential [84]. Clinical trials using erlotinib and lapatinib, a potent reversible ErbB1 and ErbB2 inhibitor, are in course.

*Insulin-like growth factor 1 receptor (IGFR)*

Targeting the IGF signaling pathway represents a promising strategy in the development of novel anti-cancer therapeutics [88-91]. The therapeutic potential of targeting the IGF signaling pathway is derived from the role it plays in the promotion of cell growth and inhibition of apoptosis. The insulin-like growth factor 1 receptor (IGF-1R) is also overexpressed in medulloblastoma cell lines and tumor samples, [92] and more than half of medulloblastoma patients express the activated, phosphorylated form of IGF-1R [93, 59]. The IGF-1R ligands, IGF-1 and IGF-2, are involved in controlling the proliferation of medulloblastoma and of cerebellar precursor cells, [94, 95] and IGF-2 is a downstream target of SHH signaling [96]. Strategies to impair the function of IGF-IR could supplement conventional therapeutic regimens against medulloblastoma by compromising the growth and survival of these cells. Inhibition of IGF-1R signaling by a dominant-negative IGF-1R mutant, or with antisense oligonucleotides against the IGF-1R mRNA, reduces medulloblastoma tumor growth [97]. Several clinical trials have investigated new therapies that target the extracellular domains of the IGF-R, but there are no studies evaluating the effect of those agents in medulloblastoma. Receptor blockade with the use of monoclonal antibodies against the IGF-1R as an anti-cancer strategy has been the most clinically investigated approach to date, and already has suggested benefits in different cancer types when used alone [98] or combined with chemotherapy [99]. Moreover, data has accumulated to suggest that a bi-directional crosstalk pathway between the ErbB family of receptors and IGF-1R pathway exists and may be responsible for resistance to targeting these receptor pathways [100].

*PDGFR*

PDGF-Rβ overexpression is associated with metastasis in medulloblastoma [101]. PDGF-Rα is also highly expressed in this malignancy and is a target of imatinib mesylate, a tyrosine kinase inhibitor that also has an effect on c-kit. C-kit (also known as CD117) is a cytokine receptor expressed on the surface of hematopoietic stem cells as well as other cell types. Signaling through c-kit, which is highly expressed in medulloblastoma, plays a role in cell

survival, proliferation, and differentiation, and altered forms of this receptor may also be associated with other types of cancer [102-104].

It has been shown that treating medulloblastoma cell lines with imatinib inhibited migration and invasion, and induced apoptosis and inhibition of cellular proliferation. This indicates that PDGF-Rβ tyrosine kinase activity is critical for migration and invasion of medulloblastoma possibly by transactivating EGFR, and therefore that imatinib may represent an important novel therapeutic agent for the treatment of medulloblastoma [105]. There is also considerable enthusiasm in adding ErbB2 and PDGF-Rα antagonists to conventional chemotherapy and radiotherapy.

It is important to mention that the relation between cellular pathways that affect both brain development and cell proliferation and differentiation cannot be analyzed as a simple phenomenon. The results of activating various growth factor receptors may occur via crosstalk among downstream effectors, since signal components can be shared between different signaling pathways. As a result, responses to one initiating signal may activate multiple simultaneous responses.

**Downstream Signaling**

*RAS/MAPK*

The RAS/mitogen activated protein kinase (RAS/MAPK) pathway is the most frequently activated downstream pathway upon ligand binding to transmembrane growth factor receptors. This pathway has been implicated in the development of medulloblastoma and recent studies have demonstrated that members of the RAS/MAPK signaling pathway are upregulated in metastatic tumors [106]. Even though there are evidences demonstrating that this mechanism contributes to tumor development, activating mutations in established mutational hotspots within the PDGFR–RAS/MAPK pathway have proven to be rare events in medulloblastoma [107]. Nonetheless, highly effective and relatively non-toxic therapies for medulloblastoma have been developed to target the RAS/MAPK pathway. One such target is farnesyltransferase, an enzyme responsible for post-translational modification of H-RAS, K-RAS and N-RAS proteins. Antisense oligonucleotides against RAS and RAF, and kinase inhibitors targeting RAS effector pathways and pathways upstream of RAS are also employed as therapy [108, 109].

*Myc Pathway*

Another important target involved in many human malignancies is the Myc transcription factor family. The myc proto-oncogenes, all members of the bHLH transcription factor family, are comprised of c-myc, N-myc and L-myc, and are involved in cell-cycle regulation, proliferation and differentiation [110, 54]. Myc genes play a major role in human

oncogenesis. The c-myc oncogene is implicated in a wide variety of human tumors, including Burkitt's lymphoma [111] and breast cancer [112]. N-myc is overexpressed in a more restricted set of malignancies, predominantly neuroblastoma and related tumors, such as medulloblastoma, while L-myc is over-expressed in small-cell lung carcinomas as a result of gene amplification [113-115]. In medulloblastoma, in addition to c-myc amplification, c-myc overexpression is also associated with poor survival [116] and often associated to the large cell/anaplastic medulloblastoma phenotype [117]. Myc activation can be caused by activation of the SHH and Wnt pathways, and PTCH1 deletion increases N-myc protein stability as a mechanism of medulloblastoma initiation and progression [118, 119]. Over 40% of medulloblastoma patients present c-myc alterations. Potential strategies that either inhibit the growth promoting effect of Myc in tumor cells and/or activate its pro-apoptotic function are presently being explored, which could have potential to combat and eradicate tumors cells in combination with conventional anti-tumor therapy [120]. It would be highly desirable to identify Myc inhibitors that can be brought to clinical trials for medulloblastoma treatment [121], however targeting Myc as a therapeutic strategy has always brought concerns that it would induce serious side-effects by inhibiting the proliferation of normal tissues. Nonetheless, a recent study has shown that mice tolerated the effects of extended Myc inhibition by dominant-interfering Myc mutant expression. Myc inhibition also triggered rapid regression of incipient and established K-Ras-induced lung tumors, suggesting that targeting Myc can be an effective, efficient and tumor-specific cancer therapy [122].

Another way of targeting Myc would be through the use of small molecules [123-128]. The small molecule 10058-F4 has been extensively studied and found to induce cell-cycle arrest, apoptosis, and differentiation in some cells [129-132]. *In vitro* data indicate that cell-cycle arrest is induced in medulloblastoma via inhibition of c-myc [133]. In neuroblastoma, direct targeting of N-myc can also be achieved by inducing differentiation using retinoic acid (RA), which led to considerable interest in retinoids. In preclinical models, RA has been shown to induce apoptosis in medulloblastoma and neuronal differentiation in cultured cell lines and in xenografted mice [134] via bone morphogenetic protein-2 (BMP-2) transcription and caspase activation [135]. RA derivatives, such as 13-cis-retinoic acid, all-trans-retinoic acid and fenretinide, are currently in development for use in clinical trials [136], with 13-cisretinoic acid already being tested in combination with chemotherapy in patients.

