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EFEITO DOS ANTIINFLAMATÓRIOS NÃO-ESTERÓIDES SOBRE A PROLIFERAÇÃO CELULAR E ATIVIDADE DAS ECTO-NUCLEOTIDASES EM LINHAGENS DE GLIOMAS

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Àqueles que são a razão da minha existência:

meus pais, meu irmão e meu namorado.

“Aprender é a única coisa que a mente nunca se cansa, nunca desiste e nunca se arrepende.”

Galileu Galilei

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RESUMO

Gliomas são os mais comuns e devastadores tumores primários do sistema nervoso central. Nucleotídeos extracelulares estão envolvidos em diversos processos patofisiológicos no sistema nervoso central. Os níveis dos nucleotídeos da adenina podem ser controlados por hidrólise através da ação de vários membros da família das ectonucleotidases. O AMP formado pelas NTPDases é hidrolizado até adenosina por ação da ecto-5'-nucleotidase (ecto-5'-NT). A enzima ciclooxigenase (COX) está surgindo como um novo alvo na prevenção e no tratamento do câncer, sendo que substanciais evidências epidemiológicas, experimentais e clínicas sugerem que os antiinflamatórios não-esteróides (AINEs) possuem propriedades anticâncer. Vários estudos têm demonstrado que certos AINEs causam efeitos antiproliferativos independentes da atividade da COX. Assim, o objetivo deste estudo foi avaliar o efeito dos AINEs em linhagens celulares de gliomas e os possíveis mecanismos envolvidos neste efeito e avaliar a influência da indometacina na cascata de enzimas que catalisam a interconversão dos nucleotídeos. Indometacina, acetaminofeno, sulfeto de sulindaco e NS-398 induziram uma inibição da proliferação celular de modo tempo e dose dependente. Indometacina causou uma redução significativa na viabilidade celular. Nenhum dos AINEs testados induziu ativação de caspase 3/7. O tratamento com indometacina diminuiu a percentagem de células na fase S, com um aumento relativo nas fases G0/G1 e/ou G2/M, indicando uma parada na progressão do ciclo celular. A exposição de células de glioma à indometacina causou um aumento nas hidrólises de AMP e ATP. Um aumento significativo nos níveis de mRNA da ecto-5'-NT/CD73 foi observado após tratamento com indometacina. Estes resultados suportam a hipótese que o aumento na atividade da ecto-5'-NT está relacionado com a superexpressão do mRNA com possíveis alterações no catabolismo das purinas extracelulares. Os dados sugerem ainda que o receptor A_3 e a enzima ecto-5'-NT estão envolvidos no efeito antiproliferativo da indometacina nas linhagens celulares de gliomas. Considerando que a via das ectonucleotidases pode representar um importante mecanismo associado com a transformação maligna dos gliomas, os AINEs podem ser clinicamente importantes na intervenção farmacológica deste tipo de tumor.

ABSTRACT

Gliomas are the most common and devastating primary tumors of the central nervous system. Extracellular nucleotides are involved in diverse pathophysiological functions in the nervous system. The levels of extracellular adenine nucleotides can be controlled by hydrolysis through the action of several members of ectonucleotidases family. The AMP formed by E-NTPDases is hydrolyzed to adenosine by action of ecto-5'-nucleotidase (ecto-5'-NT). The enzyme cyclooxygenase (COX) is arising as a new target for cancer prevention and treatment, as substantial epidemiologic, experimental and clinical evidence suggests that the nonsteroidal anti-inflammatory drugs (NSAIDs) possess anticancer properties. Several studies have demonstrated that certain NSAIDs cause antiproliferative effects independent of COX activity. Thus, the aim of this study was to evaluate the effect of NSAIDs in glioma cells lines and evaluate the possible mechanisms involved in this effect and the influence of indomethacin on the enzyme cascade which catalyses interconversion of purine nucleotides. Indomethacin, acetaminophen, sulindac sulfide and NS-398 induced a time- and concentration-dependent inhibition of cell proliferation. Indomethacin caused a significantly reduction in cell viability. None of the NSAIDs tested induced caspase 3/7 activation. The treatment of indomethacin decreased the percentage of cells in the S phase, with relative increases in the G0/G1 and/or the G2/M phase, indicating an arrest in progression of cell cycle. Exposure of glioma cells to indomethacin caused an increase in the AMP and ATP hydrolysis. A significant increase on mRNA levels of ecto-5'-NT/CD73 was observed after treatment with indomethacin. These results support the hypothesis that the increase in ecto-5'-NT activity is related to an over-expression of the mRNA with possible alterations in the catabolism of extracellular purines. The data suggest that A_3 receptors and the enzyme ecto-5'-NT are involved in the antiproliferative effect of indomethacin in glioma cell lines. Considering that the ectonucleotidases pathway may represent an important mechanism associated with maligmant transformation of gliomas, the NSAIDs may be clinically important for pharmacological intervention of this kind of tumor.

APRESENTAÇÃO

Esta dissertação é constituída por Introdução, Artigos Científicos aceitos para publicação e/ou submetidos, Discussão, Conclusões e Referências Bibliográficas.

A **Introdução** apresenta um breve embasamento teórico do trabalho. Os Resultados, Materiais e Métodos, Discussão e Referências Bibliográficas encontram-se nos respectivos artigos, apresentados na seção **Capítulos - Artigos Científicos**.

As sessões **Discussão e Conclusão** contêm uma interpretação geral, englobando os dois trabalhos, bem como as principais conclusões da dissertação.

Em **Referências Bibliográficas** há uma listagem das referências utilizadas na Introdução e Discussão desta dissertação.

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LISTA DE ABREVIATURAS

AAS - ácido acetilsalicílico (*acetylsalicylic acid)*

ACR - regiões conservadas da apirase (*apyrase conserved regions*)

ADP - adenosina difosfato (*adenosine diphosphate*)

AKT - proteína cinase B (*protein kinase B*)

AMP - adenosina monofosfato (*adenosine monophosphate*)

AINEs - antiinflamatórios não-esteróides

APCP - α,β-metileno ADP *(α,β-methilene ADP*)

ATP - adenosina trifosfato (*adenosine triphosphate*)

BHE - barreira hematoencefálica

CDK - ciclina dependente de cinase (*cyclin-dependent kinase*)

CPT - 8-ciclopentilteofilina (*8-cyclopentyltheophiline*)

CTP - citidina trifosfato (*citidine triphosphate*)

COX - ciclooxigenase (*cyclooxygenase*)

COX-1 - ciclooxigenase-1 (*cyclooxygenase-1*)

COX-2 - ciclooxigenase-2 (*cyclooxygenase-2)*

COX-3 - ciclooxigenase-3 (*cyclooxygenase-3)*

CPA - ciclopentiladenosina (*cyclopenthyladenosine*)

DPCPX – 1,3-dipropil-8-ciclopentilxantina (*1,3-diprophyl-8-cyclopentylxanthine*)

Ecto-5'-NT - ecto-5'-nucleotidase

EGF - fator de crescimento epidérmico (*Epidermal Growth Factor*)

E-NPP - *ectonucleotide pyrophosphatase/phosphodiesterase*

ERK - cinase Regulada por Sinais Extracelulares (*Extracellular Signal-Regulated Kinase*)

E-NTPDase - *ectonucleoside triphosphate diphosphohydrolase*

FBS - soro fetal bovino (*fetal bovine serum*)

FKHR - Fatores de Transcrição da Forquilha (*Forkhead transcription factors*)

GAPDH - *glyceraldehyde-3-phosphate dehydrogenase*

GBM - glioblastoma multiforme

GFAP - proteína glial fibrilar ácida (*Glial Fibrillary Acidic Protein*)

GSK-3 - glicogênio sintase cinase-3 (*Sintase glicogen kinase-3*)

GTP - guanosina trifosfato (*guanosine triphosphate*)

IGF - fator de crescimento ligado à insulina (*Insulin-like Growth Factor*)

JNK - *Jun n-terminal kinase*

LDH - *lactate dehydrogenase*

mRNA - RNA mensageiro

mTOR: *mammalian target of rapamycin*

MAPK - proteína cinase ativada por mitógenos (*Mitogen-Activated Protein Kinase*)

NF-κB - fator nuclear κB (*nuclear factor κB*)

NTPDase - *nucleoside triphosphate diphosphohydrolase*

NSAIDs - nonsteroidal anti-inflammatory drugs

PDGF - fator de crescimento derivado de plaquetas (*Plateled Growth Factor*)

PDK - cinase dependente de fosfoinositídeos (*phosphoinositide-dependent kinase*)

PG - prostaglandinas (*prostaglandins*)

PGE2 – prostaglandina E2 (*prostaglandin E2*)

PGG - prostaglandina G (*prostaglandin G*)

PGH - prostaglandina H (*prostaglandin H*)

Pi - fosfato inorgânico

PI3K - fosfatidilinositol 3-cinase (*Phosphoinositide 3-kinase*)

PI4K - fosfatidilinositol 4-cinase (*Phosphoinositide 4-kinase*)

PKA - proteína cinase A (*Protein kinase A*)

PKB - protein cinase B (*Protein kinase B)*

PKC - proteína cinase C (*Protein kinase C)*

8PT - 8-fenilteofilina (*8-phenytheophyline*)

P2X - receptor purinérgico ionotrópico

P2Y - receptor purinérgico metabotrópico

PTEN - homólogo da fosfatase e tensina deletado no cromossoma 10

(Phosphatase and Tensin Homologue Delected on Chromossome 10)

RTK - receptor tirosina cinase (*tirosine kinase receptor*)

RT-PCR - *reverse transcription-polymerase chain reaction*

SNC - sistema nervoso central

SNP - sistema nervoso periférico

UTP - uridina trifosfato (*uridine triphosphate*)

INTRODUÇÃO

1. Gliomas

Os tumores primários do sistema nervoso central (SNC) estão entre as mais prevalentes neoplasias sólidas, representando uma das mais importantes causas de mortalidade por câncer antes dos 15 anos de idade (Baylei *et al*., 1995) e correspondem a cerca de 50% de todas as neoplasias cerebrais (Hochberg & Pruitt, 1991). Dentre os vários tipos de tumores do SNC, os mais freqüentes são os gliomas, que possuem origem glial e incluem tumores constituídos predominantemente de células com características de astrócitos (astrocitomas), oligodendrócitos (oligodendrogliomas), células ependimais (ependimomas) e misturas de vários tipos de células gliais (por exemplo, oligoastrocitomas) (Kleihues & Cavenee, 2000). Há um predomínio dos gliomas de origem astrocítica, sendo que estes correspondem a mais de 60% de todos os tumores primários. Ainda existe controvérsia se os gliomas são originados de células maduras ou células-tronco no cérebro adulto mas o diagnóstico patológico depende de semelhanças entre as células tumorais e células gliais maduras não neoplásicas (Kleihues & Cavenee, 2000). Histologicamente são considerados astrocitomas os tumores que apresentem células com citoplasma abundante e marcação para GFAP (*Glial Fibrillary Acidic Protein*). De acordo com Holland e cols. (2001), ainda não foi possível demonstrar diretamente que tipo de célula dá origem aos diferentes tipos de gliomas humanos. Segundo estes autores, tanto podem derivar de uma população de células progenitoras do SNC como de células adultas que sofrem mutações e conseqüentes anormalidades nas vias de sinalização, levando as células a apresentarem um fenótipo indiferenciado.

1.1. Classificação e Patologia

O sistema de classificação da Organização Mundial de Saúde (OMS) classifica os gliomas em 4 graus de malignidade (I a IV), de acordo com critérios histopatológicos: morfologia celular, proliferação microvascular, atividade mitótica e necrose (Kleihues *et al*, 1993). Os gliomas de grau I se comportam geralmente como benignos, enquanto os gliomas de graus II a IV são malignos e se infiltram de maneira difusa no cérebro. A forma mais maligna é o glioblastoma multiforme (GBM), que possui grau IV e é um dos cânceres humanos mais agressivos, com uma sobrevivência média inferior a um ano, sendo que esta estatística não se modificou significativamente nas últimas 2 décadas (Holland, 2001; Maher *et al*, 2001). O GBM pode ser dividido em dois subtipos, baseados em características clínicas: o GBM primário e o secundário. O GBM primário surge na ausência de uma lesão pré-existente, sendo mais freqüentes em pacientes idosos, enquanto o GBM secundário se origina de um astrocitoma de baixo grau, predominando em jovens (Kleihues *et al*, 1993; Holand, 2001; Behin *et al*, 2003). Os GBM são compostos de astrócitos hiperproliferativos, infiltrativos e pleomórficos, com áreas de necrose, hemorragias e angiogênese. Estas características patológicas heterogêneas são o maior obstáculo para o manejo efetivo dos gliomas.

1.2. Terapêutica

A cirurgia representa o tratamento de primeira escolha para os pacientes com gliomas, entretanto a ressecção completa do tumor é limitada pela alta invasividade das células tumorais nos tecidos normais circundantes, o que leva a uma elevada taxa de recorrência (Behin *et al*., 2003). Portanto, a maioria dos pacientes é tratada com radioterapia e/ou quimioterapia pós-operatória (Mazeron &

Kantor, 1998). Contudo, estes agentes demonstram eficácia limitada, principalmente pela ausência de especificidade terapêutica das drogas citotóxicas contra tais neoplasias, além da quimiorresistência intrínseca destes tumores e da baixa tolerância do tecido normal aos efeitos tóxicos decorrentes a terapia (Mousseau *et al*, 1993). Além disso, a barreira hematoencefálica (BHE) limita a entrada dos quimioterápicos no SNC, de modo que apenas fármacos altamente lipofílicos (temozolomida e nitrosuréias) podem ser utilizados (Mousseau *et al*, 1993).

A pobre responsividade dos tumores primários do SNC à quimoterapia, bem como o sucesso limitado da cirurgia e radioterapia pós-operatória, justificam os esforços dedicados no desenvolvimento e aprimoramento de novas modalidades terapêuticas. Dessa forma, torna-se necessário a optimização das estratégias terapêuticas, bem como a compreensão de seu potencial e suas limitações no tratamento dos gliomas, sendo que o avanço no conhecimento das características moleculares destas neoplasias, pode contribuir neste desenvolvimento.

1.3. Características Moleculares

A tumorogênese envolve, em essência, o descontrole das funções de proto-oncogenes e genes supressores tumorais. Tais genes codificam proteínas reguladoras de complexas rotas de transdução de sinais para funções celulares essenciais, tais como: proliferação, diferenciação, apoptose, adesão, migração e angiogênese (Tronick & Aaronson, 1995; Weinstein *et al*., 1995; Weinberg, 1996). A Figura 1 esquematiza os principais eventos celulares que levam à gliomagênese.

Além da heterogeneidade patológica, os gliomas apresentam uma heterogeneidade molecular. O processo de transformação de uma célula normal em uma célula tumoral é extenso e pode prosseguir por vários caminhos. Acredita-se

que somente alguns dos processos moleculares são compartilhados por um determinado tipo de neoplasia, de modo que cada tumor possui uma característica genética e epigenética única.

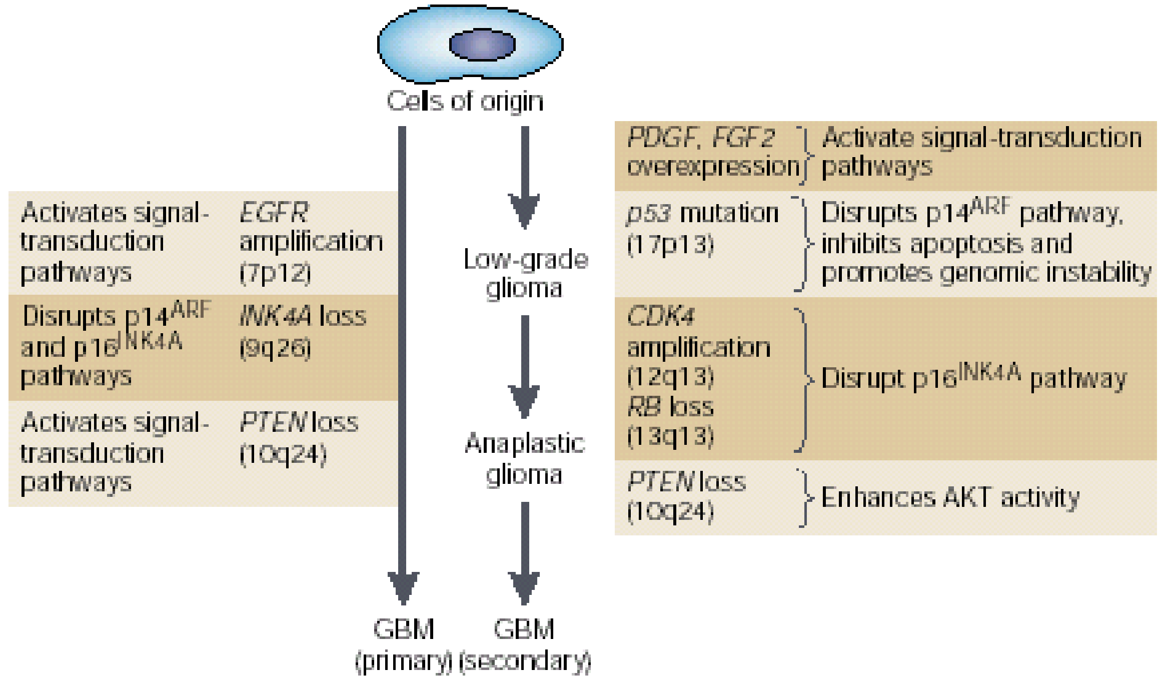

Figura 1. Alterações genéticas que levam à gliomagênese. As mutações citadas estão correlacionadas com os diferentes graus de malignidade dos gliomas. Adaptado de Holland, 2001.

No GBM, várias proteínas têm sido relacionadas com proliferação celular, invasão e metástases em relação ao cérebro normal e aos gliomas de baixo grau. Dentre as principais, pode-se citar as MAPK (*Mitogen-activated Protein Kinase*) (Bredel *et al*., 1999), PKC (*Protein Kinase C*) (Bredel *et al*., 1997), PI3K (*Phosphoinositide 3-kinase*) (Schelegel *et al*., 2000) e AKT (*Protein Kinase B*). A Figura 2 apresenta de forma sintética algumas destas vias.

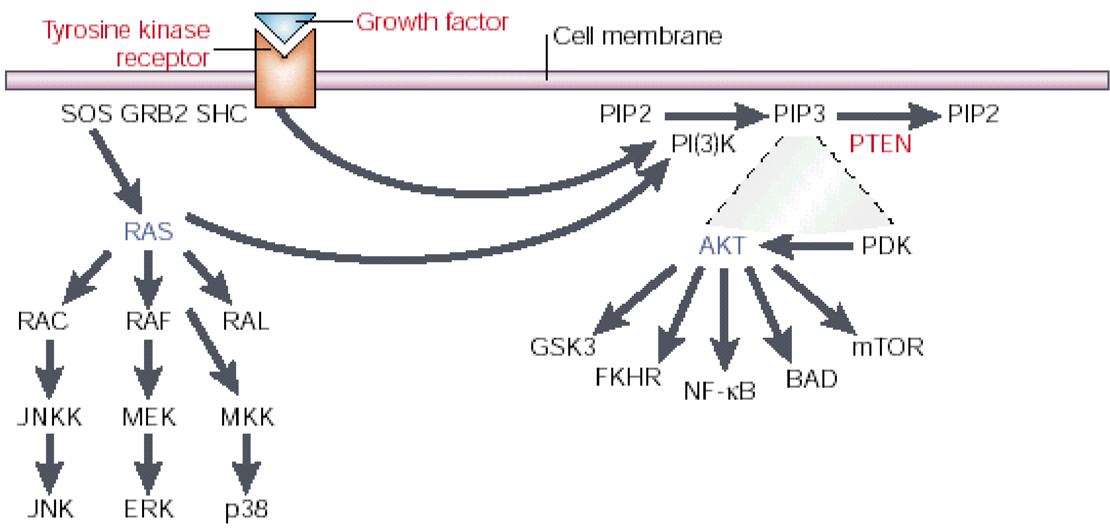

Figura 2. Principais vias de sinalização relacionadas com diferenciação, sobrevivência, apoptose e proliferação celular. Adaptado de Holland, 2001.

A cascata das MAPK (Ras→ Raf→ MEK→ ERK) não é uma simples via linear. Vários estímulos, que variam com os tipos celulares, provavelmente induzem a ativação de diversas vias, com cinéticas de ativação distintas, que acabam convergindo na ERK (*Extracellular signal-Regulated Kinase)*. Quando ativada, essa proteína é translocada ao núcleo ativando diversos fatores de transcrição que estão relacionados à proliferação e ao crescimento celular, por exemplo através do aumento da expressão de ciclinas, reguladores positivos do ciclo celular (Liu *et al*, 1996; Dirks *et al.*, 1997). Em células de tumores, a via da ERK encontra-se usualmente ativada (Hoshino *et al*, 1999).

A Ras é um ponto convergente de muitas vias de sinalização celular (Campbell *et al*, 1998). As proteínas Ras são ativadas transitoriamente em resposta a vários sinais extracelulares como fatores de crescimento, citocinas, hormônios e neurotransmissores, que estimulam receptores celulares de superfície que incluem receptores tirosina quinase (RTK) e receptores transmembrana ligados à proteína G. A atividade da Ras e da Akt encontra-se elevada na maioria dos GBMs

analisados (Guha *et al*, 1997; Holland, 2000; Choe *et* al, 2003). A via da PI3K/AKT tem um papel muito importante na gliomagênese, sendo ativada por diversos fatores de crescimento e estando principalmente envolvida na regulação da sobrevivência celular, isto é, a sua ativação produz um efeito anti-apoptótico. Essa via é controlada pela atividade de uma proteína citoplasmática que antagoniza as ações da PI3K, a PTEN (*Phosphatase and tensin homologue deleted from cromossome 10*). O nível de expressão da PTEN está diretamente relacionado com a malignidade dos gliomas: quanto maior a expressão desta proteína, menos maligno é o tumor, sendo que GBMs apresentam PTEN mutada ou deletada (Sano *et al*., 1999). Outra característica importante dos GBMs é a deleção do gene supressor tumoral, *p53*, um fator de transcrição que regula a progressão do ciclo celular e apoptose em resposta a vários fatotes extracelulares, como por exemplo dano ao DNA e mutações oncogênicas (Bogler *et al*., 1999). Quando há uma lesão no DNA, p53 interrompe o ciclo no primeiro ponto de controle, permitindo o reparo. Se esse reparo apresentar falhas, inicia-se o processo de apoptose (Bogler *et al*., 1999). As fases do ciclo celular estão esquematicamente representadas na Figura 3.

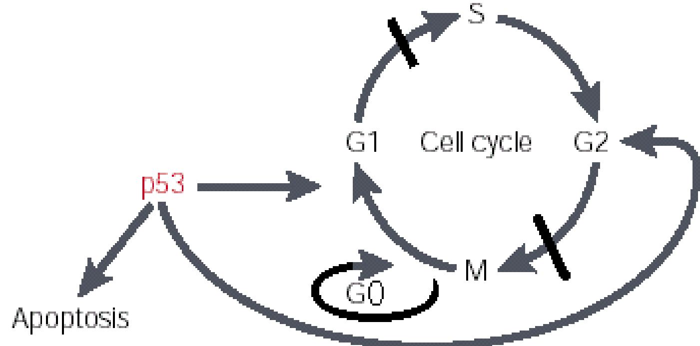

Figura 3. Diagrama do ciclo celular. O ciclo celular é composto por uma seqüência ordenada de fases: G1, S (síntese de DNA), G2 e M (mitose). G0 representa a fase em que as células não estão se dividindo, mas podem entrar novamente no ciclo. Estas fases são estritamente reguladas. Adaptado de Holland, 2001.

As PKCs são uma família de sernina/treonina/cinases que possuem um papel importante em muitas vias de transdução de sinal, sendo que em vários tumores um aumento na sua atividade está relacionado com aumento da proliferação celular (Couldwell *et al*, 1991), aumento do fenótipo invasivo (Uhm *et* al, 1996), diminuição da apoptose (Couldwell *et al*, 1994), aquisição de resistência multidroga (Chambers *et* al, 1993) e radioresistência (Zhang *et al,* 1992). Uma vez ativada, as PKCs transmitem sinais ao núcleo através da cascata das MAPK, que resultam na ativação de fatores de transcrição envolvidos na progressão do ciclo celular e diferenciação (Ueda *et al*., 1996; Seger *et al*., 1995). Os gliomas, caracteristicamente, apresentam PKC hiperativa ou superexpressa, que se relaciona com seu grau de malignidade (Baltuch & Yong, 1996). Outra proteína que se encontra superexpressa e com alta atividade em diversos tipos de tumores é a c-Src (Irby & Eatman, 2000). Além de aumentar a proliferação celular, pode regular a adesão, invasão e motilidade celulares (Roche *et al,* 1995; Frame, 2002; Jones *et al,* 2002), sendo uma molécula crucial em uma rede complexa de proteínas que interagem entre si. Além disso, amplificações ou superexpressões de genes que codificam fatores de crescimento (PDGF, EGF, IGF) ou seus receptores atingem 60% dos astrocitomas. Isso pode gerar sinais aberrantes capazes de induzir descontrole na mitogênese, migração celular, síntese de proteinases e angiogênese (Ekstrand *et al*., 1991; Takahashi *et al*., 1991; Wong *et al*., 1992). Assim, tais alterações não intensificam apenas o crescimento tumoral, mas criam condições para a expansão do tumor nos tecidos vizinhos.