*p53*

The p53 transcription factor is important in regulating cell-cycle progression and thus functions as a tumor suppressor. Established as an essential protein for interrupting DNA replication in cells with damaged DNA, it is widely involved in preventing cancer [137]. Little is known about the differences in p53 expression and function among the various embryonal

brain tumor subtypes. The *TP53* gene encoding the p53 protein is probably the most commonly mutated gene in human cancers, but it is infrequently mutated in primary childhood tumors, including medulloblastoma – studies suggested that p53 is mutated in 10% or less of medulloblastoma cases [138-141]. However, inactivation of p53 contributes significantly to medulloblastoma and neuroblastoma development in specific animal models [142]. Furthermore, individuals with the Li-Fraumeni syndrome carrying germ-line mutations of p53 may develop medulloblastoma [143]. p53 immunopositivity for accumulation has been suggested to be associated with poorer survival in medulloblastoma [144], and significantly increased p53 protein levels were found in anaplastic medulloblastoma as compared to classic and nodular medulloblastoma. Interestingly, p53 protein accumulation, which is often associated with loss of functionality, has been found by some, but not all studies. The incidence of p53 mutations, as well as of p53 inactivating binding protein MDM2, overexpression are very rare in medulloblastoma. However up to 40% of medulloblastoma express a dysfunctional p53 protein. p53 inhibition by PAX5, an early development gene, is also suggested to play a role in medulloblastoma development since PAX5 expression is deregulated in approximately 70% of cases [8].

*Protein kinase A (PKA) Pathway*

Protein kinase A (PKA) refers to a family of enzymes whose activity is dependent on the intracellular level of cyclic adenosine monophosphate (cAMP). It is now known that increased cAMP inhibits proliferation under most circumstances [145] and can also stimulate apoptosis [146]. Regarding medulloblastoma, PKA activity is influenced by pituitary adenylyl cyclase-activating peptide (PACAP), which has been shown to regulate cerebellar granule precursor proliferation *in vitro* and to act as a physiological factor that regulates the pathogenesis of SHH pathway-associated medulloblastoma [147]. One of the substrates activated by PKA is a phosphodiesterase, which quickly converts cAMP to AMP, thus reducing the amount of cAMP available to activate PKA. Rolipram, a phosphodiesterase 4 (PDE4) inhibitor, suppresses tumor cell growth *in vitro* and inhibits intracranial growth in xenograft models of malignant brain tumors. The regulation of cAMP levels may also be involved in the antiapoptotic effects of the chemokine receptor, CXCR4, which is important in the developing cerebellum [148]. A recent study shows that, besides promoting tumor growth, PDE4 is widely expressed in brain tumors and is overexpressed in the medulloblastoma cell line, Daoy. Furthermore, we have recently observed that rolipram significantly inhibits proliferation of medulloblastoma cell lines *in vitro* [149], an observation that is corroborated by Goldhoff and colleagues [150], who showed that PDE4 inhibition by rolipram suppressed the growth of glioblastoma xenografts.

*Protein kinase B (PKB)/AKT Pathway*

Protein kinase B (PKB), also known as Akt, and extracellular signal-regulated kinase (ERK) were found to be upregulated in 80% of medulloblastoma cases [151, 152]. The PKB pathway is involved in cellular proliferation and may be involved in medulloblastoma formation. Akt binds either phosphatidylinositol (3,4,5)-trisphosphate (PIP3) or phosphatidylinositol (3,4)-bisphosphate [PI(3,4)P2]. The di-phosphorylated phosphoinositide is only phosphorylated by phosphoinositide 3-kinase (PI3K), and only upon receipt of chemical messengers which tell the cell to begin the growth process. PI3K can be activated by multiple different signaling pathways, including tyrosine kinase receptors in cancer cells [153]. Elevated levels of phosphorylated Akt were found in SHH-induced tumors, suggesting that activated PI3K signaling may be one of the mechanisms that suppress apoptosis in medulloblastoma [154]. Medulloblastoma primary tumors were found to constitutively express activated Akt [151]. Moreover, activated Akt significantly increases SHH-induced medulloblastoma in mice and activation of PI3K/Akt signaling is important for the proliferation of cancer stem cells following medulloblastoma irradiation [155]. Figure 2 exemplifies the plethora of molecules, and the pathways in which they play a role, that could be targeted for treating medulloblastoma.

----------Figure 2----------

## NEW TARGETS

A better understanding of the molecular pathways involved in cancer and the recognition that there may be several alterations that occur simultaneously in medulloblastoma may allow for the stratification of treatment strategies employing new drugs that act on specific targets. Crosstalk among pathways that have a definite role in medulloblastoma development may serve as basis for elucidating other molecular targets for treating this disease, as compensatory pathways may be activated during treatment, evading cell growth control strategies. It is clear that the identification of novel promising signaling pathways in medulloblastoma is often accompanied by the quest for novel therapeutic targets that have the potential to act favorably on this disease (For review, see [9, 10]). The use of new therapeutic approaches may act as complementary treatments in order to increase the effectiveness and reduce the toxic side effects of the strategies currently in use (Table 1).

### Small Molecule Antagonists

As mentioned previously, SHH inhibitors, such as cyclopamine (GDC-0449) and HhAntag-691, are promising agents for treating medulloblastoma, as they have eliminated tumors in animal models [43-44]. Inhibition of SHH downstream effectors with small-molecule antagonists of GLI-mediated transcription are also promising treatments [156]. Specific ErbB1 and ErbB2 inhibitors, such as erlotinib and lapatinib are being studied, showing a survival benefit in the treatment of lung and breast cancer, respectively [157]. However, presence of ErbB2 in medulloblastoma is still under debate, and therefore the benefit of these antagonists has not been clearly evidenced [158].

Sorafenib, a multi-kinase inhibitor, which blocks signal transducer and activator of transcription 3 (STAT3) signaling, is also being studied. STAT3 mediates the expression of a variety of genes in response to cell stimuli, and thus plays a key role in many cellular processes such as cell growth and apoptosis. Decreased proliferation and induced apoptosis in medulloblastoma cell lines and in primary human cultures were found after sorafenib treatment [159].

Imatinib also represents a kinase inhibitor for which there is some clinical experience [160] and already demonstrated future benefits in medulloblastoma therapeutics [161]. Other types of PDGF antagonists have been described [162], such as sunitinib, which shows a tolerable toxicity profile and promising results from phase I clinical trials, despite having a broad specificity [163], and axitinib, which has shown promising results for renal cancer in a phase II trial [164]. Other RAS/MAPK pathway inhibitors are under development and have shown activity in early clinical trials [108]. The selective farnesyl protein transferase inhibitor, tipifarnib, has shown antitumor effects *in vivo* and *in vitro* [165], and MEK inhibitors have proved to be effective in inhibiting the growth of tumors in immunodeficient mice. For

example, the related inhibitor, PD184352, has been used in a large study of colon carcinomas and has recently undergone phase I clinical trials [166], while the RAF inhibitor, BAY43-9006, was shown to be well tolerated at doses that inhibit phorbol-ester-induced ERK phosphorylation in patients' peripheral-blood lymphocytes.

Inhibitors of c-myc are also under study. The 10058-F4 c-myc inhibitor has been extensively studied and found to induce cell cycle arrest, apoptosis, and differentiation in some cells [129, 130]. Indirect targeting of N-myc can be achieved by inducing differentiation using retinoic acid in neuroblastoma cells. This method is already in clinical use, both for treatment of neuroblastoma and certain lymphomas. The possibility of using retinoic acid treatment is also being explored in the treatment of medulloblastoma, and *in vitro* data indicate that cell cycle arrest is induced via inhibition of c-myc [133]. Despite the promising outlook in targeting Myc as a therapeutic approach in medulloblastoma, there are very few studies exploring this possibility.