2. Antiinflamatórios Não-Esteróides

2.1. Emprego Terapêutico

Desde 1893, quando o químico alemão Felix Hoffman motivou a Bayer a produzir o ácido acetilsalicílico (AAS), patenteado como Aspirina®, os antiinflamatórios não-esteróides (AINEs) passaram a ser as drogas mais amplamente prescritas e usadas em todo o mundo, existindo atualmente mais de 50 compostos deste grupo disponíveis no mercado com variáveis graus de atividade antiinflamatória (Fuchs & Wannmacher, 1998). Os AINEs são fármacos com propriedades analgésicas, antipiréticas e antiinflamatórias, sendo que sua principal vantagem é a ausência de dependência física ou psíquica com o uso prolongado, quando comparados aos opióides (Rang *et al*, 2001). A ação antiinflamatória dos AINEs decorre da inibição da síntese de prostaglandinas (PGs), através da inibição da ciclooxigenase (COX).

Os principais usos clínicos dos AINEs são o tratamento de doenças articulares como artrite reumatóide, artrite gotosa, osteoartrite, tendinite, além de outros processos inflamatórios em geral como lesões músculo-esqueléticas e inflamações oftálmicas. Os AINEs são classificados de acordo com suas funções químicas. A maioria dos AINEs são ácidos orgânicos e atuam como inibidores competitivos reversíveis da atividade da COX, enquanto que o AAS é o único AINE que atua de forma a modificar covalentemente a COX, resultando na inibição irreversível da atividade da enzima (Goodman & Gilman, 2003).

Embora todos os AINES sejam antipiréticos, analgésicos e antiinflamatórios, existem diferenças em suas atividades individuais. Os motivos para estas diferenças entre os AINEs não estão totalmente esclarecidos, mas provavelmente estão relacionados a mecanismos de ação secundários ainda desconhecidos ou pouco estudados.

2.2. Ciclooxigenase e Síntese de Prostaglandinas

A enzima araquidonato ciclooxigenase (COX), também chamada de prostaglandina H sintase, é responsável pela formação de PGs a partir de ácido araquidônico. Os compostos da família das PGs e dos leucotrienos são denominados de eicosanóides, em virtude de serem derivados de ácidos graxos essenciais de vinte carbonos esterificados em fosfolipídios da membrana celular. A síntese dos eicosanóides pode ser desencadeada por diversos estímulos que ativam receptores de membrana, acoplados a uma proteína regulatória (proteína G), resultando na ativação da fosfolipase A_2 e/ou elevação da concentração intracelular do Ca^{2+} (Goodman & Gilman, 2003). A fosfolipase A_2 hidrolisa fosfolipídios da membrana, particularmente fosfatidilcolina e fosfatidiletanolamina, liberando o ácido araquidônico (Carvalho et al., 2004; Carvalho *et al*., 2002). O ácido araquidônico liberado servirá como substrato para duas vias enzimáticas distintas: a via das COXs, que desencadeia a biossíntese das PGs e dos tromboxanos, e a via das lipoxigenases, responsável pela síntese de leucotrienos, lipoxinas e outros compostos (Samuelsson *et al*., 1975).

As COXs possuem duas atividades distintas. Uma atividade de endoperóxido redutase que oxida e cicliza o ácido graxo precursor não-esterificado, para formar o endoperóxido cíclico prostaglandina G (PGG), e outra atividade de peroxidase que converte o PGG em prostaglandina H (PGH) (Goodman & Gilman, 2003). Sabe-se que dois genes expressam duas isoformas distintas bastante similares da enzima: a ciclooxigenase-1 (COX-1) e a ciclooxigenase-2 (COX-2). As duas isoformas têm estrutura protéica primária similar e catalisam essencialmente a mesma reação (DeWitt *et al*., 1988; Kulkarni *et al*., 2000). A COX-1 e a COX-2 são proteínas integrais que se localizam dentro da bicamada lipídica de fosfolipídeos da

membrana. O número de aminoácidos que compõem as duas enzimas é bastante semelhante, variando de 599 aminoácidos para a COX-1 e de 604 aminoácidos para a COX-2 (Vane *et al*., 1995). A COX-1 é expressa de forma constitutiva pela maioria das células e está relacionada com inúmeras funções fisiológicas, como a modulação da formação de secreções gástricas protetoras. A COX-2 pode ser induzida por vários estímulos celulares (fatores de crescimento e citocinas), sendo superexpressa nos locais de inflamação. Estudos recentes sugerem a existência de uma terceira variante catalítica, a ciclooxigenase-3 (COX-3). A COX-3, possivelmente uma variante da COX-1, encontra-se distribuída principalmente no córtex cerebral, medula espinhal e coração. Postulou-se que a inibição da COX-3 poderia representar o mecanismo central primário pelo qual os AINEs como acetaminofeno desenvolveriam suas atividades de redução da dor e febre (Chandrasekharan *et al*., 2002).

A inibição de COX-1 é, pelo menos em parte, responsável por alguns dos efeitos adversos dos AINEs, como as toxicidades renal e gastrintestinal. Esse efeito adverso é o mais freqüente dos AINEs, estando relacionado com a ação direta sobre a mucosa e inibição da síntese de PGs locais, substâncias diretamente envolvidas na modulação da secreção gástrica e na secreção de muco citoprotetor pelo intestino. Dessa forma, visando uma diminuição dos efeitos adversos decorrentes da inibição da COX-1, novos compostos com ação seletiva sobre a COX-2 estão sendo desenvolvidos.

2.3. Ciclooxigenase e Neoplasias

Estudos recentes mostram que a via das PGs, mais especificamente a enzima COX, está despontando como um alvo farmacológico promissor na

prevenção e tratamento do câncer. O papel da COX nas neoplasias foi sugerido após a demonstração de que alguns processos neoplásicos possuem altos níveis de metabólitos do ácido araquidônico, como a prostaglandina E2 (PGE2). Vários tipos de tumor superexpressam COX-2, como tumor de cólon, de mama (Hwang *et al*., 1998), de pele (Muller-Decker *et al*., 1999) e de bexiga (Shirahama *et al*., 2000), sendo que a presença de COX-2 pode ser essencial para a vascularização e crescimento do tumor (Thun *et al*., 1991). Sugere-se inclusive que a expressão de COX-2 possa ser constitutiva em alguns tipos de tumores, como observado em áreas de necrose de gliomas humanos (Shono *et al*., 2001), sendo que a superexpressão dessa enzima está associada com gliomas clinicamente mais agressivos e de pior prognóstico para o paciente (Shono *et al*., 2001).

Numerosos estudos com linhagens de células malignas, estudos clínicos e epidemiológicos têm demonstrado o potencial dos AINEs, especialmente os inibidores seletivos da COX-2, como agentes farmacológicos de papel benéfico no tratamento e prevenção de vários tipos de câncer. Ensaios clínicos têm demonstrado que alguns AINEs podem reduzir de forma significativa o número de pólipos em doença adenomatosa pré-existente (Giardiello *et al*., 1993) e diminuir o risco de câncer coloretal (Thun *et al*., 1991).

Apesar das diversas demonstrações de efeitos antiproliferativos dos AINEs, a função desempenhada pelas PGs e pela COX na patogênese do câncer ainda não está totalmente definida. Alguns autores sugerem que a COX pode estar envolvida diretamente na regulação da proliferação celular (Sheng *et al*, 1997; Nguyen & Lee, 1993; Hial *et al*., 1997), na apoptose (Tsujii & Dubois, 1995) e na interação tumor-vasos sangüíneos (Tsujii *et al*., 1998). Howe e colaboradores (2001) demonstraram que inibidores seletivos de COX-2 reduzem a angiogênese em vários modelos

experimentais, e compostos como celecoxib e rofecoxib são capazes de estimular a apoptose e suprimir o crescimento celular em muitos carcinomas *in vitro*, incluindo culturas de cânceres humanos de estômago, esôfago, língua, cérebro, pulmão e pâncreas. As reduções mais significativas foram observadas com o uso de indometacina, diclofenaco e aspirina (Thun *et al*., 2002). Apesar de os mecanismos moleculares envolvidos não estarem ainda bem definidos, acredita-se que a COX contribua para produção de fatores pró-angiogênicos e que o aumento de sua expressão durante a tumorogênese seja provavelmente conseqüência de múltiplos efeitos (Howe *et al.,* 2001). Essa capacidade que AINEs possuem de estimular a apoptose e de inibir a angiogênese faz destes fármacos importantes candidatos para suprimir transformações malignas e diminuir o crescimento de tumores.

Os estudos que visam descrever a atuação da COX em outros eventos além daqueles já conhecidos são de extrema importância. Isso pode aumentar a abrangência de utilização dos AINEs, fármacos inibidores da COX que possuem dados farmacocinéticos bem conhecidos e são amplamente utilizados na terapêutica. Além disso, alguns trabalhos sugerem que os AINEs podem possuir outros mecanismos de ação além da inibição da COX (Tegeder *et al*., 2001; Cherukuri *et al*., 2004; Xu et al., 2005), sendo que esses mecanismos seriam os responsáveis pelos efeitos observados nos estudos com células tumorais.

3. Nucleotídeos Extracelulares

As purinas (ATP, ADP e adenosina) e pirimidinas (UTP e UDP) extracelulares são mensageiros amplamente distribuídos no organismo, promovendo diversos efeitos biológicos através de receptores denominados purinoreceptores (Ralevic & Burnstock, 1998). No SNC as principais fontes de purinas extracelulares são os

neurônios, a glia e a microglia. Além do papel já estabelecido dos nucleotídeos extracelulares como neurotransmissores e moduladores no SNC e SNP, há trabalhos que mostram que as purinas extracelulares podem atuar como fatores tróficos, regulando o desenvolvimento e a manutenção do sistema nervoso e sua resposta a doenças e injúrias (Neary *et al*, 1996; Rathbone *et al*, 1999; Ciccarelli *et al*, 2001). Vários estudos têm demonstrado que o ATP extracelular é capaz de promover a proliferação celular em astrócitos (Rathbone *et al*., 1992; Neary et al., 1994). Recentemente, um trabalho do nosso grupo de pesquisa, mostrou que o ATP e a Adenosina (Ado), aumentaram a proliferação em linhagens celulares de gliomas (Morrone *et al.,* 2003).

Os receptores que ligam nucleotídeos e nucleosídeos são divididos em receptores de Ado ou P1 e em receptores P2, como apresentado na Tabela 1.

Receptores P1		**Receptores P2**	
Ligante natural	Ado	ATP, ADP, UTP e UDP	
Tipo	Acoplado à Proteína G	Canal iônico	Acoplado à Proteína G
Subgrupo	-	P2X	P2Y
Subtipos	A_1, A_{2A}, A_{2B}, A_3	$P2X_{1-7}$	$P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$

Tabela 1. Receptores de purinas e pirimidinas. Adaptado de Ralevic e Burnstock, 1998.

3.1. Adenosina

A Ado, principal produto da hidrólise do ATP, é considerada um importante neuromodulador, podendo exercer vários efeitos através da interação com receptores específicos da família P1 (Tabela 1). Estes receptores podem ser

ativados por diferentes faixas de concentração de Ado extracelular (Fredholm *et al.*, 2001), e os níveis de Ado disponíveis no local que ligam e ativam estes receptores controlam as diferentes respostas fisiológicas deste agonista. O fato da Ado ser um metabólito imediato dos nucleotídeos da adenina, a torna uma molécula sinalizadora adequada tanto para situações fisiológicas como patológicas, que resultem em degradação dos nucleotídeos da adenina (Spychala *et al.*, 2000). Vários estudos demonstraram os efeitos da Ado na regulação do ciclo celular, proliferação e apoptose de células de origem tumoral e não-tumoral, podendo exercer efeito inibidor ou promotor da proliferação celular (Ceruti *et al.,* 2000; Ohana *et al.,* 2001; Peyot *et al.,* 2000). O efeito da Ado na proliferação de células normais depende de sua concentração extracelular, do subtipo de receptor presente na membrana, e dos mecanismos de transdução de sinais por eles ativados (Merighi *et al.*, 2003). Os mecanismos de transdução de sinais utilizados pelos receptores P1 para induzir a proliferação ou morte celular incluem as vias da ERK, PI3K, Akt, fosfolipase C, PKC e fosfolipase C (Merighi *et al*, 2003).

Considerando que os nucleotídeos podem ativar receptores purinérgicos P2 e que os produtos de degradação podem ativar os receptores P1, a regulação da quantidade relativa destes nucleotídeos/nucleosídeos torna-se parte fundamental na sinalização mediada por estes compostos. Dessa forma, mecanismos que controlam a concentração dessas substâncias, como as ecto-nucleotidases possuem um papel fundamental.

3.2. Ecto-nucleotidases

A "via das ecto-nucleotidases" apresenta um papel chave na regulação da sinalização purinérgica controlando a concentração dos nucleotídeos e

nucleosídeos. Após a liberação no espaço extracelular e a ativação de receptores específicos, os nucleotídeos da adenina podem ser metabolizados pela ação destas ecto-enzimas que fazem a conversão dos nucleotídeos até Ado. Estas enzimas incluem a família das E-NTPDase (*ectonucleoside triphospate diphosphohydrolase*), as E-NPP (*ectonucleotide pyrophosphatase/phosphodiesterase*), ecto-5'-nucleotidase e fosfatases alcalina (Zimmermann, 2001) (Figura 4).

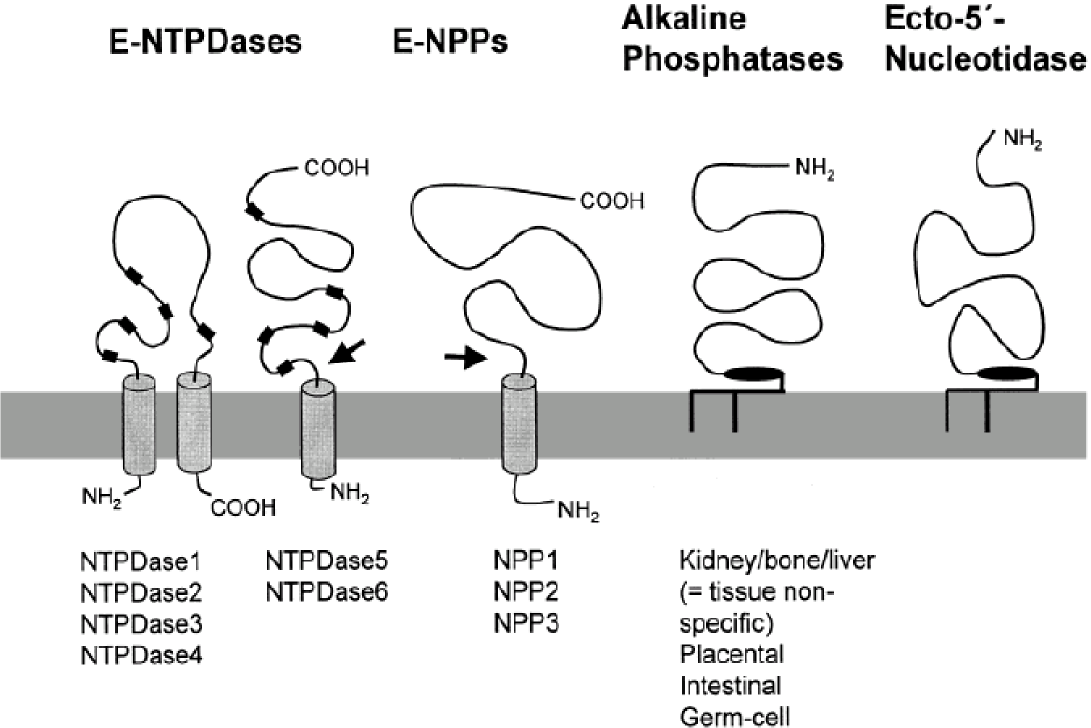

Figura 4. Topografia de membrana proposta para as nucleotidases. As NTPDases de 1 a 4 são ligadas à membrana plasmática por dois domínios transmembrana, N e C-terminal. NTPDase 5 e NTPDase 6 não possuem o domínio transmembrana C-terminal e podem ser clivadas próximo ao domínio N-terminal para formar uma proteína solúvel liberada (seta). Os quadros escuros na seqüência das NTPDases 1 a 6, representam as regiões conservadas da apirase (ACR). Todas as ecto-nucleotidases representadas são glicoproteínas. Adaptado de Zimmermann, 2001.

Em mamíferos até o momento, pelo menos oito membros da família E-NTPDase têm sido clonados e funcionalmente caracterizados. Cada membro individual, pode diferir na seqüência de aminoácidos, porém todos possuem cinco regiões conservadas, as quais foram chamadas de “regiões conservadas da apirase”, ACR (ACR 1-5 *apyrase conserved regions*) (Handa e Guidotti, 1996; Vasconcelos et al., 1996). A Tabela 2 apresenta a nova nomenclatura das E-NTPDases e os nomes previamente utilizados, bem como as diferenças consideráveis na preferência pelos substratos.

Nome Atual	Outra Nomenclatura	Preferência pelo substrato
NTPDase 1	CD39, ecto-ATP difosfoidrolase, ecto-apirase, ecto-ATPDase	ATP=ADP (1:1)*
NTPDase 2	CD39L1, ecto-ATPase	ATP>>>>ADP (30:1)*
NTPDase 3	CD39L3, HB6	ATP>ADP (3:1)*
NTPDase 4	UDPase (hLALP70v), hLALP70	UDP>GDP, CDP
NTPDase 5	CD39L4, ER-UDPase, PCPH	UDP>GDP, IDP>>>ADP, CDP
NTPDase 6	CD39L2	GDP>IDP>>UDP, CDP>>ADP
NTPDase 7	LALP1	UTP, GTP, CTP
NTPDase 8	---	ATP=ADP (1:1)*

Tabela 2. Nomenclatura e preferência de substratos dos membros da família E-NTPDases em vertebrados. * Razão de hidrólise NTD : NDP. As NTPDases de 1 a 3 hidrolisam todos os outros nucleotídeos purínicos e pirimídicos, similarmente ao ATP a ADP. Adaptado de Zimmermann (2001).

As NTPDases em conjunto com a Ecto-5’-nucleotidase/CD73 (ecto-5’-NT) constituem uma via altamente sofisticada, capaz de controlar os níveis extracelulares de ATP e Ado. A Ecto-5’-NT é expressa em vários tecidos, sendo que em SNC está predominantemente associada às células gliais, tendo sido

indentificada na membrana plasmática de astrócitos, oligodendrócitos, microglia e astrocitomas. O principal papel atribuído à ecto-5'-NT é a formação de Ado, a partir do AMP extracelular e a subseqüente ativação dos receptores de Ado (Zimmermann, 1992). Vários estudos têm demonstrado um papel da ecto-5'-NT na diferenciação de neurônios e sobrevivência celular (Maienschein e Zimmermann, 1996), bem como nos mecanismos que induzem a resistência a drogas (Ujházy *et al*., 1996). Além disso, estudos têm sugerido que a ecto-5'-NT tem características de uma molécula de adesão (Airas *et al*., 1995) e apresenta um papel na proliferação celular (Shoen *et al.,* 1988).

Um recente trabalho do nosso grupo (Wink et al., 2003) mostrou que células de gliomas apresentam um padrão alterado de hidrólise dos nucleotídeos extracelulares, quando comparado com astrócitos normais. Considerando que o ATP é reconhecido como um fator mitogênico que induz proliferação em gliomas (Morrone et al., 2003), as alterações observadas nas taxas de degradação de nucleotídeos sugerem que a via das ecto-nucleotidases poderia representar um importante mecanismo associado com a transformação maligna dos gliomas, sendo que maiores investigações poderiam tornar o sistema purinérgico um alvo de possíveis intervenções farmacológicas.

OBJETIVOS

Esta dissertação será apresentada na forma de capítulos, constituídos de artigos científicos, que visaram atingir os seguintes objetivos gerais:

Capítulo I:

Avaliar os efeitos de antiinflamatórios não-esteróides de classes químicas e especificidades diferentes em linhagens celulares de glioma humano e de rato;

Estudar os possíveis mecanismos envolvidos com o efeito antiproliferativo destes fármacos nestas linhagens.

Capítulo II:

Avaliar o efeito da indometacina, um antiinflamatório não-esteróide, na cascata de enzimas que catalizam a interconversão dos nucleotídeos (ecto-nucleotidases) em linhagens celulares de glioma humano e de rato.

CAPÍTULOS – ARTIGOS CIENTÍFICOS

4. Capítulo I

NONSTEROIDAL ANTI-INFLAMMATORY DRUGS INHIBIT THE GROWTH OF C6 AND U138-MG GLIOMA CELL LINES

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Nonsteroidal anti-inflammatory drugs inhibit the growth of C6 and U138-MG glioma cell lines

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used drugs for the treatment of inflammatory disease and have a chemopreventive effect in a variety of tumors. Several studies have demonstrated unequivocally that certain NSAIDs cause antiproliferative effects independent of cyclooxygenase (COX) activity. In this study, we investigated the effect of chemically unrelated NSAIDs in the proliferation of glioma cell lines and the possible mechanisms involved in indomethacin-mediated inhibition of proliferation in glioma cells lines. The glioma cell lines were treated with NSAIDs and proliferation was measured by cell counting. Indomethacin, acetaminophen, sulindac sulfide and NS-398 (*N*-[2-cyclohexyloxy)-4-nitrophenyl]methane-sulfonamide) induced a time- and concentration-dependent inhibition of C6 rat glioma cell proliferation. The inhibition of COX by chemically unrelated NSAIDs leads to inhibition of rat and human glioma cell proliferation. The tetrazolium reduction assay (MTT) indicated a reduction in cell viability induced by indomethacin. None of the NSAIDs tested induced caspase-3/7 activation, assayed with a fluorigenic substrate. The indomethacin-induced inhibition of C6 cells proliferation was abrogated by the use of the c-Src inhibitor, PP2 and the MEK inhibitor, PD 098059, suggesting COX-independent mechanisms. Indomethacin decreased the percentage of cells in the S phase, with relative increases in the G_0/G_1 and/or the G_2/M phase. NSAIDs may be clinically important for pharmacological intervention in gliomas.
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Keywords: Glioma; Proliferation; COX; NSAID; Indomethacin

1. Introduction

Gliomas, tumours that arise from cells of astrocytic lineage, represent 50% to 60% of brain tumors (Holland, 2001). Despite treatment, malignant gliomas recur early, leading to a mean survival of less than 12 months; these statistics have not changed significantly over the past two decades (Holland, 2001). The causes of this recurrence seem to be mainly the high proliferation, invasivity and resistance to radiation presented by these tumor cells (Avgeropoulos and Batchelor, 1999; Brandes et al., 2000).

Glioma progression is a combination of overactivated signaling pathways that control normal cell growth, aberrant expression of cellular oncogenes and deletion of tumor suppressor genes (Aaronson, 1991; Bredel and Pollack, 1999), which together lead to an abnormally high proliferative phenotype. Several signal transduction enzymes have been implicated in glioma cell proliferation, invasion and metastasis, such as mitogen-activated protein kinase (MAPK) (Bredel and Pollack, 1999), protein kinase C (PKC) (Bredel and Pollack, 1997; da Rocha et al., 2002) and phosphoinositide 3-kinase (PI3K) (Schelegel et al., 2000) pathways. It is well described that MAPK cascades can mediate cellular proliferation and differentiation in various cells, since activated extracellular-regulated protein kinase (ERK) can translocate to the nucleus and activate transcription factors that lead to the expression of genes involved in cell growth (Neary et al., 1999). Akt, also known as protein kinase B, is a downstream effector of PI3K

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and was found to be constitutively activated is some glioma cell lines (Schelegel et al., 2000).

There is a large body of evidence demonstrating that Src kinase activity are elevated in several cancers (Bjorge et al., 2000). It has been showed that c-Src is involved in Akt/PkB signaling (Buitrago et al., 2001) in several cell types, including endothelial (Okuda et al., 1999), skeletal muscle cells and astrocytes (Jacques-Silva et al., 2004), so it is possible to suppose the participation of this signaling pathway in glioma proliferation.