Regarding the PKA pathway, PDE4 has also been proved as a novel molecular target for brain tumor therapy. PDE4 inhibitors, such as rolipram, can now be evaluated in clinical trials for malignant brain tumors.

A number of small molecules targeting PKB as anticancer therapy have also been developed. These include phosphatidylinositol analogs, ATP-competitive small molecules, pseudosubstrate compounds, and allosteric inhibitors [167]. The first selective inhibitors entering clinical trials have shown efficacy in preclinical models [168, 169]. Although it has been shown that PKB is a promising target for treating medulloblastomas, there are no studies evaluating the effects of those PKB inhibitors in this malignancy.

Both Akt and ERK may contribute to the progression of medulloblastoma by triggering the mammalian target of rapamycin (mTOR) pathway and controlling translation of several cell cycle-related proteins [106-152]. It has been reported recently that the mTOR inhibitor everolimus (RAD001), is required for optimal antitumor effects in positive HER2/neu breast cancer treated with trastuzumab [170]. The fact that EGFR signals through PKC to mTOR independently of Akt in gliomas also indicates that targeting mTOR specifically may be beneficial in treating medulloblastoma [171], and recently RAD001 was tested in combination with an EGFR/VEGFR inhibitor in an in vitro malignant glioma model, showing positive results [172]. Another mTOR inhibitor, temsirolimus (CCI779) was studied in a single-arm, open-label phase II clinical trial in patients (n = 65) with glioblastoma, with 20 patients (36%) demonstrating radiographic improvement [173]. Although the use of mTOR inhibitors for treating solid malignancies is widely under investigation, there are no clinical trials to date that address its use for treating medulloblastoma specifically. Nonetheless, the fact that mTOR inhibitors synergize with drugs targeting EGFR, and the fact that they are clinical trials

assessing its use for the treatment of gliomas indicates that it may be worthwhile to investigate their potential as a treatment for medulloblastoma as well.

**Monoclonal Antibodies**

EGFR and ErbB2 specific monoclonal antibodies have also been developed. The ErbB2 humanized antibody, trastuzumab, is already licensed for treating breast cancer and might be useful for treating medulloblastoma. However, it should be taken in consideration that cancers usually develop resistance to trastuzumab and it is only effective in breast cancer where the ErbB2 receptor is overexpressed. As it was observed that expression or amplification of ErbB2 is not commonly found in medulloblastomas, it might not be a suitable target for treatment with anti-ErbB2 antibodies [158]. The EGFR antibodies, cetuximab (IgG1) and panitumumab (IgG2) are given by intravenous injection for treatment of metastatic colorectal cancer and head and neck cancer. Matuzumab, another EGFR antibody, is currently undergoing phase II clinical trials for the treatment of colorectal, lung and gastric cancer, and Nimotuzumab has been approved for squamous cell carcinoma of the head and neck and glioma.

Antibodies against IGF-1R are also promising. A phase I dose escalation study of CP-751,871, an IGF-1R monoclonal antibody, showed that it has a favorable safety profile and is well tolerated when given in continuous cycles. Furthermore, the majority of refractory solid tumor patients showed clinical benefit with relatively little adverse effects [174]. A phase I/II clinical trial comparing carboplatin and paclitaxel with and without CP-751,871 showed benefits of using this combined therapy [99]. Furthermore, several phase II studies are being planned or are ongoing to test the hypothesis of whether IGF-1R inhibition will enhance the activity of cytotoxic chemotherapy in breast cancer.

**Enzyme Inhibitors**

*Histone Deacetylases (HDAC) Inhibitors*

Histone deacetylase (HDAC) inhibitors are a promising new class of antineoplastic agents with the ability to induce apoptosis and growth arrest of cancer cells. Having been tested in phase I clinical trials, HDAC inhibitors also show a strong potential for treating medulloblastoma. HDACs are enzymes that control the levels of histone acetylation, a process involved in gene expression control. There is evidence that defective control of the histone code contributes to medulloblastoma formation, and that restoring the expression of specific genes that control methylation and acetylation patterns on histone 3, for example, reduces the proliferation of medulloblastoma *in vitro* [175]. Some HDAC inhibitors have been characterized *in vitro*, while others are already being tested in clinical trials because of their ability to inhibit cell growth and induce apoptosis in a variety of tumors. The HDAC inhibitors,

suberoyl anilide hydroxamic acid (SAHA), sodium butyrate and trichostatin A induced apoptotic cell death of medulloblastoma cells and enhanced the cytotoxic effects of ionizing radiation in the Daoy cell line, increasing the killing efficiency of TNF-related apoptosis-inducing ligand (TRAIL), a protein known to induce apoptosis. Likewise, treatment with SAHA markedly augmented the cytotoxicity of etoposide, a topoisomerase II inhibitor [176]. *In vitro*, valproic acid induced apoptosis, inhibited angiogenesis and suppressed tumor growth and, in an intracerebellar medulloblastoma xenograft model, elicited a two-fold increase in survival time [177, 178]. SAHA has been shown to induce apoptosis in medulloblastoma cultures and in mouse models. Besides being permeable to the blood-brain barrier and achieving concentrations that are at or near therapeutic levels, it can also be given safely to children. Recently, co-treatment with RA and SAHA has been shown to induce apoptosis in medulloblastoma tumor models. The efficacy of this treatment was increased with cisplatin, which has important implications for clinical trial design [179]. Taken together, these preliminary data show that HDAC inhibitors may be useful for the treatment of medulloblastoma as monotherapy, and particularly when given in combination with ionizing radiation, chemotherapy, or activation of TRAIL.

*Repressor element 1 silencing transcription factor (REST)*

Expression of the repressor element 1 silencing transcription factor (REST), also known as the neuron-restrictive silencer factor, in neural stem cells causes medulloblastoma-like cerebellar tumors by blocking neuronal differentiation, and can interact with several cellular co-repressors to regulate epigenetic modifications. There are evidences that this mechanism plays a role in the formation of human medulloblastoma [180]. Approximately 50% of human medulloblastoma express REST [181], and adenovirus-mediated expression of REST-VP16, a recombinant transcription factor that can activate REST target genes instead of repressing them, blocked the intracranial tumorigenic potential of medulloblastoma cells and inhibited growth of established tumors in nude mice, suggesting that forced expression of neuronal differentiation genes in medulloblastoma cells can interfere with their tumorigenicity [182]. Therefore, REST may serve as a new target for therapeutic interventions for medulloblastoma.