The enzyme cyclooxygenase (COX) is arising as a new target for cancer prevention and treatment, as substantial epidemiological, experimental, pathologic and clinical evidence suggests that the nonsteroidal anti-inflammatory drugs (NSAIDs) possess anticancer properties (Coogan et al., 2000). The NSAIDs exert their anti-inflammatory effects by inhibiting the synthesis of prostaglandins through inhibition of COX enzymes. There are two isoforms of COX, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). COX-1 is constitutively expressed in several cell types of normal mammalian tissues and synthesizes prostaglandins (PG) required for physiological functions like gastrointestinal cytoprotection and platelet activity; COX-2 is an inducible enzyme responsible for PG production at sites of inflammation (Smith et al., 1996, 2000). COX mediates a wide variety of derangements observed during diseases of the brain. In healthy brain, COX-1 is expressed in single macrophages/microglial cells and neurons and COX-2 is expressed by few endothelial cells (Casper et al., 2000b). COX-2 is overexpressed in several neoplasic tissues (Casper et al., 2000a), including high-grade human glioma (Joki et al., 2000). Moreover, high COX-2 expression in tumor cells is associated with clinically more aggressive gliomas and is a strong predictor of poor survival (Shono et al., 2001).

It was recently described that the NSAIDs flurbiprofen, acetaminophen, acetylsalicylate and ibuprofen, and the specific COX-2 inhibitor NS-398 (*N*-[2-cyclohexyloxy)-4-nitrophenyl] methane-sulfonamide) reduced proliferation of glioma cell lines (Joki et al., 2000; Casper et al., 2000a), but the mechanisms involved in the anti-mitogenic effect were not elucidated. Several COX-independent evidences of NSAIDs action have been raised recently, as reviewed by Tegeder et al. (2001). Therefore, the aims of the present study were: (1) to compare the effects of widely used nonprescription NSAIDs and the specific inhibitor of COX-2, NS-398, in the proliferation of human and rat glioma cells and (2) to investigate some of the mechanisms of indomethacin inhibition in glioma cells lines proliferation.

2. Materials and methods

2.1. Chemicals

Acetaminophen, indomethacin, sulindac sulfide and NS-398 were obtained from ICN Biochemicals. Signal transduction inhibitors and Propidium iodide (PI) were obtained from Sigma Chemical Co. (St. Louis, MO, USA), except by ZDEVD-CH_2F (*Z*-Asp(Ome)-Glu(Ome)-Val-Asp(Ome)), obtained from Enzyme Systems Products (Livermore, CA, USA). The drug preparations were made in cell culture-grade dimethylsulphoxide (DMSO; Sigma). The final concentrations of DMSO in the culture medium in all experiments were a maximum of 0.5% (v/v). CellTiter 96®AQ_{ueous} One Solution Cell Proliferation Assay was obtained from Promega. Cell culture media were obtained from Gibco (Gibco BRL, Carlsbad, CA, USA). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Chemicals and reagents were analytical grade or better.

2.2. Maintenance of cell line

The C6 rat glioma cell line and the U138-MG human glioma cell line were obtained from American Type Culture Collection (Rockville, Maryland, USA). The cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Carlsbad, CA, USA) containing 2.5 mg/ml Fungizone® and 100 U/l gentamicin, and supplemented with 5% (C6) or 15% (U138-MG) (v/v) fetal bovine serum (FBS; Cultilab, Campinas, SP, Brazil). Cells were kept at a temperature of 37 °C, a minimum relative humidity of 95% and an atmosphere of 5% CO_2 in air. All the experiments throughout this study were conducted in serum supplemented DMEM.

2.3. Cell counting

The C6 glioma cells were seeded at 5×10^3 cells/well in DMEM/5% FBS in 24-well plates and allowed to grown for 24 h. The medium was changed 2 h prior to treatment with NSAIDs. At the end of the evaluation treatment, 24, 48 or 72 h, the medium was removed, cells were washed with phosphate-buffered saline (PBS) and 200 µl of 0.25% trypsin/EDTA solution was added to detach the cells, which were counted immediately in a hemocytometer. The procedure was the same for the U138-MG glioma cells, except that they were seeded in DMEM/15% FBS.

To evaluate COX-independent pathways involved in indomethacin-mediated decrease in C6 cell number, we used the following inhibitors: 50 µM PD098059 (2′-amino-3′-methoxyflavone) inhibitor of MEK1; 5 µM GF 109203X (3-[1-[3-(dimethylamino)propyl]1*H*-indol-3-yl]-4-(1*H*indol-3-yl) 1*H*-pyrrole-2,5-dione), PKC inhibitor; 25 µM LY 294002 ([2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one), PI3K inhibitor; 0.5 µM PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo[3,4-*d*]pyridine), c-Src inhibitor or 30 µM ZDEVD-CH_2F (*Z*-Asp(Ome)-Glu(Ome)-Val-Asp(Ome), caspases inhibitor. The inhibitors cited above were added to the cultures 30 min before the treatment with 100 µM indomethacin.

2.4. Propidium iodide assay

Glioma cells lines are treated with indomethacin (25, 50, 100 and 400 µM) or DMSO (vehicle control) for different times (24, 48 and 72 h) in the presence of propidium iodide (6.4 µM). Propidium iodide fluorescence was excited at 515–560 nm

using an inverted microscope (Nikon Eclipse TE300) fitted with a standard rhodamine filter. Images were captured using a digital camera connected to the microscope and analyzed using Adobe Photoshop software.

2.5. Lactate dehydrogenase (LDH) activity

Cell viability was evaluated by measuring the activity of lactate dehydrogenase (LDH, ECI.1.1.27) according to the procedure of Whitaker (1969). Briefly, after 24, 48 or 72h treatment with indomethacin, the cell culture medium was collected and incubated with substrate mixture and LDH activity was determined by enzymatic colorimetric reaction. For additional comparison and to establish the limits of the method, some cells were freeze-thawed (F/T) to obtain the control of 100% of cell death. Absorption was measured at 490 nm.

2.6. MTT cell viability assay

This method provides a quantitative measure of the number cells with metabolically active mitochondria and is based on the mitochondrial reduction of a tetrazolium bromide salt (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]). Cells were plated in a 96-well plate at 10^3 /well and after 48 h treatment with indomethacin 20 µl of the CellTiter 96®AQ_{ueous} One Solution Reagent was added to culture wells and incubated for 2 h. The absorbance was read by an ELISA plate reader at 490 nm. This absorbance was linearly proportional to the number of live cells with active mitochondria.

2.7. Caspase activity assay

To measure caspase activity, C6 cell cultures were washed with PBS and then lysed on ice-cold PBS/Triton X-100 0.2%. The extract was centrifuged at 10,000×*g* for 5 min and supernatant was collected. For each reaction, 30 µg of the sample was incubated with a reaction buffer containing (g/ml) 0.1 sacarose, 0.001 CHAPS, 0.0001 BSA and 0.024 Hepes-NaOH, pH 7.5. The substrate, Ac-DEVD-AMC (Peptide Institute), had a final concentration of 0.02 mM. Caspase-3 and -7 cleave the substrate between D and AMC, releasing the fluorescent AMC, which can be quantified by fluorimetry, using an excitation wavelength of 370 nm and an emission wavelength of 460 nm. All measurements were corrected for protein concentration. The fluorescence intensity was calibrated with standard concentrations of AMC, and the caspase-3/7 activity calculated from the slope of the recorder trace and expressed in picomoles per minute per milligram of protein.

2.8. Cell cycle analysis

Analysis of cell cycle was performed by the method described by Overton and McCoy (1994), with slight modification. Cells were plated in 6-well plates and, after 2 days, were treated with 100 µM indomethacin for 24, 48 or 72 h; when the supernatant was removed, the cells were washed with PBS and directly treated with a solution containing 50 µg PI, 10 mM TRIZMA base (Sigma), 10 mM NaCl, 0.7 U RNase (Invitrogen Carlsbad, CA, USA) and 0.01% NP-40 (USB, Switzerland). After 10–30 min, data was collected of width (fl2w) and area (fl2a) of 30,000 fl2 (red–orange) pulses. Results were analyzed using Winmdi and Cylchred software (University of Wales College of Medicine, Cardiff, UK). Cells treated with DMSO were used as control.

2.9. Statistical analysis

All experiments were carried out at least three times in at least triplicate. Data were analyzed by unpaired analysis of variance (ANOVA) followed by post-hoc comparisons (Tukey test) using an Instat software package (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. NSAIDs inhibit growth of the malignant glioma cell lines C6 and U138-MG

Considering that COX-2 influences key cellular events including apoptosis, cell cycle, cell proliferation and angiogenesis (Casper et al., 2000a; Fosslien, 2001), we studied the effect of NSAIDs with different specificities over COX isoforms in the proliferation of glioma cell lines. To address the possibility that NSAIDs lead to decreased proliferation in gliomas, we performed time-course and dose–response studies with indomethacin, acetaminophen, sulindac sulfide and NS-398 in rat C6 glioma cells.

As shown in Fig. 1A, the number of C6 cells decreased over time when treated with indomethacin in the range of 25 to 400 µM. The inhibitory effect of indomethacin on cell growth increased during the 72 h of treatment evaluation. Acetaminophen, sulindac sulfide and NS-398 also inhibited cell growth in a dose-dependent manner after a 24 h treatment, and the antiproliferative effect progressively increased during the 72 h evaluation, similarly to indomethacin. An assay using sulphorhodamine B was performed as an independent method to evaluate cell growth and the results obtained were similar to cell counting data (data not shown).

In order to check if NSAIDs exhibited a growth inhibitory effect over human glioma cells, we treated U138-MG cells during 48 h with indomethacin, acetaminophen, sulindac sulfide and NS-398. As seem in Fig. 1E, a statistically significant inhibitory effect of NSAIDs was observed in U138-MG cell proliferation, indicating that these compounds exert a similar effect in rat and human malignant glioma cells.

As detected by propidium iodide incorporation, we did not observe necrotic cells in C6 (Fig. 2) and U138-MG (data not shown) treated with therapeutic doses (100 µM) of indomethacin. However, a significant increase cell damage was observed in cell treated with 400 µM indomethacin (Fig. 2).

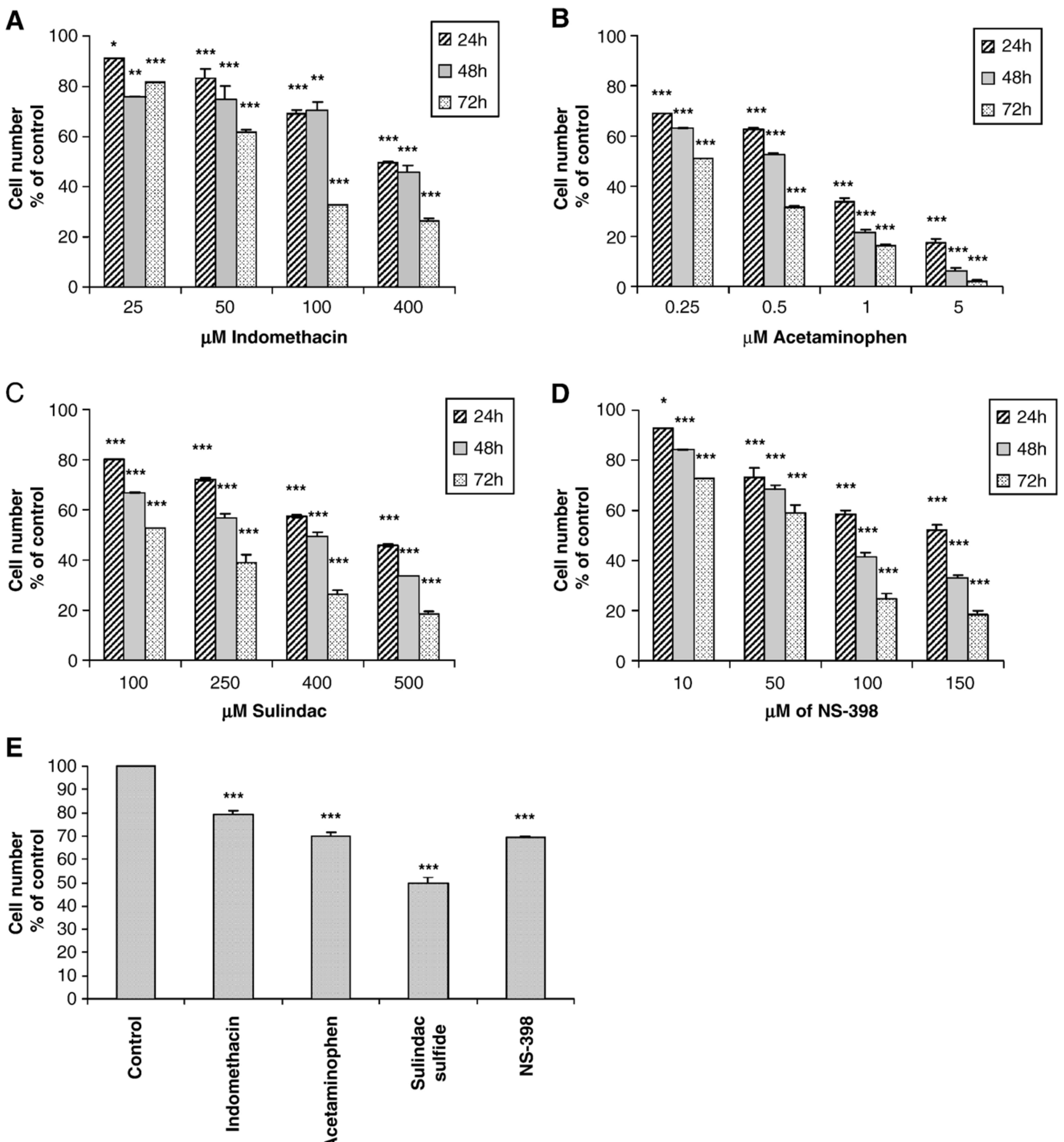

Fig. 1. Chemically unrelated NSAIDs reduce C6 and U138-MG glioma cell proliferation. Semi-confluent cultures of C6 cells were treated for the times indicated with different concentrations of (A) indomethacin, (B) acetaminophen, (C) sulindac sulfide or (D) NS-398. Cultures of U138-MG cells were treated for 48 h with 100 μM indomethacin, 500 μM acetaminophen and 400 μM sulindac sulfide or 75 μM NS-398 (E). The cells were detached with 0.25% trypsin/EDTA and counted in a hemotocytometer. Data are the means ± S.E.M. The average number of cells in untreated controls (100%) was 117,000 ± 4654 for C6 and 240,666 ± 18,502 for U138-MG. The effect was statistically significant at $*P<0.05$, $**P<0.01$ and $***P<0.001$.

3.2. Indomethacin decreases cell viability in human and rat glioma cells

To investigate whether NSAIDs modulate cell adhesion and ultimately cell viability, we used an MTT assay which measures the mitochondrial activity and, indirectly, the cell viability, even of the spontaneously detached cells in the culture medium. Cultured cells at approx. 80% confluence were incubated with various doses of indomethacin for 48 h. Analysis of MTT assay showed that indomethacin significantly diminished the cell viability at 100 μM and 400 μM, with 50% inhibition at approx. 400 μM (Fig. 3). Our data indicate that these compounds exert a similar effect in cell viability in C6 and U138-MG lines.

Cell viability was also evaluated by measuring the activity of LDH. The absence of necrotic cell death after treatment with clinically relevant doses of indomethacin (25, 50 and 100 μM)

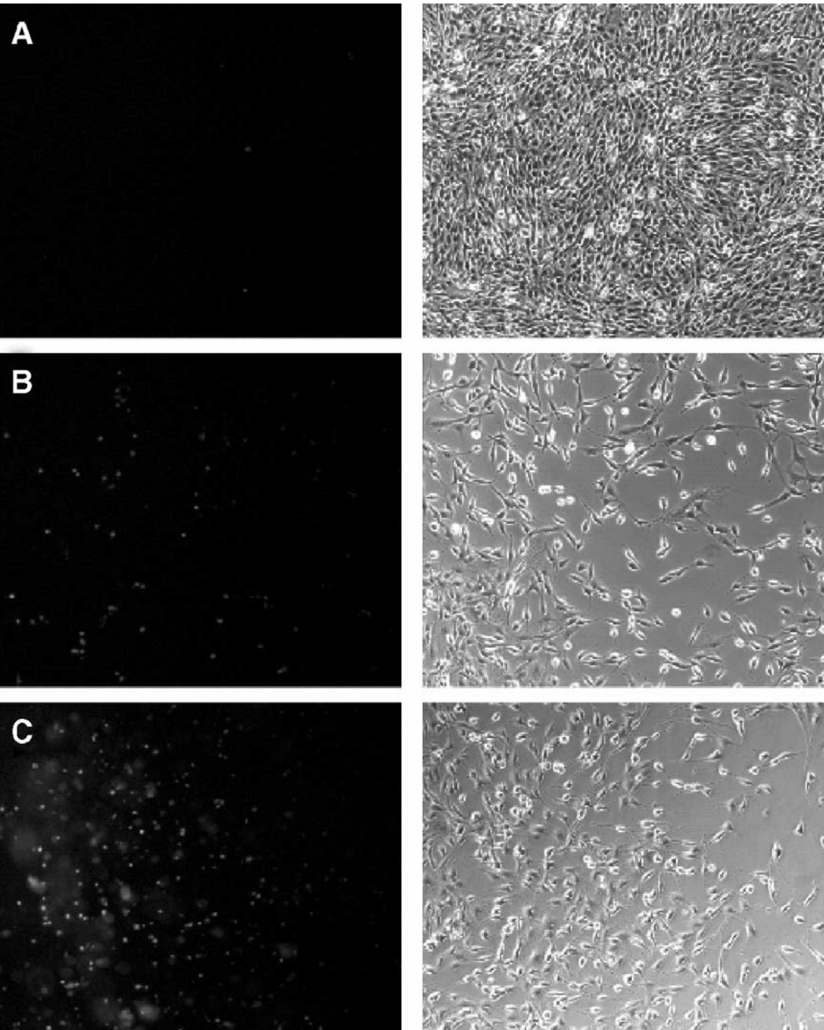

Fig. 2. Effects of indomethacin on cell death in C6 glioma cells by propidium iodide incorporation. Cells were treated with DMSO (A) or 100 μM (B) or 400 μM indomethacin (C) for 48 h in the simultaneous presence of propidium iodide (6.4 μM). Cellular death was analyzed by propidium iodide incorporation that was visualized using a Nikon inverted microscope. Panels at right of panels A, B and C are correspondent phase photomicrographs. Note the significantly decrease of total number of cells with indomethacin treatment relative to DMSO control. All experiments were carried out at least three times.

was observed by measuring the release of LDH activity to the extracellular medium. The indomethacin treatment did not increase the LDH activity in the culture supernatants of both glioma cell lines after 24, 48 and 72 h of treatment in relation to control cells. However, a significant increase in LDH activity was observed after treatment with 400 μM indomethacin for 72 h (data not shown).

3.3. NSAIDs induce caspase-independent cell death in C6 glioma cells

Cysteine-aspartate proteases (caspases) activity can indicate apoptotic cell death in brain (Meller et al., 2002). In order to verify if caspase-dependent apoptotic cell death is induced by NSAIDs in C6 cells and to support the notion that NSAIDs induce apoptosis in tumor cells, the activity of caspase-3 and -7 was evaluated. As observed in Fig. 4, indomethacin, acetaminophen, sulindac sulfide and NS-398 did not induce an increase in caspase-3/7 activity after a 48-h treatment, suggesting a caspase-3/7-independent cell death mechanism. The positive control used in this assay was LY294002-treated organotypic hippocampal slice cultures, which shows an induction in caspase-3/7 activity in comparison to the untreated organotypic slices (data not shown).

3.4. Cell cycle analysis

Flow cytometry analysis showed that the treatment with 100 μM indomethacin for 48 h induced an arrest in the G_0/G_1 and G_2/M phases in U138-MG glioma cells (Table 1), while in rat C6 cells the same treatment causes an arrest in G_0/G_1, when compared with control cells treated with DMSO. This effect was observed from 24 to 72 h.

3.5. The antiproliferative effect of indomethacin is dependent on c-Src- and ERK-mediated pathways

Because of its greater effectiveness on C6 glioma cells viability, we focused next on some mechanistic aspects of indomethacin-mediated inhibition of C6 cell proliferation.

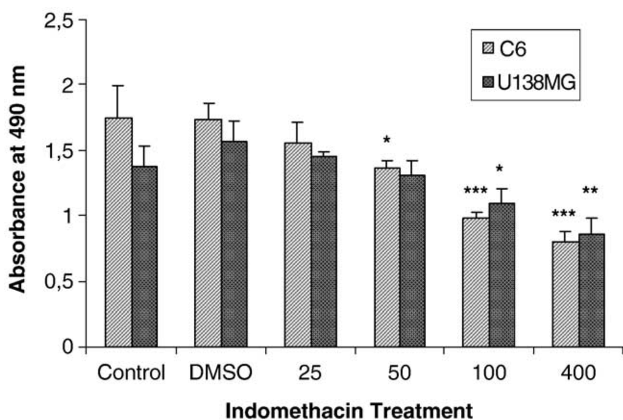

Fig. 3. Dose–response curve of indomethacin effect on cell viability by MTT accumulation in cultured U138-MG and C6 glioma cell lines. The cells were grown on a 96-well plate to reach approx. 80% confluence. Various doses of indomethacin (25 to 400 μM) were then added and the incubation continued for 48 h. The percentage viability was measured by MTT assay. Values are means± S.E.M. The effect was statistically significant at $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

Table 1
Effect of indomethacin on the cell cycle distribution of U138-MG and C6 glioma cell lines

	Time	Treatment	% G_0/G_1	% S	% G_2/M
U138-MG	24 h	DMSO	44	40	16
		Indomethacin	49	35	16
	48 h	DMSO	55	34	11
		Indomethacin	65	19	16
	72 h	DMSO	61	26	13
		Indomethacin	70	13	17
C6	24 h	DMSO	49	42	9
		Indomethacin	48	42	10
	48 h	DMSO	67	24	9
		Indomethacin	75	20	5
	72 h	DMSO	82	17	1
		Indomethacin	93	6	1

Cells were treated with 100 μM of indomethacin or DMSO (control) for the indicated times and then subjected to flow cytometry cell cycle analysis as described in Materials and methods. Data are expressed as percent of cells in G_0/G_1, S or G_2/M phases of the cell cycle. Data representative of three different experiments.

To investigate the involvement of signal transduction pathways in indomethacin-mediated inhibition of glioma cell proliferation, we treated C6 cultures with specific MEK1/2 inhibitor (50 μM PD 98059), PKC inhibitor (5 μM GF 109203X), PI3-K inhibitor (25 μM LY 294002) or c-Src inhibitor (0.5 μM PP2) 30 min prior to treatment with 100 μM indomethacin for 24 h. The treatment of C6 cultures with the inhibitors GF 109203X and LY 294002 per se decreased the number of cells by 22% and 30%, respectively ($P<0.001$), as observed in Table 2. As expected, the number of cells in cultures treated with indomethacin represented 67% of the control cultures ($P<0.001$). When cultures were pretreated with PP2 or PD98059 plus indomethacin, this effect was significantly prevented, indicating that ERK (Seger and Krebs, 1995) and c-Src are involved in the antiproliferative effect of indomethacin. Accordingly, indomethacin stimulated ERK phosphorylation (data not shown). The caspases inhibitor ZDEVD-CH_2F did not change the anti-proliferative effect of indomethacin, confirming the data obtained with caspase-3/7 activity.

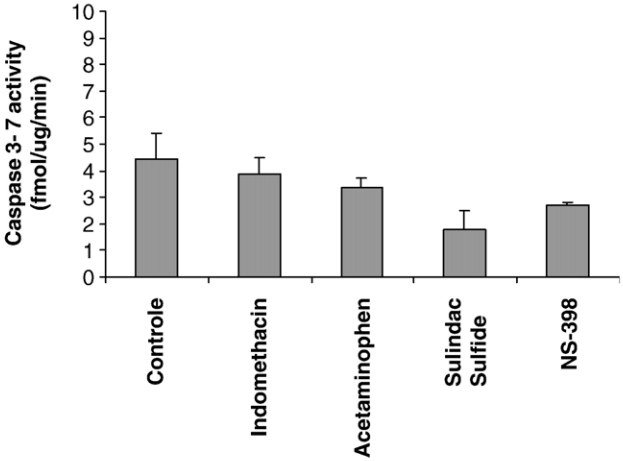

Fig. 4. Chemically unrelated NSAIDS do not induce caspase 3/7 activation in C6 glioma cells. Cells were treated for 48h with 100 μM indomethacin, 500 μM acetaminophen, 400 μM sulindac sulfide or 75 μM NS-398 and caspase 3/7 activity was determined by fluorescence intensity examination. All experiments were carried out at least three times. Data were analyzed by unpaired ANOVA.

4. Discussion

The arachidonic acid pathway and one of its key enzymes, COX (or prostaglandin H synthase), have received much attention regarding prevention and development of colon cancer. While COX-1 is constitutively expressed, COX-2 is upregulated in response to growth factors, tumor promoters, cytokines and oncogenes like *v-src*, *v-Ha-Ras*, *HER-2/neu* and *wnt* genes (Howe et al., 2001). Recently, celecoxib, a selective COX-2 inhibitor, was approved by the FDA for the prevention of colon cancer in patients with familial polyposis coli, which is a hereditary precancerous disease due to a loss of the adenomatous polyposis coli tumor suppressor gene (Tegeder et al., 2001). Enhanced PG synthesis (the main product of COX activity) may contribute to carcinogenesis in several ways,

Table 2
Effect of signal transduction pathway inhibitors on C6 cell number reduction induced by indomethacin

	% of control	
	Without indomethacin	Indomethacin-treated
Control	100±0.00	67.73±2.83 [a]
PP2	93.18±2.34	81.59±2.55 [b]
PD 98059	96.97±1.16	91.19±1.64 [c]
LY 294002	70.26±2.87 [a]	56.19±2.66
GF 109203X	77.72±1.62 [a]	55.90±0.82
ZDEVD-CH_2F	93.30±4.00	65.72±1.87

[a] Significantly different from the control group without indomethacin by ANOVA test, $P<0.001$.