## CONCLUSION

Although survival rates of medulloblastoma patients have improved, treatment strategies remain suboptimal, especially because the therapies currently employed for disease control result in an inordinately high and unacceptable rate of transient and permanent sequelae. It is true that there has been significant advance in the standard treatments, with big improvements in survival rates, but novel therapeutic approaches are necessary. An increased understanding of oncogenic cell signaling in medulloblastoma formation and progression has led to the development of highly effective and relatively non-toxic therapies for treating this disease. Much progress has been made in the identification of biological factors involved in the pathogenesis of medulloblastoma in the past years, but much has yet to be discovered. The use of agents to antagonize specific growth factor receptors or disrupt their downstream messengers has not yet shown efficacy in the few existing clinical trials, and it remains unclear how these molecular agents should be integrated into conventional therapy. Because of the complexity of cellular signaling, it seems plausible to couple new inhibitors with radiotherapy or chemotherapy, or to use a combination of these agents as a biological cocktail, therefore hitting multiple targets simultaneously. The addition of growth factor receptor antagonists to conventional chemotherapy and radiotherapy is a promising alternative to conventional treatments. Other agents, such as HDAC inhibitors, have been suggested to enhance the anticancer efficacy of classical therapeutic regimens. Moreover, the use of drugs aiming at classical cancer targets, such as PKB, as well as the use of completely new targets for medulloblastoma treatment, such as the PKA pathway, is now under investigation. Thus, new therapeutic strategies will be more effective when used in a combination of targeted inhibitors and traditional chemotherapy.

Many steps must still be taken in order to achieve the clinical use of new drugs in patients with medulloblastoma. Biological advances *in vitro* or in phase I clinical trials have yet to be integrated into the treatment of medulloblastoma in children and adults. Nonetheless, these new targets hold the promise of dramatically changing tumor stratification and of leading to new forms of cancer therapy in a near future. It is hoped that the incorporation of these biological agents which target specific signaling pathways will not only make treatment more effective, but also allow for a reduction in neurotoxic side effects.

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**Abbreviations**

**EGF** – Epidermal Growth Factor
**CNS** – Central nervous system
**NT-3** – Neurotrophin-3
**TrkC** – Tyrosine kinase C
**SHH** – Sonic hedgehog
**Wnt** – Wingless
**STAT 3** – Signal transducer and activator of transcription 3
**PDGFR** – Platelet-derived growth factor receptor
**MAPK** – Mitogen-activated protein kinase
**PTCH1** – Patched 1 receptor
**ATOH1** – Atonal homolog 1
**SMO** – Smoothened
**APC** – Adenomatous polyposis coli
**NT-3** – Neurotrophin-3
**IGF1-R** – Insulin-like growth factor 1 receptor
**PI3K** – Phosphatidylinositol 3-kinase
**RA** – Retinoic acid
**BMP-2** – Bone morphogenetic protein 2
**HDAC** – Histone deacetylase
**PKA** – Protein kinase A
**PKB** – Protein kinase B
**cAMP** – Cyclic adenosine monophosphate
**mTOR** – Mammalian target of rapamycin
**HGF** – Hepatocyte growth factor
**PDE4** – Phosphodiesterase 4
**TRAIL** – Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand

## Figure Legends

**Table 1.** Strategies for the treatment of medulloblastoma.

**Figure 1.** Established molecular pathways that contribute to medulloblastoma formation. Established molecules and their pathways are depicted in purple and grey, respectively. Direct activation and repression are represented by solid lines. Indirect activation is represented by dotted lines. Arrowheads indicate activation, whereas circles indicate inactivation or repression.

**Figure 2.** Potential molecular pathways that contribute to medulloblastoma formation. Crosstalk molecules and their pathways are depicted in green and red, respectively. Direct activation and repression are represented by solid lines. Indirect activation is represented by dotted lines. Arrowheads indicate activation, whereas circles indicate inactivation or repression.

**Table 1**

| ESTABLISHED TREATMENTS[2] | NOVEL TREATMENTS | | UNDERGOING CLINIAL TRIALS |
|---|---|---|---|
| **Chemotherapy**<br>**Radiotherapy**<br>**Stem cell rescue** | **PDGF antagonists**<br>▪ Sunitinib † [159]<br>▪ Axitinib † [160]<br>**Tyrosine kinase inhibitors**<br>▪ Imatinib † [157]<br>**ErbB1 and ErbB2 inhibitors**<br>▪ Erlotinib†<br>▪ Lapatinib†<br>**SHH inhibitors**<br>▪ Small-molecule antagonists of GLI-mediated transcription, a SHH downstream effector [153]<br>**Multi-kinase inhibitors**<br>▪ Sorafenib † [155]<br>**RAS/MAPK pathway inhibitors**<br>▪ Small molecules *[105]<br>▪ Tipifarnib, selective farnesyl protein transferase inhibitor * [161]<br>▪ PD184352 *[162]<br>▪ BAY43-9006 | **c-Myc Inhibitors**<br>▪ 10058-F4 [126, 127]<br>**PDE4 inhibitors**<br>▪ Rolipram ¥ [147]<br>**PKB pathway inhibitors** [163]<br>**ErbB2 antibodies**<br>▪ Trastuzumab †<br>**EGFR antibodies**<br>▪ Cetuximab †<br>▪ Panitumumab †<br>▪ Matuzumab *<br>▪ Nimotuzumab*<br>**IGF-1R antibody**<br>▪ CP-751,871 *[167]<br>**HDAC inhibitors** [169]<br>▪ Suberoyl anilide hydroxamic acid (SAHA) †<br>▪ Sodium butyrate †<br>▪ Trichostatin A †<br>▪ Valproic acid † | **SHH inhibitors**<br>▪ Cyclopamine: phase I [40]<br>▪ HhAntag-691: phase I [40]<br>**Retinoic Acid (RA) derivatives**<br>▪ Trial as single agent in patients with high-risk medulloblastoma, and together with radiotherapy and chemotherapy [133] |

** Undergoing or undergone clinical trials for other tumors*

† *Already in clinical use for other tumors*

¥ *Already in clinical use for other diseases*

**Figure 1**

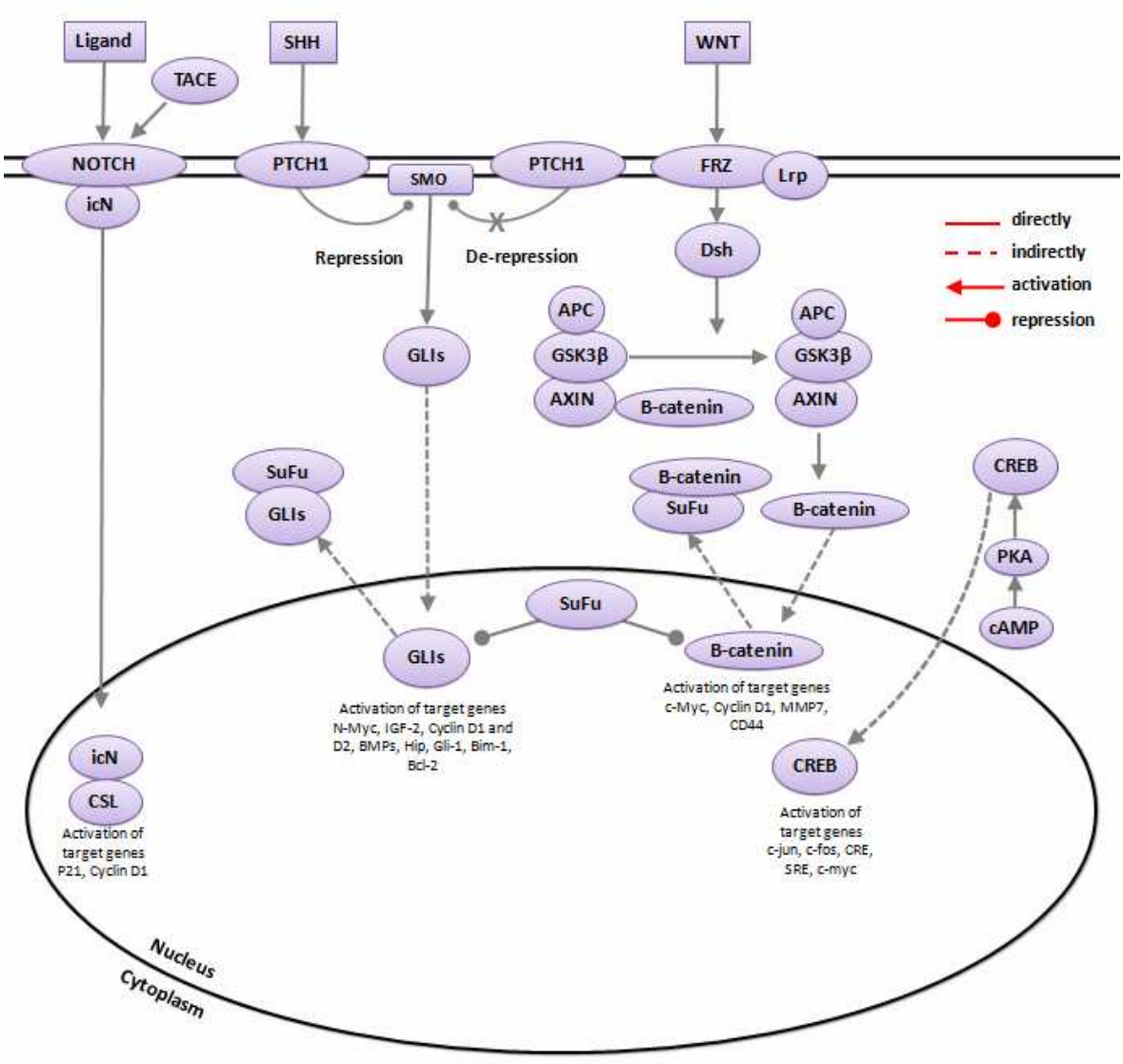
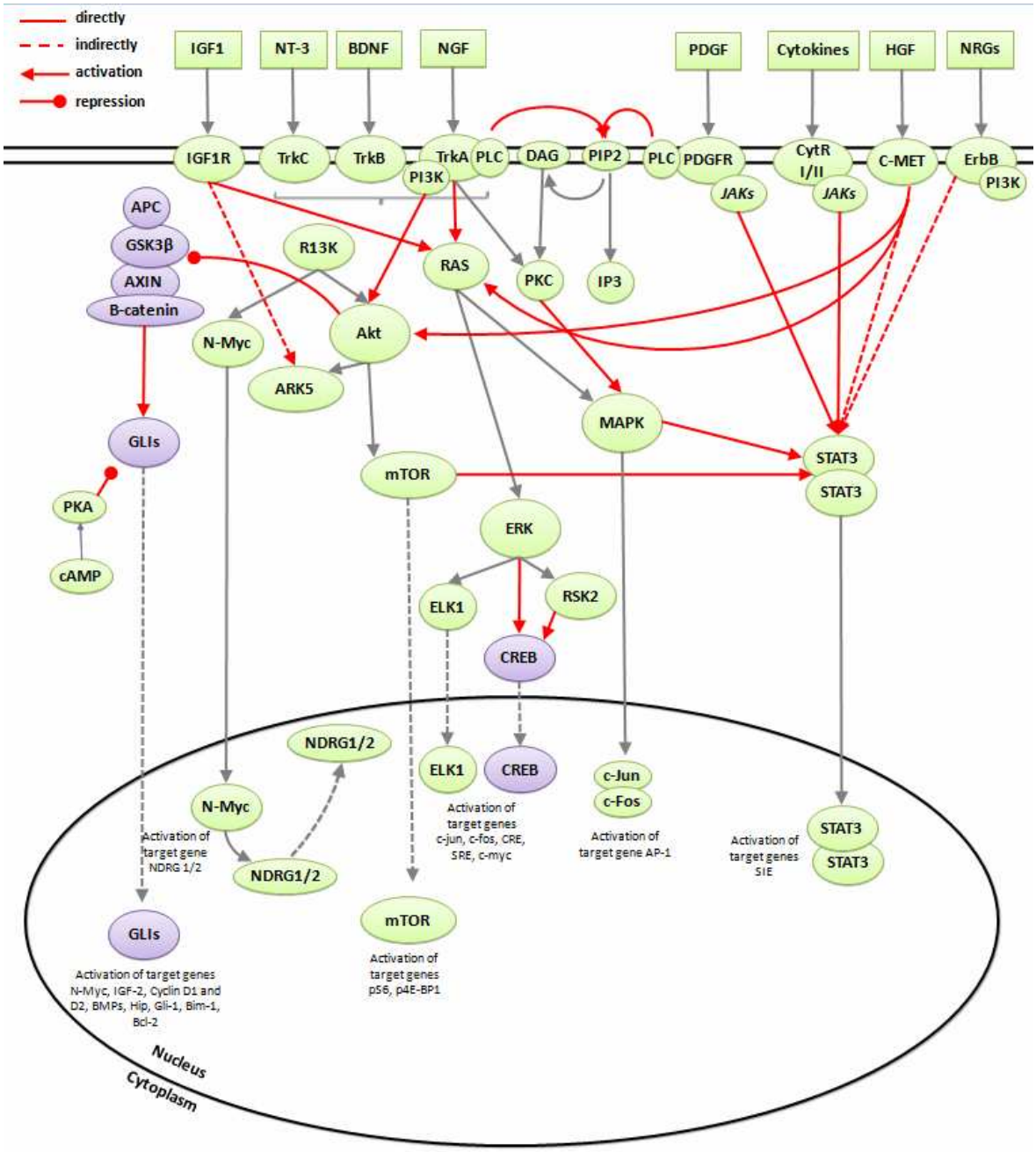

Ligand
TACE
SHH
WNT
NOTCH
icN
PTCH1
SMO
PTCH1
FRZ
Lrp
Repression
De-repression
Dsh
directly
indirectly
activation
repression
APC
GSK3β
AXIN
B-catenin
GLIs
SuFu
GLIs
B-catenin
SuFu
B-catenin
CREB
PKA
cAMP
SuFu
GLIs
B-catenin
Activation of target genes N-Myc, IGF-2, Cyclin D1 and D2, BMPs, Hip, Gli-1, Bim-1, Bcl-2
Activation of target genes c-Myc, Cyclin D1, MMP7, CD44
icN
CSL
Activation of target genes P21, Cyclin D1
CREB
Activation of target genes c-jun, c-fos, CRE, SRE, c-myc
Nucleus
Cytoplasm

**Figure 2**


directly
indirectly
activation
repression
IGF1
NT-3
BDNF
NGF
PDGF
Cytokines
HGF
NRGs
IGF1R
TrkC
TrkB
TrkA
PLC
DAG
PIP2
PLC
PDGFR
CytR I/II
C-MET
ErbB
PI3K
PI3K
JAKs
JAKs
APC
GSK3β
AXIN
B-catenin
R13K
RAS
PKC
IP3
N-Myc
Akt
ARK5
MAPK
STAT3
STAT3
GLIs
mTOR
PKA
cAMP
ERK
ELK1
RSK2
CREB
NDRG1/2
ELK1
CREB
c-Jun
c-Fos
STAT3
STAT3
N-Myc
Activation of target gene NDRG 1/2
NDRG1/2
Activation of target genes c-jun, c-fos, CRE, SRE, c-myc
Activation of target gene AP-1
Activation of target genes SIE
GLIs
mTOR
Activation of target genes N-Myc, IGF-2, Cyclin D1 and D2, BMPs, Hip, Gli-1, Bim-1, Bcl-2
Activation of target genes p56, p4E-BP1
Nucleus
Cytoplasm