[b] Significantly different from the indomethacin-treated group by ANOVA test, $P<0.05$.

[c] Significantly different from the indomethacin-treated group by ANOVA test, $P<0.001$.

including direct stimulation of cell growth, which was detected in gastric (Ristimaki et al., 1997), esophageal (Wilson et al., 1998) and lung carcinomas (Hida et al., 1998) and brain tumors (Deininger et al., 1999; Joki et al., 2001).

Because of new reports indicating COX-independent pathways by which NSAIDs can act to reduce cancer cell growth (Joki et al., 2001), the aim of this study was to evaluate the role of different NSAIDs in the proliferation of human and rat glioma cell lines and the mechanisms possible involved in the anti-mitogenic effect of indomethacin in these cells.

Indomethacin and sulindac sulfide significantly reduced C6 and U138-MG glioma cell proliferation (Fig. 1), a result previously observed in gastric carcinoma cell lines and colon cancer cells (Fujiwara et al., 1993; Yamamoto et al., 1999). Although the concentration of 500 μM of sulindac sulfide is about 10 times greater than the therapeutic doses for COX inhibition (Ravis et al., 1993), the concentration used for subsequent studies with indomethacin (100 μM) is a clinically relevant dose (Lentjes and van Ginneken, 1987). Acetaminophen is not as potent in inhibiting COX activity as are indomethacin and sulindac sulfide, but this drug also inhibited the proliferation of C6 and U138-MG cells (Fig. 1), which reinforces the role of COX-independent pathways. In the other hand, NS-398, an experimental selective COX-2 inhibitor, i.e. not for use in humans (Futaki et al., 1994), also inhibited cell proliferation, suggesting the presence of COX-dependent and independent mechanisms.

As reviewed by Williams et al. (1999), there is variability in the effects that different NSAIDs have on cancer cells: some NSAIDs, such as sulindac sulfide, potently induce apoptosis, while other NSAIDs, such as SC-58125 (selective COX-2 inhibitor), primarily induce cell cycle arrest, thus decreasing cellular proliferation rates. In addition, several studies showed that apoptosis was induced in glioma cultures treated with NS-398 or acetaminophen (Joki et al., 2000; Bae et al., 2001).

Recently, Bae et al. (2003) have reported that acetaminophen induced DNA fragmentation and an increase in LDH release after 24 h treatment. However, in this study, a very high concentration of this drug (5 mM) was used, which can be considered a cytotoxic dose to the normal cells, limiting its therapeutic use. It is important to note that in our study we obtained a decrease in cell viability after treatment with therapeutic doses of indomethacin, as observed by a MTT assay (Fig. 3). Our results are in accordance with several studies reviewed by Eli et al. (2001), in which it was showed that indomethacin reduced cell viability in carcinoma cells. The absence of necrotic cells in gliomas cell cultures treated with therapeutic doses of indomethacin was detected by propidium iodide incorporation (Fig. 2) and confirmed by the measurement of the release LDH activity (data not shown).

The measurement of caspase-3 and -7 activity, a marker of apoptotic cell death, did not present any differences between untreated and NSAIDs-treated C6 cells (Fig. 4), while DNA fragmentation, another indicator of apoptotic cell death, was induced by indomethacin treatment (data not shown),

In addition, the caspase activity data was confirmed by data presented in Table 2, where the caspase inhibitor ZDEVD-FMK was unable to prevent the effect of indomethacin. A caspase-independent cell death was reported elsewhere by NSAIDs (Biffl et al., 2003). Therefore, it is possible to suggest that indomethacin induce caspase-independent cell death in malignant glioma cells.

Recently, Parashar et al. (2005) showed an arrest in the G_2/M phase of human neuroblastoma cells using CAY10404, a highly selective COX-2 inhibitor. In our study, a significant increase in the proportion of cells in the G_0/G_1 and G_2/M phases and the concurrent decrease in the proportion of cells in the S phase after 48 h treatment with 100 μM indomethacin were observed, suggesting the arrest of the cells in G_1 and/or G_2 phase of the cell cycle (Table 1). Interestingly, in rat C6 glioma cells, indomethacin treatment causes an arrest in G_0/G_1. These results indicate that the antiproliferative effects of indomethacin in gliomas cell lines are, in part, due to inhibition of cell cycle progression. Several lines of evidence link NSAIDs effects to the cell cycle. In gliomas, celecoxib, a selectively inhibitor of COX-2, exerts antiproliferative effects via the transcriptional inhibition of two essential components of the cell cycle machinery, cyclin A and cyclin B (Kardosh et al., 2004). In human colon cancer cell, indomethacin was shown to induce cell cycle arrest in G_2 phase in a p53-dependent way, through induction of $p21^{WAF1/CIP1}$ and consequent inhibition of CDK2 and CKD4 (Xu and Zhang, 2005). Since the glioma cell lines used in this study are p53 positive, p53-dependent mechanism could be involved in the arrest of cell cycle induced by NSAIDs.

The mechanisms associated with the effectiveness of NSAIDs against cancer cells remains under investigation, but COX-2 inhibition is suggested to be the most remarkable one. It appears that the more advanced the tumor stage and grade, the greater the level of COX-2 production (Ristimaki et al., 2002), although COX-2 over-expression may be part of a complement of malignant characteristics of the carcinomatous state. The tumor environment is an optimal medium to gene transcription, as it contains deregulated oncogenes, cytokines, growth factors and hormones, all of which have been shown to cause induction of COX-2 expression (Vadlamudi et al., 1999; Xie and Herschmann, 1996). So, the precise role of COX-2, and specifically its interplay with COX-1 in carcinogenesis, is unknown. COX-3 a putative COX-1 variant (Carvalho et al., 2004), but, unfortunately, there are no specific inhibitors of COX-3 commercially available to study the importance of this enzyme in the control of glioma growth.

The mechanism by which NSAIDS caused reduction in proliferation of various cell lines is unknown. Celecoxib, a specific COX-2 inhibitor, has been shown to have antitumor effects that may be mediated through the COX-independent inhibition of AKT signaling (Yang et al., 2004). There is accumulating evidence indicating that the effectiveness of NSAIDs against cancer cells is due to COX-independent mechanisms. According to Rigas and Schiff (2000), NSAIDs most likely stimulate apoptosis via both COX-dependent and independent mechanisms. NSAIDs still have antineoplasic effects when used against COX-1 and COX-2-null mouse embryo fibroblasts (Zhang et al., 1999), and the concentrations of NSAIDs used to inhibit growth are often 10 to 100 times

higher than are required to inhibit COX activity (He et al., 1999). These and other studies have provided evidence for a number of alternative mechanisms to explain the anticancer effects of NSAIDs. Ordan et al. (2003) reported a c-Jun N-terminal kinase (JNK)-dependent mechanism for the antiproliferative effect of aspirin in melanoma cells. Others have suggested that the mechanism underlying the anticancer actions of NSAIDs may involve NF-κB, activator protein 1, p38 MAPK, ribosomal S6 kinase 2, STAT1, PPARγ and the inhibition of cell cycle progression (Tegeder et al., 2001).

Cellular proliferation and differentiation in several cell types are mediated by ERK (Meller et al., 2002) and c-Src, which are involved in the control of a variety of cellular processes such as proliferation, differentiation, motility and adhesion (Ordan et al., 2003). There is a large body of evidence demonstrating that Src kinase activity, and sometimes Src protein levels, are elevated in several cancers including colon and breast cancer, with a correlation often observed between increases in Src kinase activity and degree of malignancy (Bjorge et al., 2000). There are contradictory results concerning inhibitory or activating effects of NSAIDs on MAPK family members (Pillinger et al., 1998; Schwenger et al., 1998), which are probably dependent on the cellular context, chemical classes and concentration of the NSAIDs used. Nevertheless, our observations are in line with the findings of Elder et al. (2002), who showed that NS-398 causes sustained activation of ERKs, resulting in the induction of apoptosis in colorectal carcinoma cells. As c-Src is upstream of ERK in human astrocytoma cells (Gendron et al., 2003), we propose a mechanism where indomethacin induce the activation of c-Src that then leads to activation of ERK and, thus, reduction in C6 cell number, although we cannot exclude whether these two pathways are independently activated by indomethacin.

Despite aggressive surgical and adjuvant therapy, little progress has been made in the successful management of malignant glioma. However, research efforts have yielded some promising leads. One of which is the apparent association between NSAID use and decreased cancer risk. Considering that indomethacin, sulindac and acetaminophen have the ability to cross the blood–brain barrier (Courade et al., 2001) and they are sufficiently safe to allow large-scale administration on a chronic basis to individuals, they represent potentially useful agents for glioma chemoprevention or adjuvant therapy.

5. Conclusions

In conclusion, our data indicates that treatment of C6 and U138-MG glioma cells with indomethacin, acetaminophen, sulindac sulfide and NS-398 leads to a decrease in cell number and indicate a caspase-3/7-independent apoptotic cell death mechanism. The antiproliferative effects of indomethacin in glioma cell lines are, at least in part, due to inhibition of cell cycle progression. Indomethacin-induced decrease in cell number in C6 glioma cells is mediated by c-Src and ERK signalling. These results, in combination with the documented in literature, could have profound clinical implications for gliomas treatment and prevention.

Acknowledgments

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References

Aaronson, S.A., 1991. Growth factors and cancer. Science 254, 1146–1153.

Avgeropoulos, N.G., Batchelor, T.T., 1999. New strategies for malignant gliomas. The Oncologist 4, 209–224.

Bae, M.A., Pie, J.E., Song, B.J., 2001. Acetaminophen induces apoptosis of C6 glioma cells by activating the c-Jun NH2-terminal protein kinase-related cell death pathway. Mol. Pharmacol. 60, 547–556.

Bae, J.S., Jang, M.K., Hong, S., An, W.G., Choi, Y.H., Kim, H.D., Cheong, J., 2003. Phosphorylation of NF-kappa B by calmodulin-dependent kinase IV activates anti-apoptotic gene expression. Biochem. Biophys. Res. Commun. 13 (305), 1094–1098.

Biffl, W.L., Carnaggio, R., Moore, E.E., Ciesla, D.J., Johnson, J.L., Silliman, C. C., 2003. Clinically relevant hypertonicity prevents stored blood-and lipid-mediated delayed neutrophil apoptosis independent of p38 MAPK or caspase-3 activation. Surgery 134, 86–91.

Bjorge, J.D., Jakimiw, D.J., Fujita, D.J., 2000. Selected glimpses into the activation and function of Src kinase. Oncogene 19, 5620–5635.

Brandes, A.A., Pasetto, L.M., Monfardini, S., 2000. New drugs in recurrent high grade gliomas. Anticancer Res. 20, 1913–1920.

Bredel, M., Pollack, I.F., 1997. The role of protein kinase C (PKC) in the evolution and proliferation of malignant gliomas, and the application of PKC inhibition as a novel approach to anti-glioma therapy. Acta Neurochir. (Wien) 139, 1000–1013.

Bredel, M., Pollack, I.F., 1999. The p21-*Ras* signal transduction pathway and growth regulation in human high-grade gliomas. Brain Res. Rev. 29, 232–249.

Buitrago, C., Boland, R., Boland, A.R., 2001. The tyrosine kinase c-Src is required for 1,25-$(OH)_2$-vitamin D3 signalling to the nucleus in muscle cells. Biochim. Biophys. Acta 1541, 179–187.

Carvalho, W.A., Carvalho, R.D.S., Rios-Santos, F., 2004. Specific cyclooxygenase-2 inhibitor analgesics: therapeutic advances. Rev. Bras. Anestesiol. 54, 448–464.

Casper, D., Lekhraj, R., Yaparpalvi, U.S., Pidel, A., Jaggernauth, W.A., Werner, P., Tribius, S., Rowe, J.D., LaSala, P.A., 2000a. Acetaminophen selectively reduces glioma cell growth and increases radiosensitivity in culture. J. Neurooncol. 46, 215–229.

Casper, D., Yaparpalvi, U., Rempel, N., Werner, P., 2000b. Ibuprofen protects dopaminergic neurons against glutamate toxicity in vitro. Neurosci. Lett. 11 (289), 201–204.

Coogan, P.F., Rosenberg, L., Palmer, J.R., Strom, B.L., Zauber, A.G., Stolley, P. D., Shapiro, S., 2000. Nonsteroidal anti-inflammatory drugs and risk of digestive cancers at sites other than the large bowel. Cancer Epidemiol. Biomark. Prev. 9, 119–123.

Courade, J.P., Besse, D., Delchambre, C., Hanoun, N., Hamon, M., Eschalier, A., Caussade, F., Cloared, A., 2001. Acetaminophen distribution in the rat central nervous system. Life Sci. 69, 1455–1464.

da Rocha, A.B., Mans, D.R.A., Regner, A., Schwartsmann, G., 2002. Targeting protein kinase C: new therapeutic opportunities against high-grade malignant gliomas? The Oncologist 7, 17–33.

Deininger, M.H., Weller, M., Streffer, J., Mittelbronn, M., Meyermann, R., 1999. Patterns of cyclooxygenase-1 and -2 expression in human gliomas in vivo. Acta Neuropathol. 98, 240–244.

Elder, D.J.E., Halton, D.E., Playle, L.C., Paraskeva, C., 2002. The MEK/ERK pathway mediates COX-2-selective NSAID-induced apoptosis and induced COX-2 protein expression in colorectal carcinoma cells. Int. J. Cancer 99, 323–327.

Eli, Y., Przedeeki, F., Levin, G., Kariv, N., Raz, A., 2001. Comparative effects of indomethacin on cell proliferation and cell cycle progression in tumor cells grown in vitro and in vivo. Biochem. Pharmacol. 61, 565–571.

Fosslien, E., 2001. Review: molecular pathology of cyclooxygenase-2 in cancer induced angiogenesis. Ann. Clin. Lab. Sci. 31, 325–348.

Fujiwara, Y., Tarnawski, A., Fujiwara, K., Arakawa, T., Kobayashi, K., 1993. Inhibitory effects of indomethacin on growth and proliferation of gastric carcinoma cells KATO III. J. Physiol. Pharmacol. 44, 147–153.

Futaki, N., Takahashi, S., Yokoyama, M., Arai, I., Higuchi, S., Otomo, S., 1994. NS-398, a new anti-inflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (COX-2) activity in vitro. Prostaglandins 47, 55–59.

Gendron, F., Neary, J.T., Theiss, P.M., Sun, G.Y., Gonzalez, F.A., Weisman, G. A., 2003. (a) Mechanisms of $P2X_7$ receptor-mediated ERK1/2 phosphorylation in human astrocytoma cells. Am. J. Cell Physiol. Cell Phys. 284, C571–C581.

He, T.C., Chan, T.A., Volgelstein, B., Kinzler, K.W., 1999. PPAR-gamma is an APC-regulated target of nonsteroidal antiinflammatory drugs. Cell 99, 335.

Hida, T., Yatabe, Y., Achiwa, H., Muramatsu, H., Kozaki, K., Nakamura, S., Ogawa, M., Mitsudomi, T., Sugiura, T., Takahashi, T., 1998. Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas. Cancer Res. 58, 3761–3764.

Holland, E.C., 2001. Gliomagenesis: genetic alterations and mouse models. Nature 2, 120–129.

Howe, L.R., Subbaramaiah, K., Brown, A.M.C., Dannenberg, A.J., 2001. Cyclooxygenase-2: a target for the prevention and treatment of breast cancer. Endocr. -Relat. Cancer 8, 97–114.

Jacques-Silva, M.C., Rodnight, R., Lenz, G., Liao, Z., Kong, Q., Tran, M., Kang, Y., Gonzales, F.A., Weisman, G.A., Neary, J.T., 2004. P2X7 receptors stimulate AKT phosphorylation in astrocytes. Br. J. Pharmacol. 141, 1107–1118.

Joki, T., Heese, O., Nikas, D.C., Bello, L., Zhang, J., Kraeft, S.K., Seyfried, N. T., Abe, T., Chen, L.B., Carroll, R.S., Black, P., 2000. The cyclooxygenase-2 inhibitor celecoxib induces apoptosis by blocking Akt activation in human prostate cancer cells independently of Bcl-2. J. Biol. Chem. 275, 11397–11403.

Joki, T., Carroll, R.S., Dunn, I.F., Zhang, J., Abe, T., Black, P.M., 2001. Assessment of alterations in gene expression in recurrent malignant glioma after radiotherapy using complementary deoxyribonucleic acid microarrays. Neurosurgery 48, 195–2001.

Kardosh, A., Blumenthal, M., Wang, W.J., Chen, T.C., Schonthal, A.H., 2004. Differential effects of selective COX-2 inhibitors on cell cycle regulation and proliferation of glioblastoma cell lines. Can. Biol. Ther. 3, 55–62.

Lentjes, E.G., van Ginneken, C.A., 1987. Pharmacokinetics of flufenamic acid in man. Int. J. Clin. Pharmacol. Ther. Toxicol. 25, 185–189.

Meller, R., Skradski, S.L., Simon, R.P., Henshall, D.C., 2002. Expression, proteolysis and activation of caspases 6 and 7 during rat C6 glioma cell apoptosis. Neurosci. Lett. 324, 33–36.

Neary, J.T., Kang, Y., Bu, Y., Yu, B., Akong, K., Peters, C.M., 1999. Mitogenic signaling by ATP/P2Y purinergic receptors in astrocytes: involvement of a calcium-independent protein kinase C, extracellular signal-regulated protein kinase pathway distinct from the phosphatidylinositol-specific phospholipase C/calcium pathway. J. Neurosci. 19, 4211–4220.

Okuda, M., Takahashi, M., Suero, J., Murry, C.E., Traub, O., Kawakatsu, H., Berk, B.C., 1999. Shear stress stimulation of $p130^{Cas}$ tyrosine phosphorylation requires calcium-dependent c-Src activation. J. Biol. Chem. 274, 26803–26809.

Ordan, O., Rotem, R., Jaspers, H., Flescher, E., 2003. Stress-responsive JNK mitogen-activated protein kinase mediates aspirin-induced suppression of B16 melanoma cellular proliferation. Br. J. Pharmacol. 138, 1156–1162.

Overton, W.R., McCoy Jr., J.P., 1994. Reversing the effect of formalin on the binding of propidium iodide to DNA. Cytometry 16, 351–356.

Parashar, B., Shankar, L.S., O'Guin, K., Butler, J., Vikram, B., Shafit-Zagardo, B., 2005. Inhibition of human neuroblastoma cell growth by CAY10404, a highly selective COX-2 inhibitor. J. Neuro-Oncol. 71, 141–148.

Pillinger, M.H., Capodici, C., Rosenthal, P., Kheterpal, N., Hanft, S., Philips, M. R., Weissmann, G., 1998. Modes of action of aspirin-like drugs: salicilates inhibit ERK activation and integrin-dependent neutrophil adhesion. Proc. Natl. Acad. Sci. U. S. A. 95, 14540–14545.

Ravis, W.R., Diskin, C.J., Campagna, K.D., Clark, C.R., McMillian, C.L., 1993. Pharmacokinetics and dialyzability of sulindac and metabolites in patients with end-stage renal failure. J. Clin. Pharmacol. 33, 527–533.

Rigas, B., Shiff, S.J., 2000. Is inhibition of cyclooxygenase required for the chemopreventive effect of NSAIDs in colon cancer? A model reconciling the current contradiction. Med. Hypothesis 54, 210–215.

Ristimaki, A., Hunkanen, A., Jankala, H., Sipponen, P., Harkonen, M., 1997. Expression of cyclooxygenase-2 in human gastric carcinoma. Cancer Res. 57, 1276–1280.

Ristimaki, A., Sivula, A., Lundin, J., Lundin, M., Salminen, T., Haglund, C., Joensuu, H., Isola, J., 2002. Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. Cancer Res. 62, 632–635.

Schelegel, J., Piontek, G., Budde, B., Neff, F., Kraus, A., 2000. The Akt/PKB-dependent anti-apoptotic pathway and the MAPK cascade are alternatively activated in human glioblastoma multiforme. Cancer Lett. 158, 103–108.

Schwenger, P., Alpert, D., Skolnick, E.Y., Vilcek, J., 1998. Activation of p38 mitogen-activated protein kinase by sodium salicylate leads to inhibition of tumor necrosis factor-induced IkappaB alpha phosphorylation and degradation. Mol. Cell. Biol. 18, 78–84.

Seger, R., Krebs, E.G., 1995. The MAPK signaling cascade. FASEB J. 9, 726–735.

Shono, T., Tofilon, P.J., Bruner, J.M., Owolabi, O., Lang, F.F., 2001. Cyclooxygenase-2 expression in human gliomas: prognostic significance and molecular correlations. Cancer Res. 61, 4375–4381.

Smith, W.L., Garavito, R.M., DeWitt, D.L., 1996. Prostaglandin endoperoxidase H synthases (cyclooxygenases)-1 and -2. J. Biol. Chem. 271, 33157–33160.

Smith, W.L., DeWitt, D.L., Garavito, R.M., 2000. Cyclooxygenases: structural, cellular and molecular biology. Annu. Rev. Biochem. 69, 145–182.

Tegeder, I., Pfeilschifter, J., Geisslinger, G., 2001. Cyclooxygenase-independent actions of cyclooxygenase inhibitors. FASEB J. 15, 2057–2072.

Vadlamudi, R., Mandal, M., Adam, L., Steinbach, G., Mendelsonh, J., Kumar, R., 1999. Regulation of the cyclooxygenase pathway by the HER-2 receptor. Oncogene 18, 305–314.

Whitaker, J.F., 1969. A general colorimetric procedure for the estimation of enzymes witch are linked to the $NADH-NAD^+$ system. Clin. Chim. Acta 24, 23–27.

Williams, C.S., Mann, M., DuBois, R.N., 1999. The role of cyclooxygenases in inflammation, cancer and development. Oncogene 18, 7908–7916.

Wilson, K.T., Fu, S., Ramanujan, K.S., Meltzer, S.J., 1998. Increase expression of inducible nitric oxide synthase and cyclooxygenase-2 in Barrett's esophagus and associated adenocarcinomas. Cancer Res. 58, 2929–2934.

Xie, W., Herschmann, H.R., 1996. Transcriptional regulation of prostaglandin synthase-2 gene expression by platelet derived growth factor and serum. J. Biol. Chem. 271, 131742–131748.

Yamamoto, Y., Yin, M.J., Lin, K.M., Gaynor, R.B., 1999. Sulindac inhibits activation of the NF-κB pathway. J. Biol. Chem. 274, 27307–27314.

Yang, H.M., Kim, K.W., You, H.J., Jeon, S.I., Youn, S.W., Kim, S.H., Oh, B.H., Lee, M.M., Park, Y.B., Walsh, K., 2004. Celecoxib, a cyclooxygenase-2 inhibitor, reduces neointimal hyperplasia through inhibition of AKT signaling. Circulation 110 (3), 301–308.

Xu, M., Zhang, G.Y., 2005. Effect of indomethacin on cell cycle proteins in colon cancer cell lines. World J. Gastroenterol. 11911, 1692–1696.

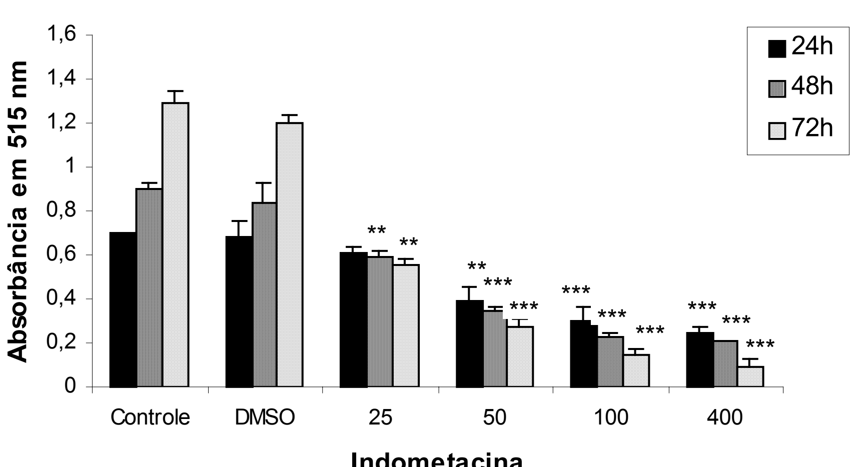
Zhang, X., Morhan, S.G., Langenbach, R., Young, D.A., 1999. Malignant transformation and antineoplasic actions of non steroidal antiinflammatory drugs (NSAIDs) on cyclooxygenase-null embryo fibroblast. J. Exp. Med. 190, 451.