## 4 DISCUSSÃO

O meduloblastoma é o tipo mais comum de tumor intracranial em crianças (CBTRUS, 2007-2008), altamente metastático (CRAWFORD *et al.*, 2007) e permite uma sobrevida ainda muito baixa dos pacientes tratados (TAYLOR *et al.,* 2003). O tratamento padrão, com radioterapia e quimioterapia, ainda causa fortes efeitos tóxicos, em especial nos pacientes mais jovens, com impacto significativo sobre a qualidade de vida dos sobreviventes, como dificuldades neurocognitivas (PALMER; REDDICK; GAJJAR, 2007), entre outros efeitos.

Já está claro que a desregulação de vias de sinalização essenciais para o desenvolvimento cerebral, como as vias sonic hedgehog (SHH), Wnt e Notch, desempenham um papel importante na patogênese dos meduloblastomas (CARLOTTI *et al.,* 2008). No entanto, alterações nessas vias não representam todas as possibilidades de formação de meduloblastomas existentes. Outras vias de sinalização celular têm sido investigadas, a fim de compreender a patogênese desses tumores, em especial, vias de sinalização que desempenham papel importante durante o desenvolvimento, como as dos receptores de neurotrofinas.

No presente estudo, nós avaliamos a expressão de GRPR e o efeito dos agonistas GRPR, bombesina (BB) e GRP, do antagonista GRPR RC-3095, do inibidor de fosfodiesterase 4 (PDE4) rolipram combinado ou não com GRP e de BDNF sobre a viabilidade celular de diferentes linhagens de meduloblastoma humano.

Embora a expressão de BDNF e seu receptor TrkB tenham sido detectados em amostras de biópsia de meduloblastoma (TAJIMA et al., 1998; WASHIYAMA et al., 1996), estudos anteriores não verificaram se o BDNF influencia a proliferação e viabilidade de células de meduloblastoma. Nesse trabalho, mostramos evidências de que o tratamento com BDNF recombinante inibe significativamente a viabilidade de duas linhagens de meduloblastoma que expressam TrkB.

Esses achados sugerem que o BDNF pode, sob certas circunstâncias, reduzir ao invés de estimular a sobrevivência e proliferação de célula de meduloblastoma. Embora BDNF seja conhecido por seu efeito em células neuronais, onde o BDNF promove a sobrevivência celular e neurogênese (BATH & LEE, 2006; HUANG & REICHARDT, 2003), alguns estudos anteriores indicaram que BDNF pode também

reduzir a proliferação celular no sistema nervoso central. Sendo assim, BDNF inibe a proliferação de céulas progenitoras neuronais através de um mecanismo envolvendo óxido nítrico sintase neuronal em cérebro de camundongos (CHENG et. Al., 2003). Além disso, foi demonstrado que BDNF reduz a proliferação de células de retina, sendo que um antagonista do receptor Trk foi capaz de bloquear esse efeito (DOS SANTOS et. al., 2003). Ainda precisa ser determinado o motivo pelo qual BDNF afeta a viabilidade celulas em doses baixas, mas não em doses altas na linhagem DAOY. Estudos anteriores encontraram padrões de dose-resposta bifásicos para os efeitos de BDNF. Por exemplo, uma dose baixa de BDNF promoveu, enquanto uma dose alta inibiu a regeneração axonal em motoneurônios de rato (BOYD & GORDON et. al., 2002).

Em estudos investigando a expressão do receptor TrkC em meduloblastomas, o melhor resultado de pacientes mostrando níveis mais altos de TrkC tem sido associado com uma possível atividade promotora de diferenciação de NT-3. O significado do efeito inibitório de BDNF sobre a viabilidade celular observado no presente estudo para a progressão tumoral em meduloblastomas ainda precisa ser determinado. Um entendimento mais profundo do papel de BDNF e TrkB na influência sobre o crescimento de meduloblastomas pode levar a uma validação do receptor TrkB como um novo alvo terapêutico em meduloblastomas.

Estudos anteriores sobre o papel do GRPR na proliferação e viabilidade de células de câncer mostraram que agonistas do GRPR aumentam, enquanto antagonistas inibem a proliferação de vários cânceres experimentais, *in vitro* e *in vivo* (revisado em CORNELIO et al. 2007). A bombesina estimula e antagonistas GRPR como o RC-3095 reduzem a proliferação de células de glioma *in vitro*, quando utilizado em doses e tratamentos similares aos utilizados no presente estudo (DE OLIVEIRA et al. 2009; FLORES et al. 2008; KIARIS et al. 1999; PINSKI et al. 1994b). Em células de neuroblastoma Neuro2A, recentemente foi mostrado que o RC-3095 pode inibir ou promover a proliferação celular dependendo da dose (ABUJAMRA et al., 2009). Os mecanismos envolvidos na inibição da proliferação de células de câncer pelo antagonismo de GRPR podem incluir a expressão alterada ou liberação de neurotrofinas (FARIAS et al. 2009). Surpreendentemente, nossos resultados *in vitro* indicam que as linhagens DAOY, D283 e ONS76 de meduloblastoma humano, apesar de expressarem mRNA e proteína do GRPR, não

têm sua viabilidade celular afetada por por GRP, bombesina ou RC-3095, que têm como alvo a região extracelular de GRPR.

Embora não tenhamos examinado a expressão de GRP nas linhagens de meduloblastoma usadas nesse estudo, evidências anteriores indicam que células de tumores cerebrais que não expressam GRP podem ainda assim expressar GRPR e responder a seus ligantes. Sendo assim, Kiaris e colaboradores (1999) descreveram expressão de GRPR, efeitos estimulatórios de GRP e efeitos inibitórios de antagonistas GRPR (incluindo RC-3095 em células de glioma humano que não expressam mRNA para GRP. Sendo assim, é possível que GRP aja como um fator de crescimento parácrino ao invés de autócrino em células de tumor cerebral.

Uma possibilidade é que, em meduloblastomas, o GRPR aja regulando a diferenciação ao invés de agir como um fator de crescimento promotor de mitogênese. Tal papel do GRP como um agente que contribui para a diferenciação tumoral foi proposto para câncer de cólon (GLOVER et al. 2005). Dado que o GRPR está agora estabelecido como um alvo terapêutico em câncer e que antagonistas GRPR têm sido desenvolvidos como agentes anti-câncer em potencial, será importante determinar se a sinalização por GRPR está envolvida ou não na formação e progressão de meduloblastomas.

Receptores acoplados a proteína G como GRPRs e vias de sinalização de proteínas cinases têm sido propostos como novos alvos terapêuticos anticâncer e contra doenças neurodegenerativas (CHEN *et al*., 1998; 2002; HELMBRECH & RENSING, 1999; PATEL *et al*., 2006; CORNÉLIO *et al.,* 2007; DORSAM & GUTKIND, 2007; ROBERTS & DER, 2007). Nos últimos 10 anos, antagonistas seletivos para GRPR e os mecanismos moleculares associados a eles têm sido estudados com a finalidade de se tornarem drogas experimentais para o tratamento de tumores (PINSKI *et al.*, 1994b; KIARIS *et al*., 1999; KIM *et al.*, 2000; THOMAS *et al*., 2005; SCHWARTSMANN *et al.*, 2006; ZHANG *et al*., 2007). Foi mostrado recentemente que o GRP não afeta a viabilidade de células de glioblastoma humano em cultura quando dado sozinho, mas é capaz de promover viabilidade celular quando combinado com rolipram ou outros estimuladores da sinalização por cAMP (FARIAS et al. 2008). Por esta razão analisamos também se o GRP poderia afetar a viabilidade de células de meduloblastoma quando combinado com rolipram. O rolipram inibe a PDE4, levando a um aumento nos níveis de cAMP intracelular e estimulação da via de sinalização da PKA. É sabido que a via da PKA é importante

para funções celulares tais como motilidade, adesão, interação célula a célula, captação e transdução de sinais externos (KONDRASHIN et al., 1999). Goldhoff e colaboradores (2008) mostraram recentemente que o rolipram promove regressão tumoral e aumento de sobrevivência em camundongos xenotransplantados com a linhagem de glioblastoma humano U87-MG. Além disso, esses autores demonstraram que a superexpressão de PDE4A1 em meduloblastomas estimula o crescimento tumoral. De maneira consistente com esses achados e nossos próprios resultados anteriores com a linhagem de meduloblastoma DAOY (SCHMIDT et al. 2009), o rolipram induz uma inibição significativa na viabilidade das três linhagens de meduloblastoma humano estudadas: DAOY, D283 e ONS76. O efeito inibitório do rolipram em células DAOY não foi modificado pelo co-tratamento com GRP, indicando que, em células de meduloblastoma, o GRP não promove sobrevivência celular ou viabilidade.

Os resultados do presente trabalho indicam que o BDNF pode inibir a viabilidade de células de meduloblastoma humano *in vitro*; que o GRPR pode não estar envolvido na regulação da viabilidade de células de meduloblastoma e inibidores de PDE4, como o rolipram, são capazes de inibir a viabilidade de células de meduloblastoma apesar de uam estimulação concomitante de GRPR.

Nossos resultados indicam um possível envolvimento de GRPR na diferenciação de células de medulloblastoma, assim como o envolvimento do receptor TrkB e PDE4 na regulação do crescimento de meduloblastomas. Estudos futuros serão necessários para avaliar se esses alvos podem realmente desempenhar um papel importante na terapia para essa malignidade em pacientes, com redução de seqüelas do tratamento e benefícios para sua qualidade de vida.

## 5 CONCLUSÃO

Este trabalho objetivou avaliar o efeito de agonistas e antagonista do receptor de GRP, do ativador da via de AMPc/PKA rolipram e de BDNF sobre a viabilidade celular de linhagens de meduloblastoma humano *in vitro*, uma vez que esses podem ser alvos moleculares promissores para o desenvolvimento de alternativas terapêuticas para meduloblastomas.

Este trabalhou mostrou que as linhagens de meduloblastoma humano DAOY, D283 e ONS76 expressam o receptor GRPR. No entanto, os agonistas GRPR bombesina e GRP e o antagonista GRPR RC-3095 não tiveram efeito sobre a viabilidade celular dessas linhagens.

Neste trabalho identificamos que o inibidor de PDE4, rolipram, inibe a viabilidade celular de linhagens de meduloblastoma humano *in vitro*, representando a primeira evidência na literatura de que rolipram é capaz de inibir a proliferação de células de meduloblastoma. Também mostramos que o efeito inibitório de rolipram não foi modificado pelo co-tratamento com GRP em DAOY, diferente do que acontece em certas condições, em que rolipram pode aumentar ao invés de diminuir a proliferação de células de câncer no co-tratamento com GRP.

Também foi demonstrado que BDNF inibe a viabilidade das linhagens de meduloblastoma humano DAOY e D283, sem efeito na linhagem ONS76, apesar de todas expressarem o receptor de BDNF, TrkB.

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## ANEXO A - CURRICULUM VITÆ resumido

## SCHMIDT, A. L.

### 1. DADOS PESSOAIS

**Nome:** Anna Laura Schmidt

**Local e data de nascimento:** Porto Alegre, Rio Grande do Sul, Brasil, 04 de agosto de 1985.

**Endereço profissional:** Hospital de Clínicas de Porto Alegre - Rua Ramiro Barcelos, 2350, 2º andar – Centro de Pesquisas Experimentais – Laboratório de Pesquisas em Câncer – CEP: 90035-003 – Bom Fim – POA – RS - Brasil

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### 2. FORMAÇÃO

Bacharelado em Biomedicina (Universidade Federal do Rio Grande do Sul, 2004-2007).

### 3. ESTÁGIOS

**Universidade Federal do Rio Grande do Sul – Departamento de Bioquímica – (2004-2007)**

Bolsa AT e PIBIC CNPq

Atividades de Iniciação Cientifica em projetos de pesquisa do Grupo de Erros Inatos do Metabolismo:

- Efeitos dos metabólitos acumulados em acidemias orgânicas sobre o metabolismo energético cerebral de ratos.
- Efeitos dos metabólitos acumulados em algumas acidemias orgânicas sobre o sistema glutamatérgico de ratos.
- Estudo sobre os mecanismos de neurotoxicidade de metabólitos acumulados em acidemias orgânicas.

Orientador: Prof. Dr. Moacir Wajner

**Universidade Federal do Rio Grande do Sul– Centro de Ecologia (2007)**

Atividades de estágio curricular:

- Área de Análises Físico-químicas
- Área de Análises Microbiológicas
- Área de Ecotoxicologia
- Área de Bioindicação Vegetal

Responsável: Prof. Dra. Maria-Teresa Raya Rodriguez

## 4. PRÊMIOS E DISTINÇÕES

1. Láurea Acadêmica, Graduação em Biomedicina, Universidade Federal do Rio Grande do Sul, 2008.
2. Destaque no XIX Salão de Iniciação Científica da UFRGS, Universidade Federal do Rio Grande do Sul - Pró-Reitoria de Pesquisa, 2007.