4.1. Anexos Capítulo I

Dados não mostrados no **"Capítulo I"**, mas ciatados na **"Discussão".**

Figura 1

A



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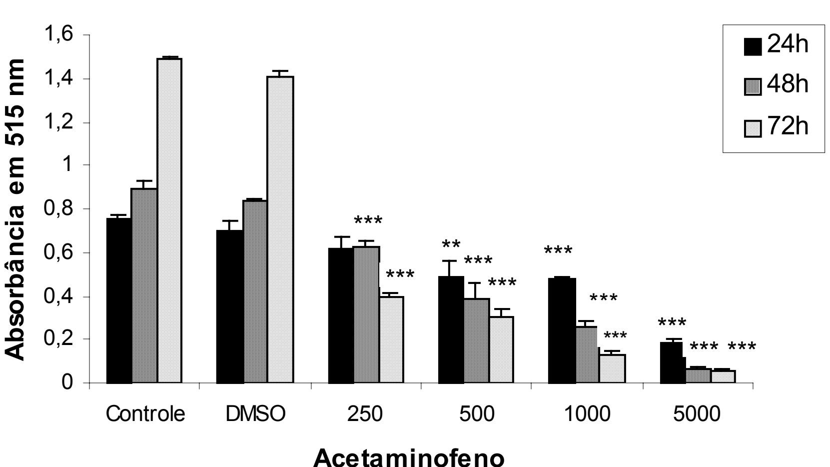

Figura 1

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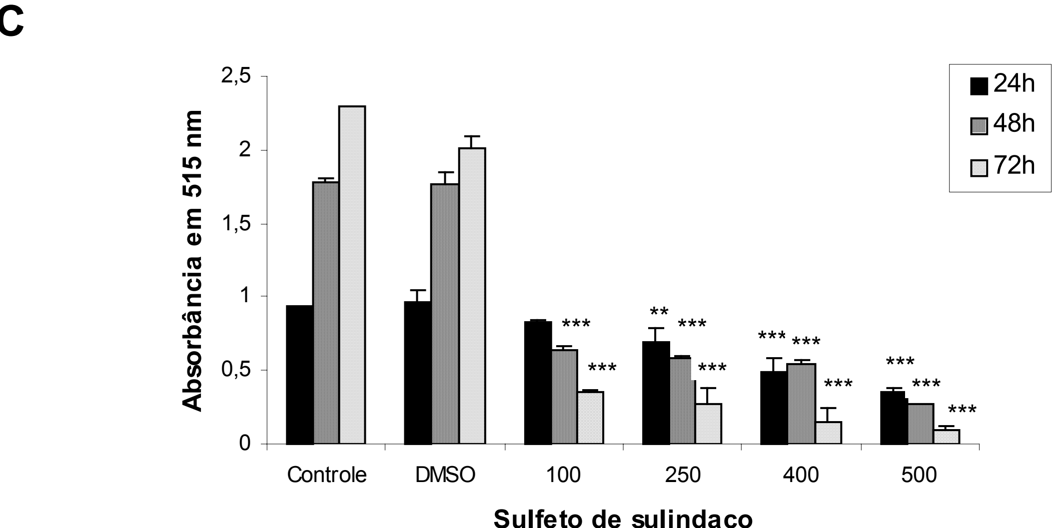

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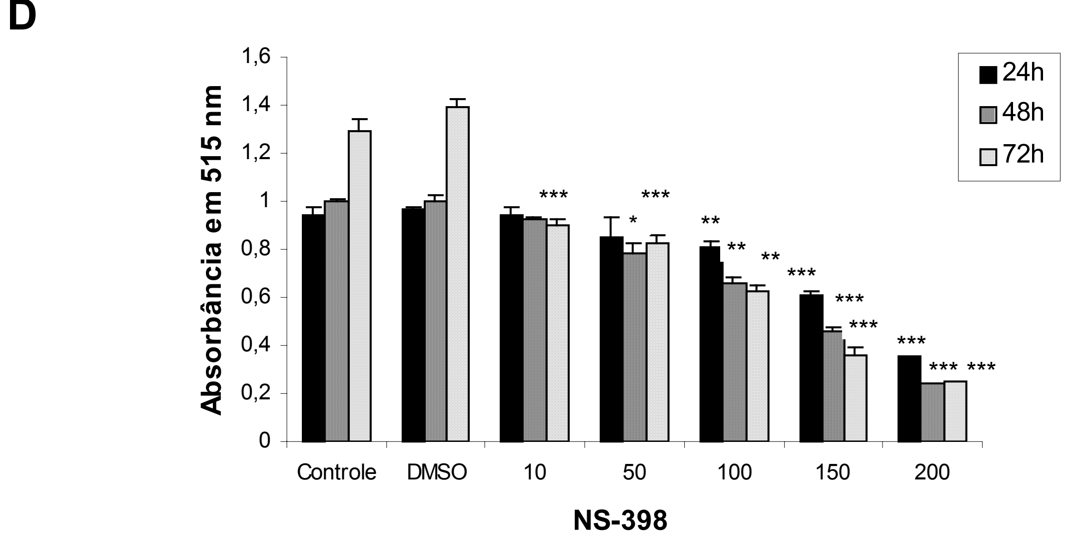


Figura 1. Inibição da proliferação celular na linhagem de glioma C6. A linhagem de glioma C6 foi tratada com os AINEs indometacina (A), acetaminofeno (B), sulfeto de sulindaco (C) e NS-398 (D) por 24, 48 ou 72h nas concentrações indicadas (μM). A metodologia utilizada foi o ensaio da Sulforrodamina B, onde os valores de absorbância em 515 nm são diretamente proporcionais à quantidade de proteína. O efeito foi estatisticamente significativo em comparação aos controles, sendo ***p<0.0001; **p<0.01; *p<0.05. A análise estatística utilizada foi ANOVA seguida do Teste de Tukey (n = 3).

Figura 2

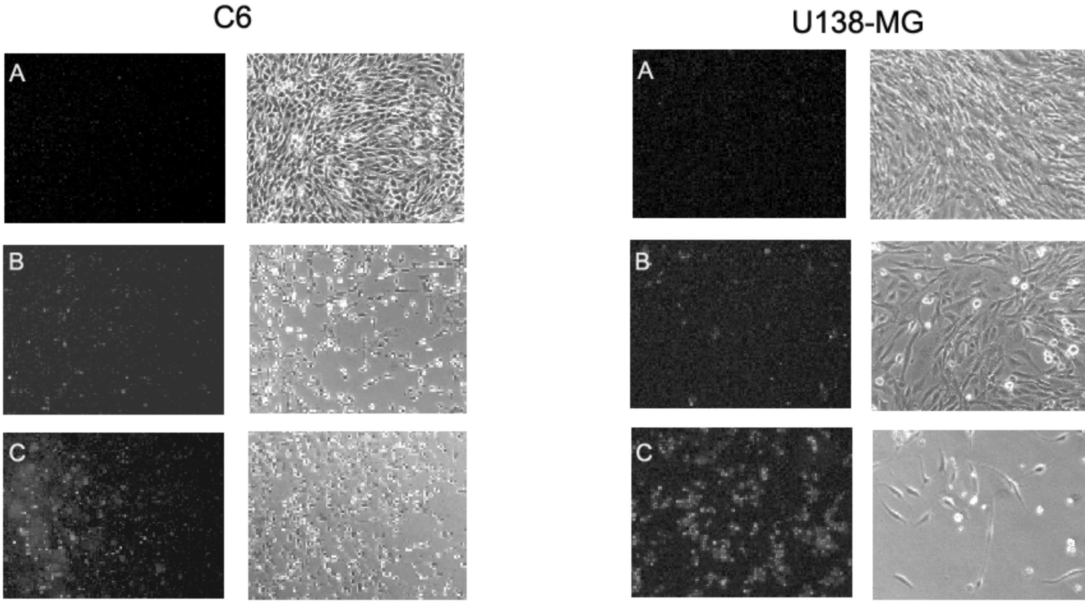
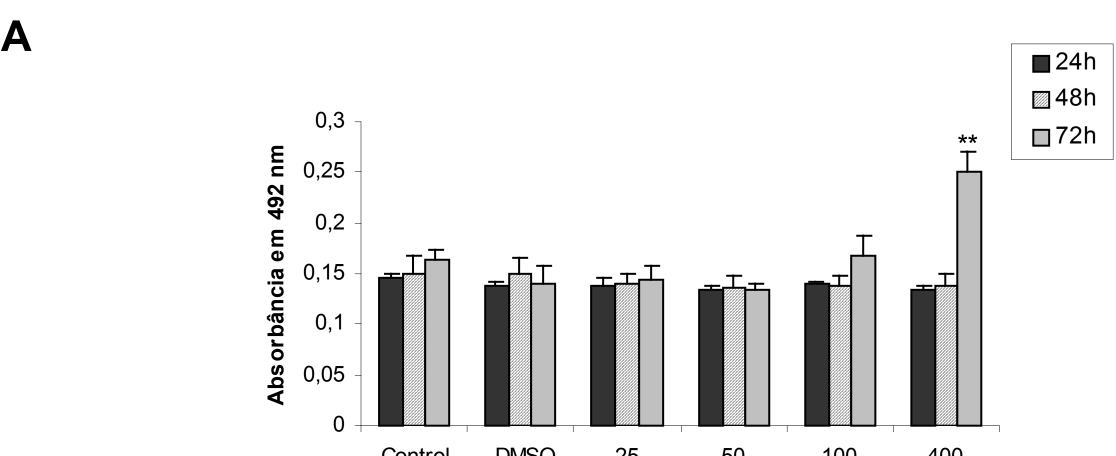


Figura 2. Efeito da indometacina na incorporação de iodeto de propídeo nas linhagens de gliomas C6 e U138-MG. As células foram tratadas com DMSO (A), 100 μM (B) ou 400 μM indometacina (C) por 48h. A morte celular foi analisada pela incorporação de iodeto de propídeo (6.4 μM), que foi visualizada utilizando um microscópio invertido (Nikon Eclipse TE 300). As fotos à direita de A, B e C são correspondentes à visualização no microscópio de contraste de fase. Observe o significativo decréscimo do número de células no tratamento com indometacina em relação aos respectivos controles. As fotos são representativas de 3 experimentos. Observação: os resultados com a linhagem C6 já foram mostrados no Capítulo I.

Figura 3

A



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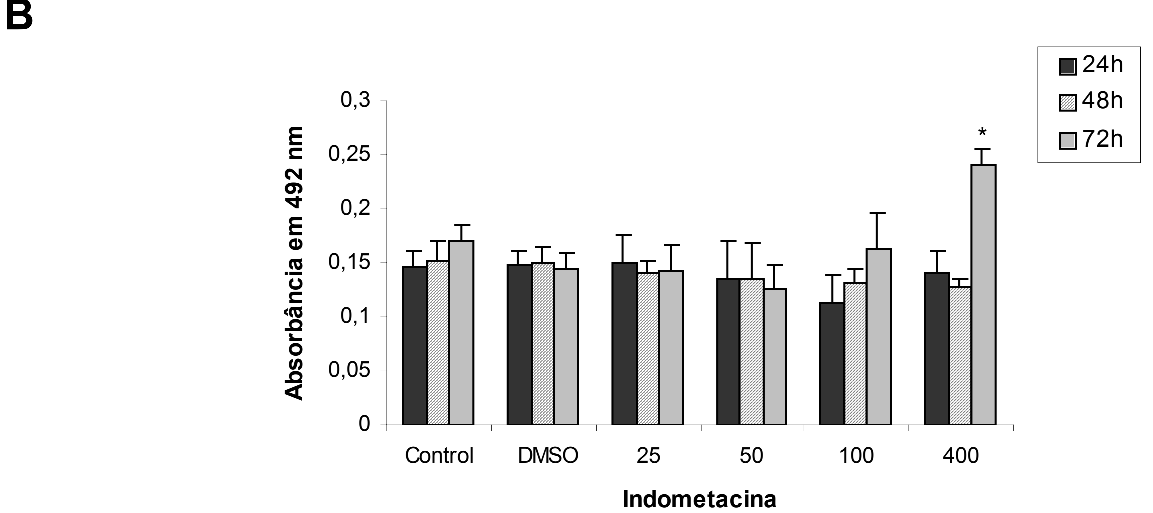

Figura 3. Liberação da lactato desidrogenase (LDH). A morte celular das linhagens de glioma C6 (A) e U138-MG (B) foi medida pela liberação da LDH nas células tratadas com indometacina nas concentrações indicadas (μM) por 24, 48 ou 72h. Como controle positivo do experimento foram utilizadas células de glioma rompidas por congelamento/descongelamento em nitrogênio líquido (valor de 0,910 ± 0,0112). O efeito foi estatisticamente significativo em comparação aos controles, sendo **p<0.01; *p<0.05. A análise estatística utilizada foi ANOVA seguida do Teste de Tukey (n = 3).

Figura 4

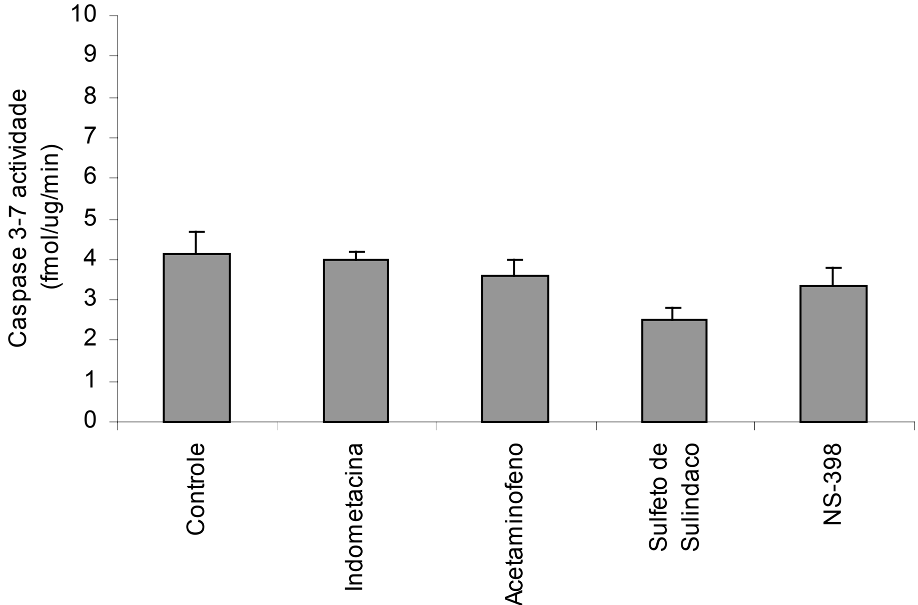

Figura 4. AINEs não induzem a ativação de caspases 3/7 na linhagem de glioma U138-MG. As células foram tratadas por 48h com concentrações correspondentes ao IC_{50} de cada AINE: indometacina (100 μM), acetaminofeno (500 μM), sulfeto de sulindaco (400 μM) ou NS-398 (75 μM). A atividade das caspases 3/7 foi determinada pela avaliação da intensidade de fluorescência. Nenhuma das leituras de fluorescência foram significativas em relação ao controle. A análise estatística utilizada foi ANOVA seguida do Teste de Tukey (n = 3).

Figura 5

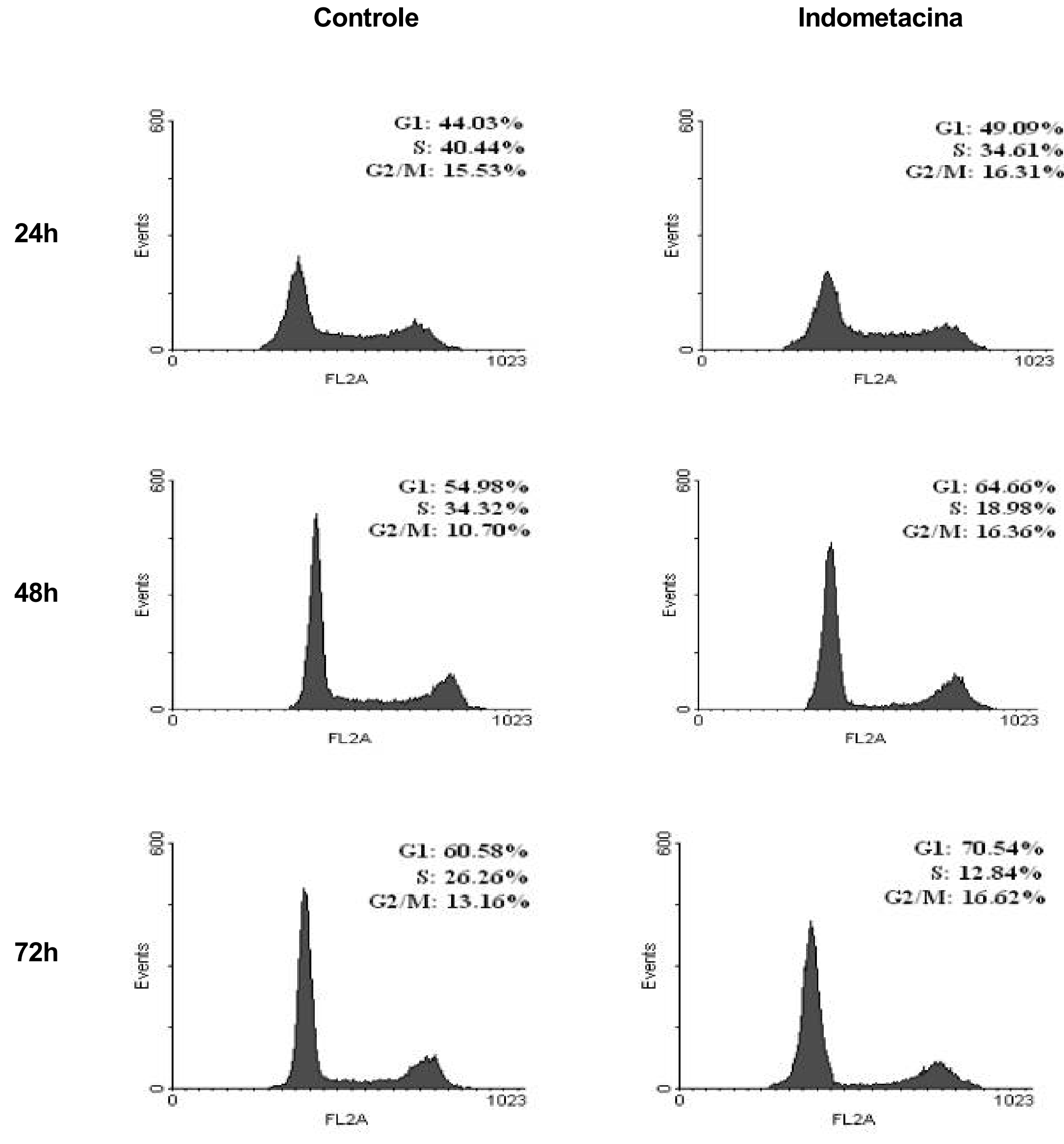

Figura 5. Efeito da indometacina na distribuição do ciclo celular na linhagem de glioma U138-MG. As células foram tratadas com 100 μM de indometacina (à direita) e o Controle com DMSO (à esquerda) por 24, 48 ou 72h e submetidas à análise do ciclo celular por citometria de fluxo como descrito nos Materiais e Métodos (Capítulo I). Os dados são expressos como percentual de células nas fases G0/G1, S, ou G2/M do ciclo celular. Os dados são representativos de três experimentos independentes com resultados similares.

Figura 6

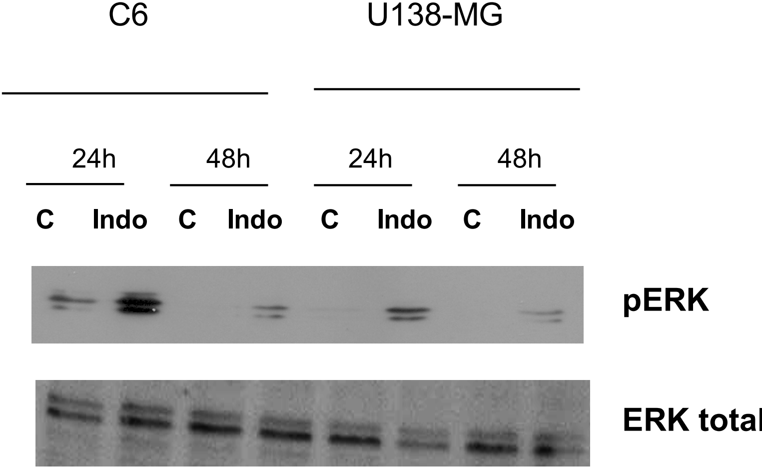

Figura 6. Indometacina induz a fosforilação da MAPK p42/44 (ERK) nas linhagens de glioma C6 e U138-MG. As células foram tratadas com 100 μM de indometacina (Indo) e o controle com DMSO (C) pelos tempos indicados (24 ou 48h). O lisado das células contendo a mesma quantidade de proteína foi submetido à eletroforese (SDS-PAGE/12% acrilamida), seguido de análise por *Western Blotting* usando anticorpos específicos fosfo-p42/44 MAPK (pERK) e total-p42/44 MAPK (ERK total). Os dados são representativos de três experimentos independentes com resultados similares.

5. Capítulo II

INDOMETHACIN STIMULATES ACTIVITY AND EXPRESSION OF ECTO-5’-NUCLEOTIDASE/CD73 IN GLIOMA CELL LINES

Artigo submetido para publicação na Revista *Cancer Letters*.

Ms. Ref. No.: CAN-D-06-00031

Title: Indomethacin stimulates activity and expression of ecto-5'-nucleotidase/CD73 in glioma cell lines

Cancer Letters

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INDOMETHACIN STIMULATES ACTIVITY AND EXPRESSION OF ECTO-5'-NUCLEOTIDASE/CD73 IN GLIOMA CELL LINES

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Abstract

Considering that the enzymes involved in the purine nucleotide catabolism may represent an important mechanism associated with maligmant transformation of gliomas, we investigated the influence of indomethacin on the ectonucleotidases in U138-MG and C6 glioma cell lines. Exposure of cells to therapeutic doses indomethacin caused an increase in the AMP and ATP hydrolysis. A significant increase on mRNA levels of ecto-5'-nucleotidase/CD73 also was observed. Our data suggest that A_3 receptors and the enzyme ecto-5'-nucleotidase/CD73 are involved in the antiproliferative effect of indomethacin in glioma cell lines. The NSAIDs may be clinically important for pharmacological intervention of this kind of tumor.

Keywords: Indomethacin, gliomas, ecto-5´-nucleotidase/CD73, adenosine.

1. Introduction

Brain tumors are the third most frequent cause of cancer-related death in adults and the second most common cause of cancer-related death in children [1]. Glioblastomas, tumors that arise from cells of astrocytic lineage, are the most common and the most maligmant form of primary brain tumor, with an average survival of <1 year [2]. Malignant gliomas are composed of multiple cell types displaying a wide spectrum of heterogeneity in terms of morphology, biological aggressiveness, invasive potential, and radiation sensitivity [3].

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most widely used drugs for the treatment of inflammatory diseases [4]. Numerous experimental, epidemiological, and clinical studies suggest that NSAIDs have promise as anticancer agents. Recent studies have provided evidence for an additional role for NSAIDs in preventing growth of cancer cells by inhibiting cyclooxygenase (COX) enzymes [5,6]. Other studies revealed that the antiproliferative effect of NSAIDs may be, in part, independent of cyclooxygenase inhibition [4].

The powerful NSAID indomethacin is an indolic acid derivative, a potent inhibitor of both cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) and has been reported to repress the growth of carcinoma cells [7]. We have recently shown that the treatment with indomethacin inhibited cell proliferation and reduced cell viability in glioma cell lines [8].

Adenine nucleotides and nucleosides represent an important class of extracellular molecules involved in modulation of signaling pathways that are crucial for normal functioning of the nervous system [9]. Besides the well established physiological effects of ATP, there is evidence showing that extracellular ATP can

stimulate mitogenesis and cellular proliferation and cause cytotoxic effects on tumor cells, depending on the cell type and the ATP concentration [10]. The events induced by extracellular adenine nucleotides are controlled by the action of ecto-enzymes, which play a central role by modulating the extracellular levels of these important signaling molecules. E-NTPDases (Ectonucleoside triphosphate diphosphohydrolase) are enzymes that hydrolyze nucleosides tri- and diphosphates with different preference for substrate, generating its respective nucleosides monophosphates. The molecular properties, functional roles and nomenclature of nucleotidases have been reviewed [11]. Ecto-5'-nucleotidase (ecto-5'-NT/CD73), the final enzyme in the extracellular nucleotide pathway, hydrolyses nucleoside monophosphates to the respective nucleosides and is a key enzyme in the nucleotide degradation pathway [12].

Adenosine, the product of ATP hydrolysis, is a physiological regulator of various cellular activities such as cell growth, differentiation, and cell death [13]. Several studies have proposed that adenosine acts as a potent regulator of normal and tumor cell growth. These effects are closely related to extracellular adenosine concentrations, cell surface expression of different adenosine receptors subtypes, and signal transduction mechanisms activated following the binding of specific agonists [14]. Adenosine can act on four distinct G-protein-coupled receptors (A_1, A_{2A}, A_{2B} and A_3 receptors), which have been identified on both neurons and glial cells [15,16]. The A_{2A} and A_3 receptors have been specifically implicated in modulation of cell death in cardiomyocytes and brain astrocytes [17].

Considering that the exact mechanisms involved in the antiproliferative effect of indomethacin are not completely elucidated and that we have previously showed that alterations in the ecto-nucleotidases pathway may represent an important

mechanism associated with malignant transformation of glioma cell lines [18], the aim of the present study was to investigate the effect of indomethacin on ecto-nucleotidases activities in C6 and U138-MG glioma cell lines.

2. Material and Methods

2.1. Chemicals

Indomethacin was obtained from ICN Biochemicals. Cell culture medium, penicillin/streptomycin, 0,25% trypsin/ EDTA solution were obtained from Gibco (Gibco BRL, Carlsbad, CA, USA); fetal bovine serum (Cultilab, Campinas, SP, Brazil); Trizol LS reagent (Life Technologies); M-MLV RT, dNTPs (Promega), Taq polymerase (CenBiot-UFRGS), oligonucleotides (Invitrogen). MRS 1220 was obtained from Tocris Cookson (UK). CPT (8-cyclopentyltheophiline), DPCPX (1,3-diprophyl-8-cyclopentylxanthine), CPA (cyclopenthyladenosine), 8PT (8-phenytheophyline), APCP (α,β-methilene ADP) and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All the other chemicals and reagents were analytical grade.

2.2. Maintenance of cell lines

The human glioblastoma cell line U138-MG (derived from spontaneously occurring human maligmant gliomas) and the rat glioma cell line C6 (derived from N-nitrosomethylurea-induced glioma in rat) were obtained from American Type Culture Collection (Rockville, Maryland, USA). The cells were grown and maintained in

Dulbecco´s modified Eagle´s medium (DMEM) containing antibiotics penicillin/streptomycin 0.5 U/mL, and supplemented with 5% (C6) or 15% (U138-MG) (v/v) fetal bovine serum (FBS). Cells were kept at a temperature of 37°C, a minimum relative humidity of 95%, and an atmosphere of 5% CO_2 in air. All the experiments throughout this study were conducted in serum supplemented DMEM.

2.3. Indomethacin exposure

The indomethacin preparation was made in cell culture-grade dimethylsulphoxide (DMSO; Sigma). The final concentrations of DMSO in the culture medium in all experiments were at a maximum of 1.0 % (v/v).

The rat C6 glioma cells were seeded at 5 X 10^3 cells *per* well in DMEM/5% FBS in 24-well plates, and allowed to grown for 24h. The procedure was the same for the U138-MG glioma cells, except that they were seeded at 1 x 10^4 cells/ well in DMEM/15% FBS. The medium was changed 2h prior to treatment with indomethacin. The cell cultures were exposed to indomethacin (25, 50, 100 and 400 μM) for different times (24, 48 and 72 hours). In experiments made in presence of adenosine receptor agonists/antagonists, these were added to culture medium 30 min before the indomethacin treatment. Control cultures were treated with vehicle, i.e., 1% of DMSO.

2.4. Ecto-nucleotidases assays

2.4.1. Ecto-NTPDase activity

To determine the ATPase and ADPase activities, the 24 multiwell plates containing glioma cells were washed three times with incubation medium in absence of nucleotides. The enzymatic reaction was started by the addition of 200 μL of incubation medium containing 2 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM Hepes, pH 7.4 and 1 mM of ATP or ADP as substrates, at 37 ^oC. After 30 min of incubation [18] the reaction was stopped by collecting an aliquot of the incubation medium and transferring it to eppendorf tubes containing trichloroacetic acid (final concentration 5% w/v), previously placed on ice. The release of inorganic phosphate (Pi) was measured by the malachite green method [19], using KH_2PO_4 as a Pi standard. The non-enzymatic Pi released from nucleotides into the assay medium without cells was subtracted from the total Pi released during the incubation, giving net values for enzymatic activity. All samples were run in triplicate. Specific activity was expressed as nmol Pi released/min/mg of protein. The cell integrity was evaluated by measuring the activity of Lactate dehydrogenase (LDH) in another aliquot of the incubation medium.

2.4.2. Ecto-5'-nucleotidase activity

The incubation medium was the same as the one used for ecto-NTPDase activity, except that 2 mM $MgCl_2$ was used instead of $CaCl_2$ plus 2 mM AMP as substrate. The time of reaction used in this case was 10 min [18]. The other conditions were the same as used to determine the ATP and ADP hydrolysis.

2.5. Protein determination

Cells in the 24-well microplates were solubilized with 100 μL NaOH (1.0 N) and frozen overnight. An aliquot was collected and protein was measured by the Coomassie blue method [20] using bovine serum albumin as standard.

2.6. RT-PCR analysis

Total RNA from C6 rat glioma and U138-MG human glioma cell lines treated with indomethacin or DMSO (control) was isolated with Trizol LS reagent (Life Technologies), according to the manufacturer's instructions. The cDNA species were synthesized with M-MLV Reverse Transcriptase (Promega) from 5 μg of total RNA in a final volume of 25 μL with a random hexamer primer in accordance with the manufacturer's instructions. cDNA reactions were performed for 1h at 37°C and stopped by cooling at 4°C. 1 μl of cDNA was used as a template for PCR with primers specific for ecto-5´-nucleotidase or primers specific for NTPDases. As a control for cDNA synthesis, β-actin- PCR (to C6 cell line) and GAPGH-PCR (to U138-MG cell line) were performed. 1 μl of the RT reaction mix was used for PCR in a total volume of 25μl using a concentration of 0.5 μM of each primer indicated below and 50 μM of dNTP and 1 U Taq polymerase (CenBiot-UFRGS) in the supplied reaction buffer. The anneling temperature was 60°C (C6) and 52°C (U138-MG) for both primers: ecto- 5'-nucleotidase/CD73 and β-actin. The PCR cycling conditions were as follows: 1 min at 95°C, 1 min at 94°C, 1 min at anneling temperature, 1 min at 72°C. All PCRs were carried out for 35 cycles and included a final 10 min extension at 72°C. 10 μl of the PCR reaction was analysed on a 1.0 % agarose gel. The following set of primers were used: for ecto- 5'-nucleotidase/CD73; 5'CCC GGG GGC CAC TAG CAC CTC A3' and 5'GCC TGG ACC ACG GGA ACC TT3' (amplification

product 403 bp), for β-actin; 5’TAT GCC AAC ACA GTG CTG TCT GG3’ and 5’TAC TCC TGC TTC CTG ATC CAC AT3’ (amplification product 210 bp) and for GAPDH: 5´CAA AGT TGT CAT GGA TGA CC 3´ and 5´ CCA TGG AGA AGG CTG GGG 3´ (amplification product 210 bp). Oligonucleotides were obtained from Invitrogen. Negative controls were performed with templates substituted by DNAse, RNAse free distilled water for each PCR reaction. Ecto-5´-nucleotidase mRNA expression was determined as the ratio of the ecto-5´-nucleotidase to β-actin band density (C6) or GAPDH band density (U138-MG).

2.7. Cell Counting

At the end of 48h treatment with indomethacin and/or adenosine receptor agonists/antagonists the medium was removed, cells were washed with phosphate buffered saline (PBS) and 200 μL of 0.25% trypsin/EDTA solution was added to detach the cells, which were counted immediately in a hemocytometer.

2.8. Statistical analysis

All experiments were carried out at least 3 times in triplicate. Data were analyzed by unpaired ANOVA followed by *post-hoc* comparisons (Tukey test) using an Instat software package (GraphPad Software, San Diego, CA, USA). The differences were considered significant at $P < 0.05$.

3. Results

3.1. Indomethacin stimulates ATPase activity of glioma cell lines

In order to evaluate the effect of indomethacin on ecto-nucleotidase activities, we performed an enzymatic assay after the NSAID treatment with incubation times and substrate concentration established in a previous work [18]. Cells were treated with increasing concentrations of indomethacin (25, 50, 100 and 400 μM) for 24, 48 or 72 hours and incubated with ATP and ADP as substrate. The inorganic phosphate released was determined under the conditions described in Materials and Methods. Our results showed that the treatment for 24h with 400 μM of indomethacin caused an increase of 95% ($p<0.01$) in the ATP hydrolysis in C6 glioma cell line compared with control cultures (Fig. 1A). A similar effect was observed in both cell lines since 48h of treatment (Fig. 1B). A significant increase of 148% ($p<0.001$) for C6 and 85% ($p<0.001$) for U138-MG in the ATP hydrolysis was observed when cells were treated for 72 hours with 400 μM of indomethacin (Fig. 1C). Interestingly, the increase in the ATP hydrolysis with the therapeutic dose of 100 μM (75% with $p<0.01$) was observed only when human U138-MG cells were treated for 72h (Fig. 1C). Additionally, indomethacin treatment in all concentrations tested for all times did not change the ADPase activity (data not shown).

3.2. Indomethacin stimulates ecto-5´-nucleotidase/CD73 activity of human and rat glioma cell lines

To investigate the indomethacin effect on the ecto-5'-NT activity, cells were treated with increasing concentrations of indomethacin (25, 50, 100 and 400 μM) for 24, 48 or 72 hours. After that, the glioma cells were incubated with AMP as substrate and the inorganic phosphate was determined under the conditions described in

Materials and Methods. Our results showed that 400 μM indomethacin significantly increases the ecto-5'-NT activity in both glioma cell lines starting at 24h of treatment in relation to control cells (Fig. 2A). A significant increase of 52% ($p<0.05$) for C6 and 62% ($p<0.05$) for U138-MG in the AMP hydrolysis was observed when cells were treated for 48h with therapeutic doses of indomethacin (100 μM) compared with control cultures (Fig. 2B). Interestingly, the increase in the AMP hydrolysis with the lowest concentration (50 μM) was observed only after 72h of treatment in human U138-MG cells (Fig. 2C). The treatment with different final concentrations of indomethacin added to the culture medium for 24, 48 or 72 hours resulted in a dose and time-dependent increase in ecto-5´-NT activity (Fig. 2).

3.3. Indomethacin increases ecto-5'-nucleotidase/CD73 mRNA expression in glioma cells

Since indomethacin promoted a significant increase in AMP hydrolysis, the mRNA levels of ecto-5'-nucleotidase/CD73 were measured in order to verify whether this increase was a result of an enhanced expression of the protein induced by indomethacin. C6 and U138-MG glioma cells (treated with indomethacin or DMSO) revealed specific signal (403 bp fragment) corresponding to mRNA for ecto-5'-NT/CD73 (Fig. 3). Moreover, this figure shows that treatment with indomethacin (100 μM) for 48h increase the expression of the ecto-5'-NT/CD73 mRNA by 2.3 fold for C6 and 1.9 fold higher for U138-MG when compared to expression under control conditions. These results support the hypothesis that the increase in ecto-5'-nucleotidase/CD73 activity is related to an increased expression of CD73 mRNA levels.

3.4.The antiproliferative effect of indomethacin is prevented by pretreatment with A_3 antagonist and 5'NT inhibitor

Considering the stimulatory effect of indomethacin on ecto-nucleotidase cascade (ecto-ATPase and ecto-5'-NT) showed here and that adenosine is a product of ATP hydrolysis, we investigated the involvement of adenosine receptors in indomethacin-mediated inhibition of glioma cell proliferation. Glioma cells cultures were treated with A_3 antagonist (1 μM MRS1220), A_1 and A_2 antagonist (0.2 μM CPT), A_1 antagonist (1 μM DPCPX), A_1 agonist (1 μM CPA), unspecific adenosine receptor antagonist (1 μM 8PT) or ecto-5'NT inhibitor and A_1 agonist (1 μM α,β-methilene ADP) for 30 min prior to treatment with 100 μM indomethacin for 48 h. The treatment with α,β-methilene ADP (APCP) alone (without indomethacin) caused a significant reduction in cell proliferation while the treatment with the other agonists or antagonists alone did not decrease the number of cells (data not shown). The number of cells in cultures treated with indomethacin was reduced by 41% ($p<0.001$) for C6 and 44% ($p<0.001$) for U138-MG in relation to control cultures. When the cells were pretreated with α,β-methilene ADP (APCP) plus indomethacin, the inhibition in cell proliferation was reduced to 15% ($p<0.01$) and 17% ($p<0.01$) for C6 and U138-MG, respectively (Fig. 4). In addition, the pretreatment with MRS1220 plus indomethacin, reduced significantly ($p<0.001$) the inhibition of cell proliferation in relation to indomethacin group in both cell lines. Therefore, the pretreatment of glioma cells with an ecto-5'-NT inhibitor/ A_1 agonist and an adenosine A3 receptor antagonist was significantly effective in reducing the inhibition of cell proliferation

induced by indomethacin. In contrast, the other agonists/antagonists tested did not change the antiproliferative effect of indomethacin (Fig. 4).

4. Discussion

There is a growing interest in understanding the mechanisms mediating the antiproliferative effects of NSAIDs and in determining whether these effects are linked to their cyclooxygenase-inhibiting properties or independent of them. The biochemical mechanism generally ascribed to this effect is the inhibition of COX enzymes. However, several studies have demonstrated that certain NSAIDs such as indomethacin cause anti-inflammatory and antiproliferative effects independent of cyclooxygenase activity and prostaglandin synthesis inhibition [21]. As previously observed by our group, the antiproliferative effects of indomethacin in glioma cell lines are, at least in part, mediated through COX-independent pathways and inhibition of cell cycle progression. In C6 glioma cells, the indomethacin-mediated decrease in cell number is mediated by c-Src and ERK (Extracellular signal regulated protein kinase) signaling [8].

The extracellular levels of adenine nucleotides can be controlled by hydrolysis via several members of ectonucleotidases family, which are located on the cell surface or may also be found as soluble enzymes in the interstitial medium [11]. Among them, ecto-5'-NT/CD73 may reveal a variety of different functions, depending on its cell and tissue expression. Adenosine, the purine nucleoside product of ecto-5'-NT enzyme activity, has a role in many physiological and pathological events [11]. One unique characteristic of adenosine is to differentially modulate normal and transformed cell growth, depending on its extracellular concentration, the expression

of adenosine cell surface receptors, and the physiological state of the target cell [22]. ATP and adenosine are known to produce potent effects when released from neuron and astrocytes [17].

Considering that the exact mechanisms involved in the effect of indomethacin against cancer cells were not completely elucidated and that studies from our laboratory have showed that glioma cells present an altered extracellular adenine nucleotides catabolism when compared with astrocyte cultures [18], the aim of the present study was to investigate the effect of indomethacin on ecto-nucleotidases activities in C6 rat and U138-MG human glioma cell lines.

Recently, Wink et al. [18] reported that glioma cells presented an undetectable ATP- and ADP- hydrolysis activity. Our results showed a significant increase in the ATP hydrolysis when both cell lines were treated with 400 μM of indomethacin, although the human cell line presented a significant increase in ATP hydrolysis with the therapeutic dose of 100 μM. Considering that these enzymatic activities were extremely low in glioma cells as compared to astrocytes (Wink et al., 2003), an important finding of the present work is the stimulatory effect of indomethacin on ATPase activity (Fig. 1). According with data from literature, ATP is recognized as a mitogenic factor that induces proliferation in human glioma cells [23] and the substantial decrease in ATP and ADP hydrolysis represent an important mechanism associated with maligmant transformation of glioma cell lines [18]. Therefore, the effect of indomethacin against glioma cells might be, at least in part, by the increased catabolism of extracellular purines. Since the NSAIDs are known to regulate transcription, we investigated whether the increase of ATP hydrolysis was a consequence of positive modulation of NTPDases mRNA levels. However, an

increase in mRNA expression of these proteins was not observed (data not shown), indicating a non-genomic effect of indomethacin over ATPase activity.

The highly variable level of expression of ecto-5'-NT in human and animal tissues and cells suggests tissue-specific mechanisms controlling the expression of this enzyme [24]. The number of potential regulatory elements within the ecto-5'-NT promoter suggests that there might be a high level of complexity in the interactions between binding factors, especially between those localized immediately 5' to the transcription start site and those further upstream [24]. The genomic DNA upstream from the ecto-5'-NT start codon is high in G+C content, with one cAMP-responsive element (CRE) and five consensus Sp-1 binding sites [25]. Sp-1 is essential for many genes that regulate the cell survival, growth and angiogenesis [26]. Sp-1 signalling pathway may contribute to tumor development and progression [26] but the mechanism underlying in different levels of Sp-1 is unknown [26]. However, a recent study showed that Sp-1 activity can be modulated by several factors, thus activating ERK/MAPK signaling and c-JunNH2-terminal kinase-related signal pathways [27]. It may also involve many other cellular factors, including the functional status of oncogenes and tumor suppressor genes [26]. Recently, Han et al. [28] showed that NSAIDs (Nimesulide and NS-398) affected integrin alpha5 gene transcription through increase in Sp1 protein and ERK activation in human lung carcinoma cells. Considering that we observed in previous results that indomethacin caused activation of ERK [8] and that our results showed an increase of expression of ecto-5'-NT/CD73, we suggest that indomethacin may be causing ecto-5'-NT promoter activation, perhaps through induction of the Sp1 protein.

The increase of enzymatic activity and expression of ecto-5'-nucleotidase/CD73 could result in a higher adenosine formation in the extracellular

medium. Considering that indomethacin inhibits cell proliferation and increases ecto-5'-NT activity in glioma lines, we hypothesize that high extracellular adenosine could be produced and consequently could be involved in glioma cell death, probably via the A_3 adenosine receptor. To confirm the specific involvement of the A_3 receptors on the effect of indomethacin, the A_3 receptor antagonist MRS1220 was used. Accordingly to our proposal, when cell cultures were pretreated with MRS1220 plus indomethacin, the antiproliferative effect was significantly prevented in both the cell lines (Fig. 4). Although MRS1220 can also affect A_1 and A_2 receptor subtypes with very low efficacy [28], the lack of effect of A_1 and A_2 antagonist (CPT and DPCPX) indicate that the effect of adenosine is mediated mainly by the A_3 receptor subtype. α,β-methilene ADP (APCP), an ecto-5'NT inhibitor prevented the inhibition of cell proliferation induced by indomethacin (Fig. 4). Interestingly, the treatment with α,β-methilene ADP (APCP) alone caused an inhibition of cell proliferation, which is in agreement with the literature that suggest the participation of ecto-5'-NT in cell survival [12,30,31]. Taken together, the results presented here lead us to suppose that the increase in ecto-5'-NT activity can produce more adenosine in the extracellular milieu, which could be involved in the antiproliferative effects of indomethacin on glioma cells. According to literature, exposure of various cell types to different A_3 receptor agonists showed inhibition of cell proliferation in a dose-dependent manner, thus suggesting a cytostatic rather than apoptotic effect, mediated through A_3 adenosine receptors [32,33,34]. One of the different mechanisms through which A_3 adenosine receptors are able to inhibit cell proliferation was found to involve inhibition of telomerase activity and a cell cycle arrest in the G_0/G_1 phase, leaving to a cytostatic effect [32,33]. Furthermore, it has been demonstrated that the antigrowth signal exerted by A_3 receptors blocks cells

into G_1 late cell cycle phase [34]. Hence, the extracellular adenosine concentration may be a crucial factor in determining the cell progression pathway, either by apoptosis or by a cytostatic state [35]. Interesantly, in previous work from the group [23] adenosine was reported to have a stimulatory effect over thymidine incorporation and increase in cell number in the U138-MG cell line. However, the experiments were performed in starved cells (quiescent) and 100 μM of adenosine was added to the media, while in this case we are observing the effect of endogenously generated adenosine by an increase in the activity of ecto-5'-NT/CD73 by indomethacin, in cells cultured under normal conditions (with serum supplemented DMEM), which possibly would produce amounts of adenosine far lower than 100 μM. Therefore, the rapid, localized small amount of adenosine generated by CD73 could produce the inhibitory effect observed in our experiments. A detailed dose-response of the effects of adenosine is under investigation.

The dual role of A_3 agonists on tumor and normal cells is very interesting. These receptors inhibits the growth of various tumor cells while promoting the proliferation of bone marrow cells [22,34]. However, there is still much to learn about the precise pathways involved, e.g., there is only preliminary evidence for the involvement of ERK and PKB (Protein Kinase B), and other signaling pathways many also be involved [14]. Studies demonstrated that A_3 adenosine receptor stimulation leads to activation of PKC (Protein Kinase C) in human embryonic kidney cells (HEK-293) [14]. So, since that PKC signaling can activate the transcription of specific genes increasing the activity of a variety of proteins [36], including the ecto-5'-NT/CD73 [37], we speculate that the increase of expression/activity ecto-5'-nucleotidase/CD73 mediated by indomethacin in glioma cells might be due to a PKC signaling. However, additional investigations should be performed to confirm this

hypothesis, since adenosine present dual characteristics: cell protection and cell death, depending on activation of distinct receptor subtypes and specific pathophysiological conditions [17].

In conclusion, here we demonstrated that indomethacin upregulates the expression and activity of ecto-5'-NT/CD73 protein, enhancing the extracellular nucleotides cascade degradation. Therefore, the effect of indomethacin against cancer cells might be, in part, by increased the catabolism of extracellular purines. Considering that indomethacin has the ability to cross the blood-brain-barrier [38] and it is sufficiently safe to allow large-scale administration, it represents potentially useful agent for glioma chemoprevention or adjuvant therapy. These and other studies have provided evidence for a number of alternative mechanisms to explain the anticancer effects of NSAIDs.

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References

[1] S.L. Parker, T. Tong, S. Bolden, P.A. Wingo, Cancer statistics, CA Cancer J.Clin. 47 (1997) 5-27.

[2] S.H. Bigner, J. Mark, P.C. Burger, M.S. Mahaley, D.E. Bullard, L.H. Muhlabir, D.D. Bigner, Specific chromosomal abnormalities in maligmant human gliomas, Cancer Res. 48 (1988) 405 -411.

[3] A. Saxena, M.L. Shriml, M. Dean, U.I. Ali, Comparative molecular genetic profiles of anaplasic astrocytomas/glioblastomas multiformes and their subsequent recurrences, Oncogene 18 (1999) 1385 -1390.

[4] J.S. Baek, C.L. Wilson, C.H. Lee, E.T. Eling, Dual function of nonsteroidal anti-inflammatory drugs (NSAIDs): inhibition of cyclooxygenase and induction of NSAID-activated gene, Pharmacology and Experimental Terapeutics 301 (2002)1126 -1131.

[5] C.J. Grubbs, R.A. Lubet, A.T. Koki, K.M. Leahy, J.L. Masferrer, V.E. Steele, G.J. Kelloff, D.L. Hill, K. Seibert, Celecoxib inhibits N-butyl-N-(4-hydroxybutyl)-nitrosamine-induced urinary bladder cancers in male B6D2F1 mice and female Fischer-344 rats, Cancer Res. 60 (2000) 5599 -5602.

[6] C.S. Williams, A.J.M. Watson, H. Sheng, R. Helou, J. Shao, R.N. DuBois, Cancer Res. 60 (2000) 6045 -6051.

[7] Y. Eli, F Przedeeki, G. Levin, N. Kariv, A. Raz, Comparative effects of indomethacin on cell proliferation and cell cycle progression in tumor cells grown *in vitro* and *in vivo,* Biochemical Pharmacology 61 (2001) 565 -571.

[8] A. Bernardi, M.C. Jacques-Silva, A. Delgado-Cañedo, G. Lenz, A.M.O. Battastini, Nonsteroidal anti-inflammatory drugs inhibit the growth of C6 and U138-MG glioma cell lines, European Journal of Pharmacology 532 (2006) 214 - 222.

[9] V. Ralevic, G. Burnstocck, Receptors for purines and pyrimidines, Pharmacol. Rev. 50 (1998) 413 -492.

[10] R. Lemmens, L. Vanduffel, H. Teuchy, O. Culic, Regulation of proliferation of LLC-MK2 cells by nucleosides and nucleotides: the role of ecto-enzymes, Journal of Biochemestry 316 (1996) 551 -557.

[11] H. Zimmermann, Ectonucleotidases: Some Recent Developments and a note on Nomenclature, Drug Development research 52 (2001) 44 -56.

[12] H. Zimmermann. 5'-Nucleotidase: Molecular structure and functional aspects, Biochem. J. 285 (2002) 345 - 354.

[13] M.P. Abbracchio, P1 and P2 receptors in cell growth and differentiation, Drug Dev. Res. 38 (1996) 393 -406.

[14] S. Merighi, P. Mirandola, K. Varini, S. Gessi, E. Leung, P.G. Baraldi, M.A. Tabrizi, P.A. Borea, A glance at adenosine receptors: novel target for antitumor therapy, Pharmacol Ther. 1 (2003) 31 -48.