## 5. ARTIGOS COMPLETOS PUBLICADOS

1. SCHMIDT, A.L.; FARIAS, C.B.; ABUJAMRA, A.L.; KAPCZINSKY, F.; SCHWARTSMANN, G.; BRUNETTO, A.L.; ROESLER, R. BDNF and PDE4, but not the GRPR regulate the viability of human medulloblastoma cells. Journal of Molecular Neuroscience, 2009. In Press.
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4. ROSA, R. B.; DALCIN, K. B.; SCHMIDT, A. L.; GERHARDT D.; RIBEIRO, C. A. J.; FERREIRA, G. D.; SCHUCK, P. F.; WYSE, A. T. S.; PORCIÚNCULA, L.; SOUZA, D. O.; WAJNER, M. Evidence that glutaric acid reduces glutamate uptake by cerebral cortex of infant rats. *Life Sciences*, v. 81, p. 1668-1676, 2008.
5. PETTENUZZO, L. F.; FERREIRA, G. D.; SCHMIDT, A. L.; DUTRA-FILHO, C. S.; WYSE, A. T. S.; WAJNER, M. Differential inhibitory effects of

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## 6. RESUMOS E TRABALHOS APRESENTADOS EM CONGRESSOS

1. SCHMIDT, A. L.; DE FARIAS, C. B.; ABUJAMRA, A. L.; SCHWARTSMANN, G.; BRUNETTO, A. L.; ROESLER, R. As linhagens de meduloblastoma DAOY e D283 expressam o receptor do peptídeo liberador de gastrina (GRP-R), mas são refratárias ao seu antagonista, RC-3095. XI Congresso Brasileiro de Oncologia Pediátrica, 2008.
2. SCHMIDT, A. L.; DE FARIAS, C. B.; ABUJAMRA, A. L.; SCHWARTSMANN, G.; BRUNETTO, A. L.; ROESLER, R. BDNF inibe a viabilidade de linhagens celulares de meduloblastoma humano. XI Congresso Brasileiro de Oncologia Pediátrica, 2008.
3. ROSA, R. B.; SCHMIDT, A. L.; DALCIN, K. B.; WAJNER, M. Glutaric and 3-hydroxyglutaric acids decrease [3H]glutamate binding to synaptic membranes in brain of developing rats. In: 21st Biennial Meeting of the International Society for Neurochemistry and the 38th Annual Meeting of the American Society for Neurochemistry, 2007, Cancun. Journal of Neurochemistry, 2007. v. 102. p. 57-57.
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6. LEIPNITZ, G.; SCHUMACHER, C.; DALCIN, K. B.; SCUSSIATO, K.; SOLANO, A.; SCHMIDT, A. L.; SEMINOTTI, B.; DUTRA-FILHO, C. S.; WYSE, A. T. S.; WANNMACHER, C. M. D.; LATINI, A.; WAJNER, M. In vitro evidence for an antioxidant role of 3-hydroxykynurenine in brain. XXI Reunião Anual da FeSBE - Federação de Sociedades de Biologia Experimental, 2006, Águas de Lindóia. Anais da XXI Reunião Anual da FeSBE - Federação de Sociedades de Biologia Experimental, 2006. p. 101-101.
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8. VIEGAS, C. M.; FERREIRA, G. D.; SCHUCK, P. F.; TONIN, A.; GRANDO, V.; SCHMIDT, A. L.; LATINI, A.; WYSE, A. T. S.; WANNMACHER, C. M. D.; DUTRA-FILHO, C. S.; VARGAS, C. R.; WAJNER, M. Efeito da administração crônica de ácido glutárico sobre a cadeia respiratória em córtex cerebral e músculo esquelético de ratos jovens. XXI Reunião Anual da FeSBE - Federação de Sociedades de Biologia Experimental, 2006, Águas de Lindóia. Anais da XXI Reunião Anual da FeSBE - Federação de Sociedades de Biologia Experimental, 2006. p. 75-75.
9. SCHMIDT, A. L.; LEIPNITZ, G.; SEMINOTTI, B.; DALCIN, M. B.; ROSA, R. B.; DALCIN, K. B.; LATINI, A.; SOLANO, A.; SCUSSIATO, K.; WANNMACHER, C. M. D.; DUTRA-FILHO, C. S.; WAJNER, M. Efeito in vitro do ácido 3-hidroxi-3-metil-glutárico sobre parâmetros de estresse oxidativo em córtex cerebral de ratos jovens. 26ª Semana Científica do Hospital de Clínicas de Porto

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12. SCHMIDT, A. L.; PETTENUZZO, L. F.; FERREIRA, G. D.; WYSE, A. T. S.; DUTRA-FILHO, C. S.; WANNMACHER, C. M. D.; WAJNER, M. Effect of methylmalonic acid on the respiratory chain complex activities in kidney of young rats. XXXIV Reunião Anual da SBBq - Sociedade Brasileira de Bioquímica e Biologia Molecular, 2005, Águas de Lindóia. Anais da XXXIV Reunião Anual da SBBq - Sociedade Brasileira de Bioquímica e Biologia Molecular, 2005. p. 82-82.
13. SCHMIDT, A. L.; ROSA, R. B.; DALCIN, K. B.; WINTER, J. S.; TONIN, A.; VIEGAS, C. M.; SOUZA, D. O.; WAJNER, M. Evidência de que o ácido 3-hiroxiglutárico interfere na ligação de glutamato a membranas plasmáticas sinápticas de cérebro de ratos em desenvolvimento. XVII Salão de Iniciação Científica - UFRGS, 2005, Porto Alegre. Livro de Resumos do XVII Salão de Iniciação Científica - UFRGS, 2005. p. 488-488.
14. ROSA, R. B.; DALCIN, K. B.; SCHMIDT, A. L.; WINTER, J. S.; LATINI, A.; LEIPNITZ, G.; RIBEIRO, C. A. J.; SOLANO, A.; ARAUJO, J. H. B.; WAJNER, M. Glutaric and 3-hydroxyglutaric acids decrease [3H] glutamate binding to

synaptic membranes in brain of developing rats. V Congreso Latinoamericano de Errores Innatos del Metabolismo y Pesquisa Neonatal, 2005, San José. Libro del V Congreso Latinoamericano de Errores Innatos del Metabolismo y Pesquisa Neonatal. p. 225-225.

15. MARIA, R. C.; SCHUCK, P. F.; TONIN, A.; FERREIRA, G. D.; GRANDO, V.; SCHMIDT, A. L.; PERRY, M. L. S.; WAJNER, M. Efeito in vitro do ácido antranílico sobre o metabolismo energético cerebral de ratos jovens. 57ª Reunião Anual da SBPC - Sociedade Brasileira para o Progresso da Ciência, 2005, Fortaleza. Programa da 57ª Reunião Anual da SBPC. p. 36-36.
16. SCHMIDT, A. L.; ROSA, R. B.; DALCIN, K. B.; WINTER, J. S.; TONIN, A.; VIEGAS, C. M.; SOUZA, D. O.; WAJNER, M. Interferência do ácido 3-hidroxiglutárico sobre a ligação de glutamato a receptores e transportadores de membranas plasmáticas sinápticas de cérebro de ratos. 25ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2005, Porto Alegre. Revista HCPA - Anais da 25ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2005. v. 25. p. 233-233.
17. WINTER, J. S.; DALCIN, K. B.; ROSA, R. B.; SCHMIDT, A. L.; SOUZA, D. O.; WAJNER, M. Interferência do ácido glutárico sobre a ligação de glutamato a receptores de membranas plasmáticas sinápticas em cérebro de ratos. 25ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2005, Porto Alegre. Revista HCPA - Anais da 25ª Semana Científica do Hospital de Clínicas de Porto Alegre, 2005. v. 25. p. 233-234.
18. SCHMIDT, A. L.; PETTENUZZO, L. F.; WYSE, A. T. S.; WANNMACHER, C. M. D.; DUTRA-FILHO, C. S.; WAJNER, M. Efeito in vitro do ácido metilmalônico sobre as atividades enzimáticas complexo II em homogeneizado de fígado, rim, hipocampo e estriado de ratos jovens. XVI Salão de Iniciação Científica - UFRGS, 2004, Porto Alegre. Livro de Resumos do XVI Salão de Iniciação Científica - UFRGS, 2004. p. 381-381.