[15] B.B. Fredholm, M.P. Abbracchio, G. Burnstock, G.R. Dubyak, T.K. Harden, K.A. Jacobson, U. Schwabe, M. Williams, Towards a revised nomenclature for P1 and P2 receptors, Trends Pharmacol. Sci. 18 (1997) 79- 82.

[16] P. Schubert , T. Ogata, C. Marchini, S. Ferroni, K. Rudolphi, Protective mechanisms of adenosine in neurons and glial cells, Ann. N.Y.Acad. sci., 825 (1997) 1 -10.

[17] K.A. Jacobson, C. Hoffmann, F. Cattabeni, P. Abbracchio, Adenosine-induced cell death: evidence for receptor-mediated signalling.,Apoptosis 4 (1999) 197 - 311.

[18] M.R. Wink, G. Lenz, E. Braganhol, A.S.K. Tamajusuku, G. Schwartsmann, J.J.F. Sarkis, A.M.O. Battastini, Altered estracellular ATP, ADP and AMP catabolism in glioma cell lines, Cancer Letters 198 (2003) 211-218.

[19] K. Chan, D. Delfert, K.D. Junger, A direct colorimetric assay for Ca^{2+} - ATPase activity, Anal Biochem 157 (1996) 375-380.

[20] M.M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Anal Biochem 72 (1976) 218 -541.

[21] I. Tegeder, J. Pfeilschifter, G. Geisslinger, Cyclooxygenase-independent actions of cyclooxygenase inhibitors, FASEB J 15 (2001) 2057-2072.

[22] G. Ohana, S. Bar-Vehuda, F. Barer, P. Fishman, Differential effect of adenosine on tumor and normal cell growth: focus on the A_3 adenosine receptor, Journal of Cellular Physiology 186 (2001)19 -23.

[23] F.B. Morrone, M.C. Jacques-Silva, A.P. Horn, A. Bernardi, G. Schwartsmann, R. Rodnight, G. Lenz, Extracellular nucleotides and nucleosides induce proliferation and increase nucleoside transport in human glioma cell lines, Journal of Neurooncology, 64 (2003) 211 -218.

[24] J. Spychala, A.G. Zimmermann and B.S. Mitchell, Tissue-specific Regulation of the Ecto-5'-nucleotidase Promoter, The Journal of Biological Chemistry 274 (1999) 22705 -22712.

[25] K.R. Hansen, R. Resta, C.F. Webb and L.F. ThompsoN, Isolation and chacarterization of the promoter of the human 5'-nucleotidase 9CD730-encoding gene, Gene 167 (1995) 307-312.

[26] J. Zhang, Z.G. Zhu, J. Ji, F. Yuan, Y.Y. Yu, B.Y. Liu, Y.Z. Lin, Transcription factor Sp1 expression in gastric cancer and its relationship to long-term prognosis, World Gastroenterology, 11 (2005) 2213 -2217.

[27] S. Yoshida, H. Harada, H. Nagai, K. Fukito, A. Teramoto, M. Emi, Head-to-head juxtaposition of Faz-associated phosphatase-1 (FAP-1) and c-Jun NH2-terminal Kinase 3 (JNK3) genes: genomic structure and seven polymorphisms of the FAP-1 gene, J Hum Genet. (2002) 614 -619.

[28] S. Han, J. Roman, COX-2 inhibitors suppress integrin alpha-5 expression in human lung carcinoma cells through activation of ERK: involvement of Sp1 and AP-1 sites, Int J Cancer., 116 (2005) 536 -546.

[29] J. Linden, Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection, Annu. Rev.Pharmacol.Toxicol. 41(2001) 775-787.

[30] J. Turnay, N. Olmo, G. Reence, K. Von der Mark, M.A. Lizarbe, 5'-Nucleotidase actrivity in cultured cell lines: effect of different assay conditions and correlation with cell proliferation, In Vitro Cell Dev. Biol. 25 (1989) 1055-1061.

[31] J.M. Navarro, N. Olmo, J. Turnay, M.T. López-Conejo, M.A. Lizarbe, Ecto-5'-Nucleotidase from a human colon adenocarcionoma cell line: correlation between enzyme activity and levels in intact cells, Moll Cell Biochem. 187 (1998) 121-131.

[32] P. Fishman, S. Bar-Yehuda, L. Wagman, Adenosine and other low molecular weight factors released by muscle cells inhibit tumor cell growth: possible explanation for the rarity metastases in muscle, Cancer Res. 58 (1998) 3181 – 3187.

[33] R. Brambilla, F. Cattabeni, S. Ceruti, D. Barbieri, C. Franceschi, Y.C. Kim, K.A. Jacobson, K.N. Klotz, M.J. Lohse, M.P. Abbrachio, Activation of the A_3 adenosine recptor affects cell cycle progression an cell growth, Naunyn-Schmiedeberg's Arch Pharmacol 361 (2000) 225 -234.

[34] S. Merighi, K. Varani, S. Geni, E. Cattabriga, V. Lannotta, C. Ulouglu, E. Leung, P.A. Borea, Pharmacological and biochemical characterization of adenosine receptors in the human maligmant melanoma A375 cell line, Br J. Pharmacol. 34 (2002a) 1215-1226.

[35] S.L. Bajaj, J. Insel, F. Quagliata, R. Hirschhorn, R. Silber, Adenosine and adenosine analogues are more toxic to chronic lymphocytic leukemia than to normal to normal lymphocytes, Blood 62 (1983) 75 – 80.

[36] M. Hunter, M. Karin, The regulation of transcription by phosphorilation, Cell 70 (1992) 375 –387.

[37] K. Node, M. Kitakaze, T. Minamino, M. Tada, M. Inoue, M. Hori, T. Kamada, Activation of ecto-5'-nucleotidase by protein kinase C and its role in ischaemic tolerance in the canine heart, Br J Pharmacol. 120 (1997) 273 – 281.

[38] J.P. Courade, D. Besse, C. Delchambre, N. Hanoun, M. Hamon, A. Eschalier, F. Caussade, A. Cloared, Acetaminophen distribution in the rat central nervous system. Life Sci 69 (2001)1455 –1464.

Legends to the figures

Fig. 1. Effect of Indomethacin on ATPase activity in glioma cells. After semi-confluence, U138-MG and C6 cells were treated with indomethacin (25 to 400 μM) during 24 (A), 48 (B) or 72h (C). The treated and control cells were incubated with 1 mM of ATP for 30 minutes. Specific activity values are expressed as nmol Pi/min/mg protein. The values were represented as means ± SE of three independent experiments with quadruplicate determination in each. The effect was statistically significant in relation to control at $^{**}p<0.01$ and $^{***}p<0.001$ as determined by unpaired ANOVA followed by *post-hoc* comparisons (Tukey test).

Fig. 2. Dose-response curve for activation on ecto-5'-NT by indomethacin. After semi-confluence, U138-MG and C6 cells were treated with increasing concentrations of indomethacin (25 to 400 μM) during 24 (A), 48 (B) or 72h (C). After that, the cells were incubated with AMP 2 mM for 10 minutes. Specific activity values are expressed as nmol Pi/min/mg protein. Data means ± SE were obtained from three independent experiments with quadruplicates determinations in each.The effect was statistically significant in relation to control at $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ as determined by unpaired ANOVA followed by *post-hoc* comparisons (Tukey test).

Fig. 3. Ecto-5´-NT/CD73 expression in glioma cultures, evaluated by semi-quantitative RT-PCR. After treatment with 100 μM indomethacin or DMSO (control) for 48h, RNA was extracted and processed for expression analysis of ecto-5´-nucleotidase/CD73. The PCR products were separated on a 1% agarose gel. The

expression was evaluated by determining the CD73 to β-actin mRNA ratio (C6) or GAPDH mRNA ratio (U138-MG).

Fig. 4. Effect of adenosine agonists/antagonists on glioma cell proliferation inhibition induced by indomethacin. C6 (A) and U138-MG (B) glioma cells were treated for 48h with indomethacin (100 μM) without or pretreated with agonists/antagonists: DPCPX (1 μM), CPT (0.2 μM), 8PT (1 μM), CPA (1 μM), APCP (1 μM) or MRS1220 (1 μM). After that the cells were detached with 0.25% trypsin-EDTA and counted in hemocytometer. The values were represented as means ± SE of three independent experiments with triplicate determination in each. Data were analyzed by unpaired ANOVA followed by *post-hoc* comparisons (Tukey test).

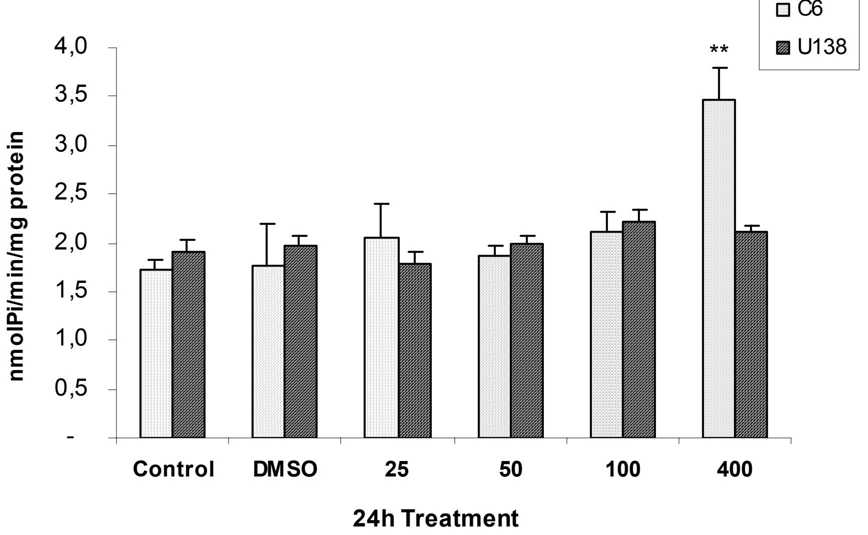
[a] Significantly different from the control group ($p<0.001$)

[b] Significantly different from the indomethacin treated group ($p<0.01$)

[c] Significantly different from the indomethacin treated group ($p<0.001$)

Figure 1

A



B

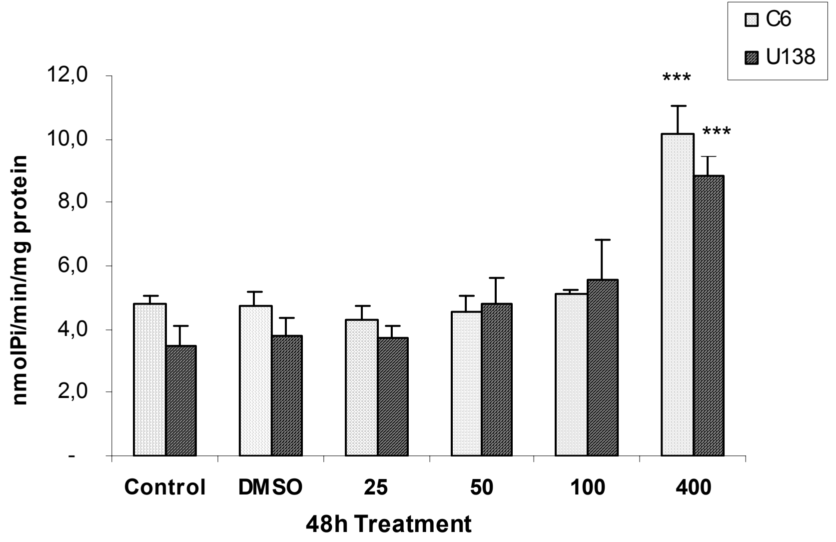


Figure 1

C

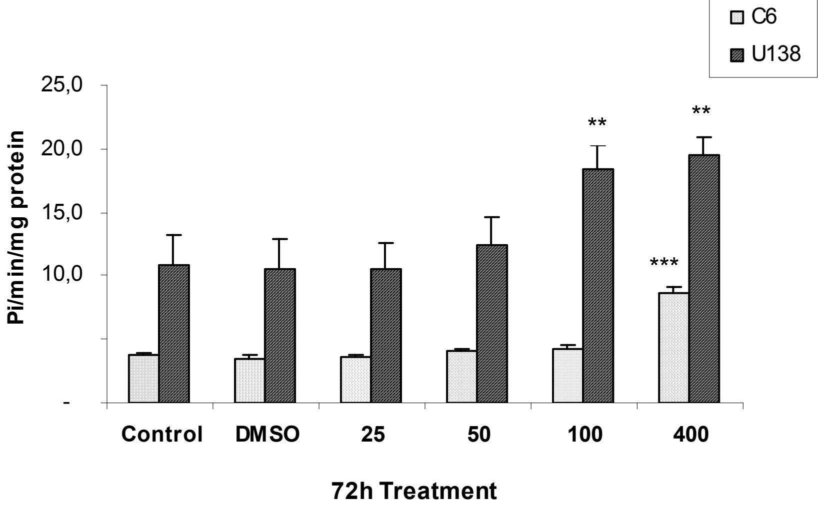
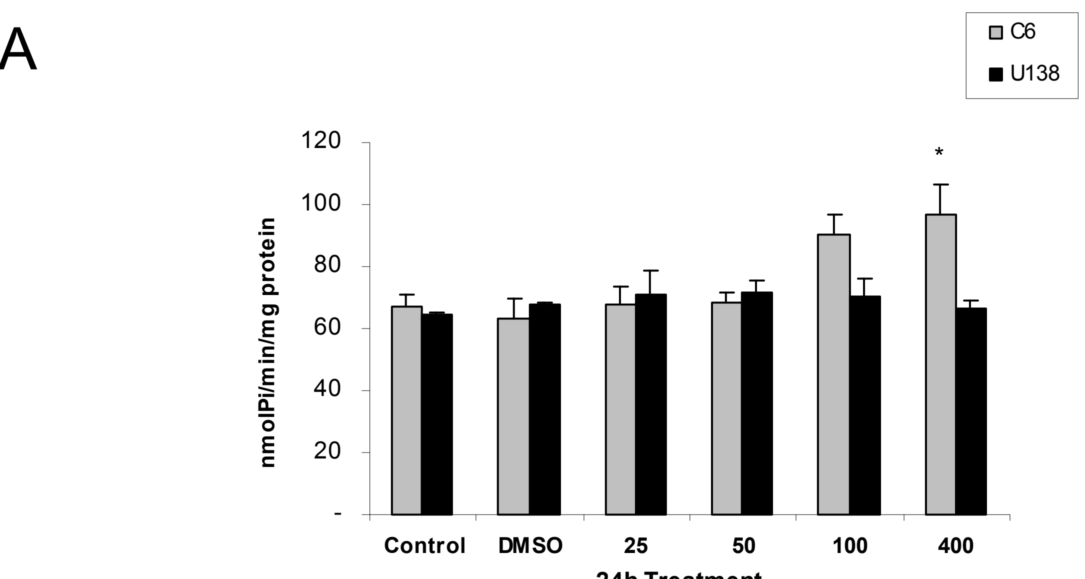


Figure 2

A



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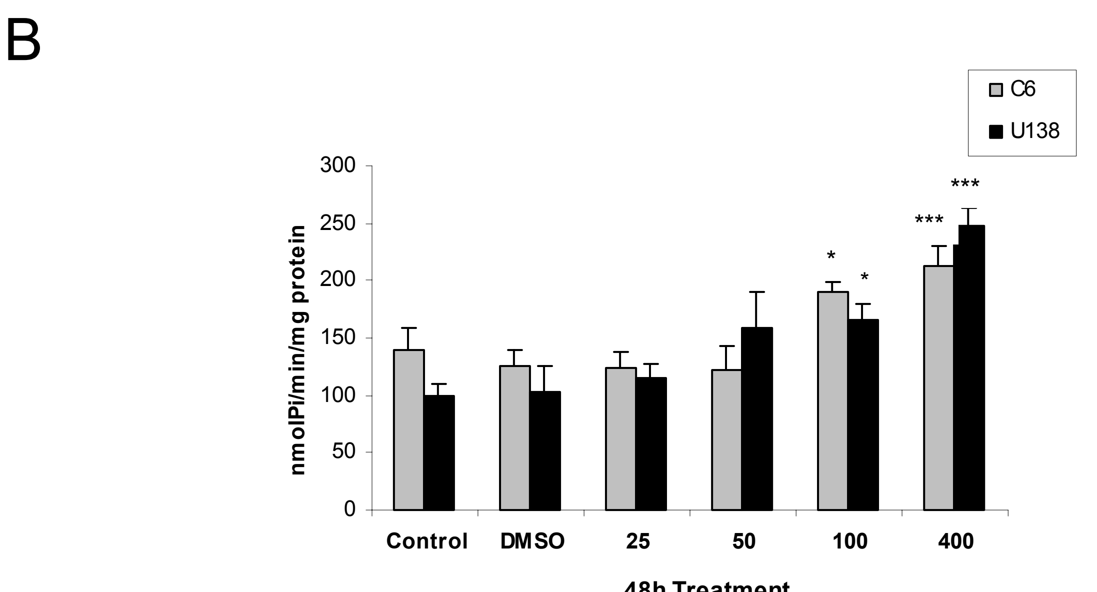


Figure 2

C

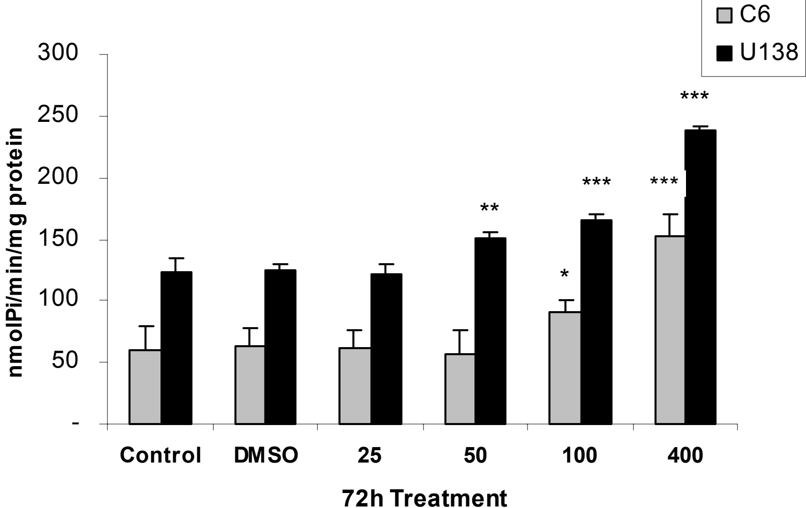
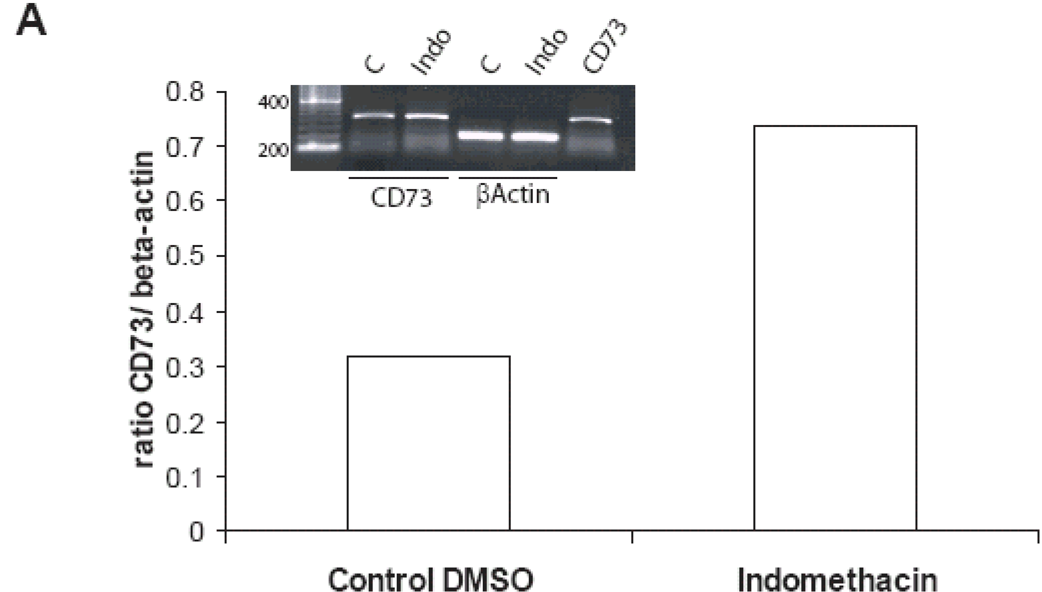


Figure 3



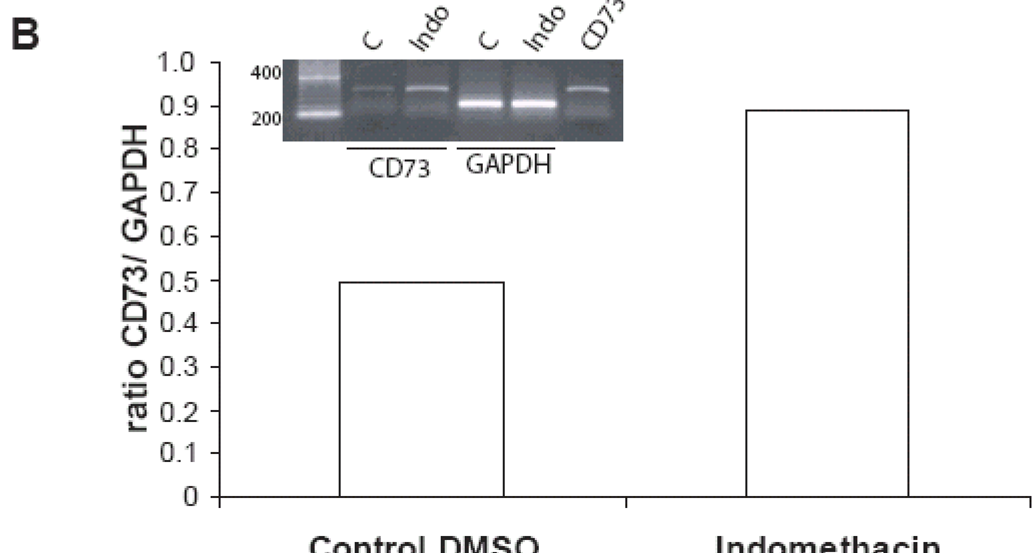
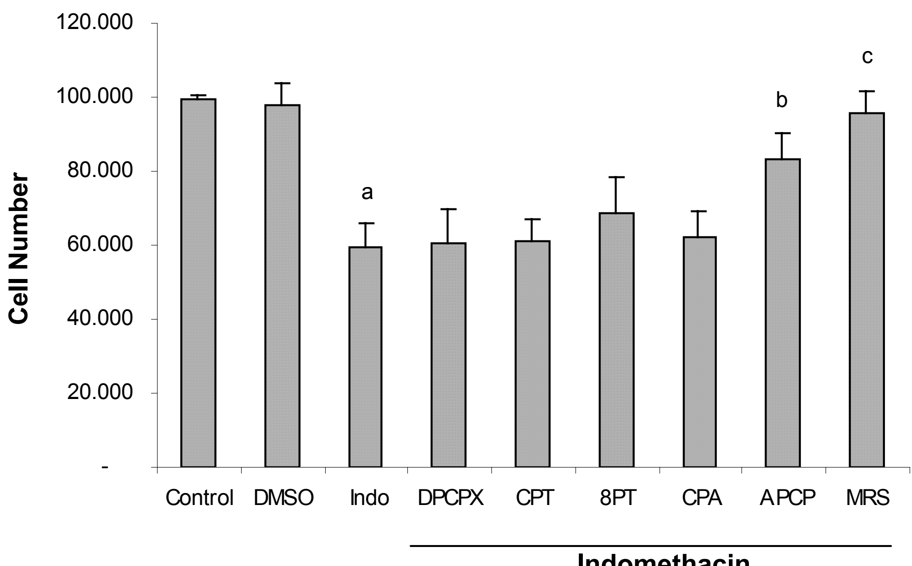


Figure 4

A



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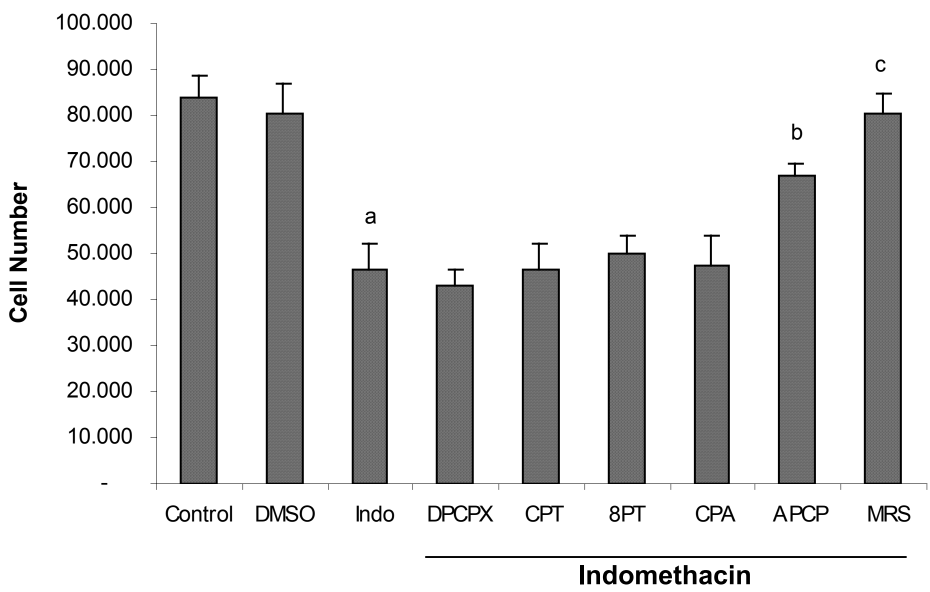


5.1. Anexos Capítulo II

Dados não mostrados no **“Capítulo II”**, mas ciatados na **“Discussão”.**

Figura 1

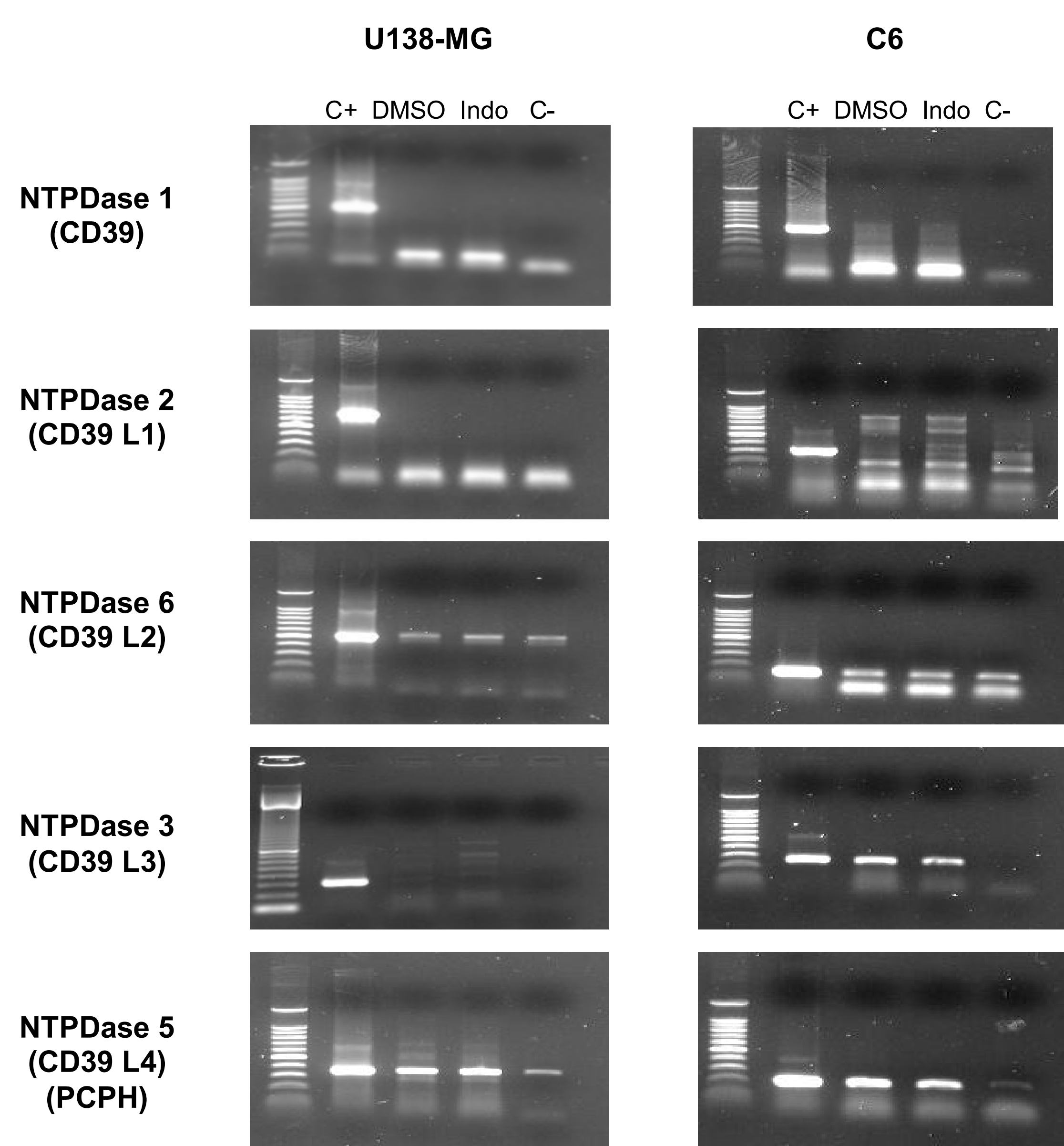
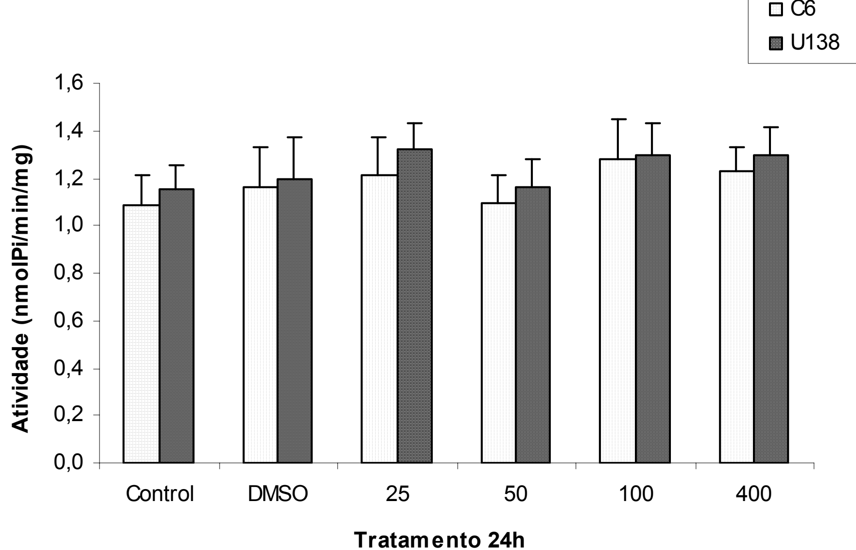


Figura 1. Análise da expressão da NTPDases por RT-PCR nas linhagens de glioma C6 e U138-MG. As células foram tratadas com 100 μM de indometacina (Indo) ou com DMSO (controle). Após 48h de tratamento, o RNA total foi extraído e o cDNA foi analisado por PCR com os "primers" específicos das NTPDases: NTPDase 1 (543 bp), NTPDase 2 (331 bp), NTPDase 6 (156 bp), NTPDase 3 (267 bp) e NTPDase 5 (210 bp), como descrito nos Materiais e Métodos (Capítulo I). Plasmídios contendo seqüências das NTPDases foram utilizados como controles positivos (C+) em todas as reações. Os controles negativos (C-) foram realizados com ausência de cDNA. Os dados são representativos de dois experimentos independentes (cDNAs diferentes) com resultados similares.

Figura 2

A



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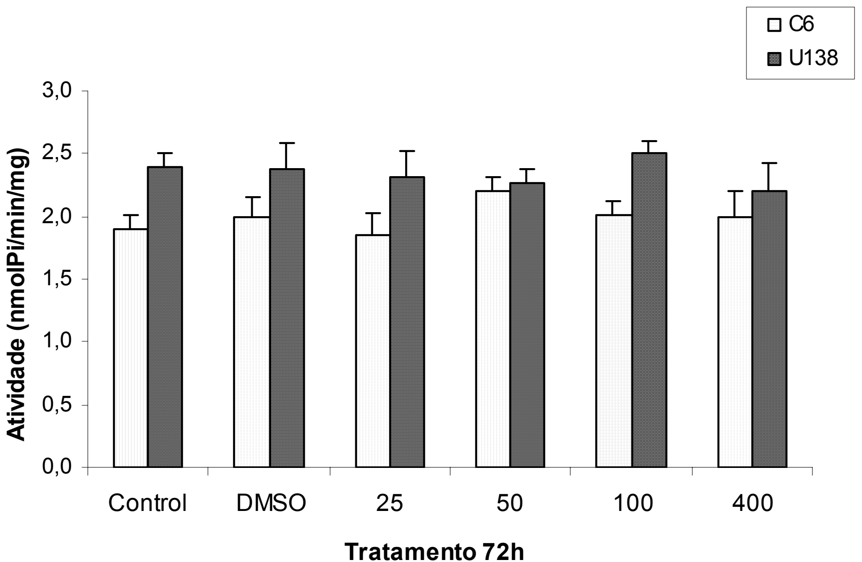

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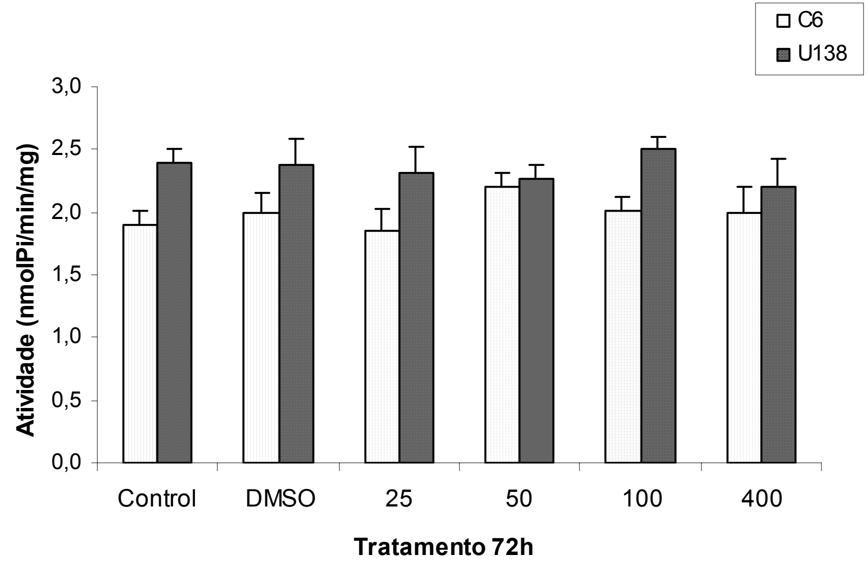
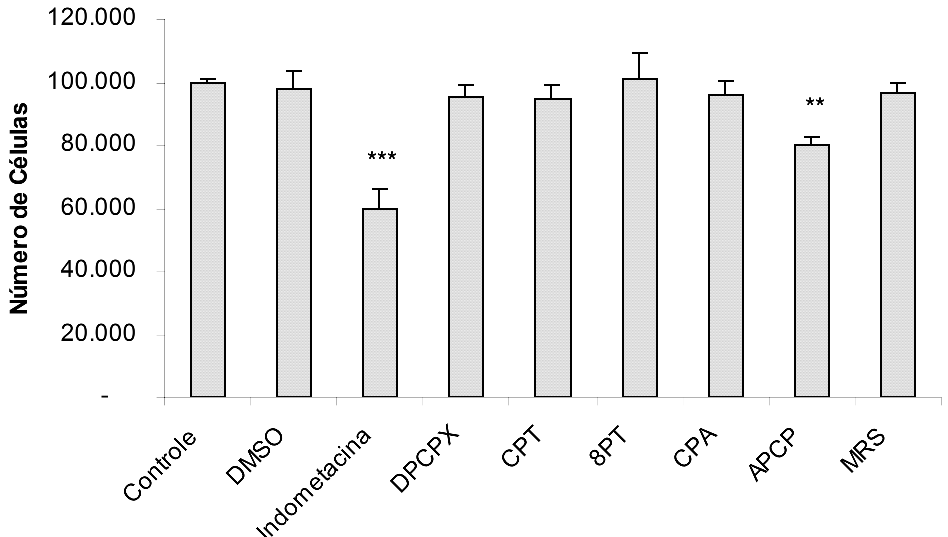


Figura 2. Efeito da indometacina sobre a atividade ADPásica nas células de glioma. Após a semi-confluência as células foram tratadas com indometacina (25, 50, 100 ou 400 µM) durante 24 (A), 48 (B) ou 72h (C). As células tratadas e os controles foram incubados com 1 mM de ADP por 30 minutos. Valores de atividade específica são expressos em nmol Pi/min/mg proteína. Os valores são representativos de três experimentos independentes determinados em quadruplicata. Nenhum dos tratamentos foi estatisticamente significativo em relação ao controle. A análise estatística utilizada foi ANOVA seguida do Teste de Tukey.

Figura 3

A



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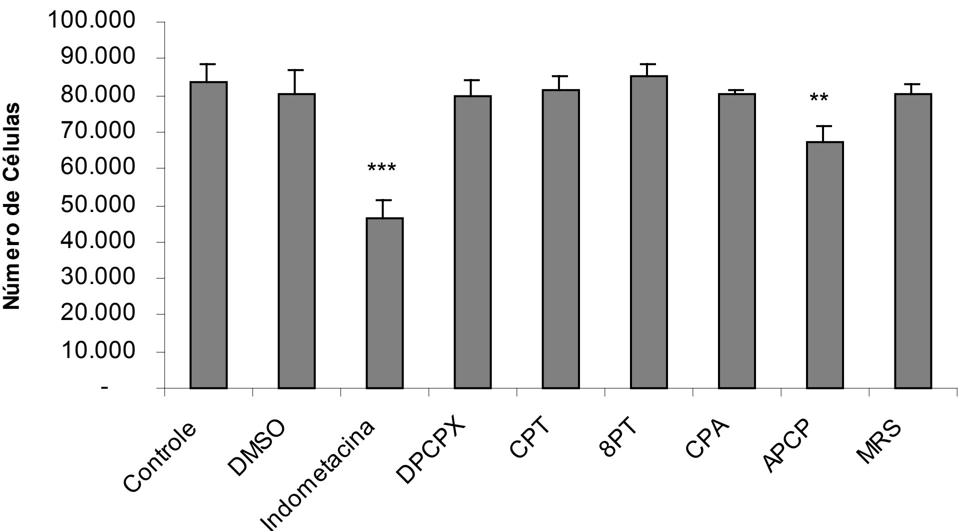

Figura 3. Efeito dos agonistas/antagonistas de adenosina na proliferação celular das linhagens de glioma C6 e U138-MG. As células C6 (A) e U138-MG (B) foram tratadas com os agonistas/antagonistas (sem a presença simultânea da indometacina): DPCPX (1 μM), CPT (0.2 μM), 8PT (1 μM), CPA (1 μM), APCP, também inibidor da ecto-5'-NT (1 μM) ou MRS1220 (1 μM) por 48h. Após esse período as células foram soltas com tripisina/EDTA e contadas em hemocitrômetro. Os valores são representativos de três experimentos independentes em triplicata. O efeito foi estatisticamente significativo com ***$p<0.0001$ (indometacina em relação ao controle); **$p<0.01$ (APCP em relação à indometacina). A análise estatística utilizada foi ANOVA seguida do Teste de Tukey.

DISCUSSÃO

Numerosos estudos experimentais, epidemiológicos e clínicos têm sugerido os AINEs como promissores agentes antitumorais. Os primeiros estudos foram realizados com câncer coloretal, mas há um crescente número de investigações com outros tipos de cânceres. Estudos epidemiológicos documentaram um índice de 40-50% na redução de incidência de câncer coloretal em indivíduos que utilizam AINEs (Smalley and DuBois 1997), sendo que o FDA (*Food and Drug Administration*) já aprovou o uso do celecoxib para pessoas que apresentam história familiar de câncer de cólon do tipo pólipo adenomatoso (Tegeder *et al*., 2001). Embora a atividade quimiopreventiva e antitumorigênica dos AINEs no câncer coloretal e outros tipos de tumores esteja bem estabelecida, os mecanismos moleculares responsáveis por estas propriedades ainda não estão elucidados (Baek *et al*., 2002). Vários estudos apresentam evidências que os AINEs previnem o crescimento de células canceroras por inibir a enzima COX (Grubbs *et al*., 2000; Wechter *et al*., 2000; Williams *et al*., 2000). Outros autores sugerem que as propriedades antitumorais podem ser independentes da atividade da COX (Hanif *et al*., 1997; Piazza *et al*., 1997; Murphy *et al*., 1998; Trifan *et al.*, 1999; Smith *et al*., 2000). Dessa forma, há um crescente interesse em elucidar os mecanismos envolvidos no efeito antiproliferativo destes fármacos.

A COX-1 é expressa de forma constitutiva pela maioria das células e estando relacionada com inúmeras funções fisiológicas, enquanto a COX-2 é a forma induzível. Em cérebros sadios a COX-1 é expressa nos macrófagos e células da microglia, enquanto a COX-2 é expressa nos neurônios e em algumas células endoteliais (Deininger *et al*., 1999). Tem sido demonstrado que gliomas humanos

superexpressam as enzimas COX-1 e COX-2 quando comparados com células normais (Matsuo *et al*., 2001; Prayson *et al*., 2002). Além disso, é descrito que a superexpressão da COX-2 está relacionada com o alto índice proliferativo dos gliomas (Prayson *et al*., 2002), bem como com o seu grau de malignidade (Joki *et al*., 2000). Em um recente estudo clínico, pacientes com tumores cerebrais malignos (GBM, astrocitoma anaplásico, meningioma maligno e meduloblastoma) apresentam elevadas concentrações de PGE2 no plasma, enquanto pacientes com tumores cerebrais benignos (meningioma benigno e adenoma de pituitária) não apresentaram este aumento (Loh *et al*., 2002).

As linhagens de gliomas têm sido extensivamente utilizadas como modelos para estudos de gliomas *in vitro* com as mais diversas abordagens. Essas células são consideradas excelentes modelos de estudo uma vez que apresentam diversas características biológicas dos tumores *in vivo*, como alto poder de invasão e proliferação (Masters *et al*., 2000; Grobben *et al.,* 2002). Considerando: 1) a constante busca de estratégias terapêuticas mais eficazes para o tratamento dos gliomas; 2) que os estudos têm sugerido os AINEs como potenciais agentes antitumorais; e 3) que os efeitos e os mecanismos destes fármacos nos gliomas não estão totalmente elucidados, o objetivo geral deste trabalho foi avaliar o efeito dos AINEs na proliferação celular em linhagens de gliomas humano (U138-MG) e de rato (C6), bem como os possíveis mecanismos envolvidos com este efeito antiproliferativo. Neste estudo, as linhagens utilizadas são representativas do glioblastoma multiforme e são COX-1 e COX-2 positivas (Deininger et al., 1999).

No primeiro capítulo, o objetivo foi avaliar o efeito de vários AINEs na proliferação celular dos gliomas, bem como alguns possíveis mecanismos envolvidos com o efeito antiproliferativo observado. Em um primeiro momento,

através do método de contagem de células, verificou-se que os AINEs (indometacina, acetaminofeno, sulfeto de sulindaco e NS-398) foram capazes de inibir significativamente a proliferação celular em ambas as linhagens celulares estudadas, sendo este efeito tempo e dose dependente (Figura 1, Capítulo I). A proliferação celular também foi avaliada por outra metodologia, o ensaio da Sulphorrodamina, onde se observou um efeito semelhante com todos os AINEs testados (Figura 1, Anexos Capítulo I). Estes resultados estão de acordo com trabalhos da literatura, que mostram o efeito antiproliferativo dos AINEs em outros tipos celulares (Fujiwara *et al*., 1993; Yamamoto *et al*., 1999). O fato de fármacos da mesma classe terapêutica, porém com especificidades e classes químicas diferentes causarem um efeito semelhante, sugere que, provavelmente, este efeito seja um mecanismo de ação comum a este grupo de fármacos e não apenas um efeito químico. Além disso, é importante ressaltar que o acetaminofeno não é um potente inibidor da COX, como a indometacina e o sulfeto de sulindaco, e mesmo assim foi capaz de inibir significativamente a proliferação celular, o que sugere a existência de um possível mecanismo independente de COX.

Na seqüência deste estudo, utilizou-se a indometacina como representante de um dos AINEs onde inicialmente foi observado um efeito antiproliferativo, uma vez que este fármaco é um dos mais potentes desta classe terapêutica, além de não possuir todos os seus mecanismos de ação totalmente estabelecidos. Considerando esse efeito de inibição da proliferação e conseqüente morte celular, foram utilizadas metodologias para tentar avaliar os possíveis mecanismos envolvidos neste efeito, bem como caracterizar esta morte celular como necrótica ou apoptótica. Através da detecção da incorporação do iodeto de propídeo pode-se observar que ambas as linhagens celulares tratadas com concentrações

terapêuticas de indometacina (100 μM) apresentaram ausência de necrose, sendo que apenas as células tratadas com concentrações acima da faixa terapêutica (400 μM) apresentaram incorporação do corante, indicando presença de necrose (Figura 2, Capítulo I e Figura 2, Anexos Capítulo I). Através da medida da LDH (lactato desidrogenase), foi confirmada a ausência de necrose nas células tratadas nas mesmas condições, uma vez que esta enzima é citosólica e apresentou-se aumentada apenas no tratamento com 400 μM do fármaco (Figura 3, Anexos Capítulo I). Para avaliar se o tratamento com indometacina estaria alterando a viabilidade mitocondrial e, conseqüentemente, a viabilidade celular, foi utilizado o método do MTT onde se observou uma diminuição da viabilidade das células após o tratamento com este AINE (Figura 3, Capítulo I). A ausência de ativação de caspases 3/7 nos gliomas tratados com os AINEs (Figura 4, Capítulo I e Figura 4, Anexos Capítulo I), sugere uma morte celular não-apoptótica, porém não se pode descartar a possibilidade de uma morte por apoptose independentemente da ativação das caspases, uma vez que alguns autores demonstram que este tipo de apoptose pode ocorrer (Biffe *et al.,* 2003). A principal importância deste conjunto de resultados é que estes efeitos foram observados com doses terapêuticas do fármaco, uma vez que muitos trabalhos da literatura mostram resultados semelhantes mas com doses consideradas citotóxicas para as células normais, o que limitaria o seu uso na terapêutica.

Recentemente Parashar *et al.* (2005) mostrou que várias linhagens celulares de neuroblastoma tratadas com o AINE CAY10404 apresentaram alterações na progressão do ciclo celular. Outros trabalhos da literatura sugerem que alterações em células tratadas com AINEs envolvendo ciclo celular se devem a mecanismos independentes da inibição da COX (Tegeder *et al*., 2001). No presente estudo, as

células de glioma humano quando tratadas com indometacina (100 μM) apresentaram um aumento na proporção de células nas fases G0/G1 e G2/M e diminuição de células na fase S, sugerindo uma parada na progressão do ciclo celular em G1 e/ou G2 (Tabela 2, Capítulo I). Da mesma forma, as células de glioma de rato apresentaram uma parada na progressão do ciclo celular, porém apenas em G1. Gráficos representativos da citometria de fluxo são apresentados na Figura 5, Anexos Capítulo I. De acordo com dados da literatura, a progressão do ciclo celular da fase quiescente (Go) para a fase de síntese (S) envolve a ativação e/ou produção de uma variedade de proteínas regulatórias (Murray *et al*., 1989). As ciclinas representam o maior grupo de proteínas reguladoras do ciclo celular (Sherr *et al*., 1994; Morgan *et al*., 1995). Pai e cols. (2000) mostraram que a indometacina causa uma parada na progressão do ciclo celular por redução da fosforilação de pRb e aumento da proteína p21 em linhagem de célula endotelial. A atividade de pRb é supostamente regulada por fosforilação/defosforilação durante o ciclo celular (Weinberg *et al*., 1995). Assim sugere-se que a redução na fosforilação de pRb está associada com o decréscimo na expressão da proteína ciclina D1 e que isso leva à inibição da proliferação celular (Pai *et al*. 2000). De acordo com Kardosh e cols. (2004), celecoxib, um seletivo inibidor de COX-2, exerce um efeito antiproliferativo independente de COX-2 em várias linhagens de gliomas (LN229, LN18, A172, U87-MG), através da inibição de dois componentes essenciais da maquinaria do ciclo celular, ciclina A e ciclina B. No presente trabalho, os resultados mostraram que, o efeito antiproliferativo da indometacina se deve, pelo menos em parte, por uma parada na progressão do ciclo celular, mas o mecanismo exato permanece em investigação, sendo que mais estudos são necessários para avaliar o efeito da indometacina nas proteínas que regulam a progressão do ciclo celular.

Considerando que a literatura descreve, em vários tipos celulares, que uma diminuição da proliferação por conseqüência de uma parada na progressão do ciclo celular é um mecanismo COX-independente (Grosch *et al*., 2001; Goel *et al*., 2003; Kardosh *et al*., 2004; Cherukuri *et al*., 2004; Ding *et al*., 2005), provavelmente esse seja o efeito da indometacina nas linhagens de gliomas aqui estudadas.

Como revisado por Tegeder e cols. (2001), além da inibição da progressão do ciclo celular, os AINEs poderiam ter mecanismos adicionais envolvendo NF-κB, p38 MAPK, ativador de proteína 1, cinase ribossomal S6, STAT1 e PPARγ. Dessa forma, foram utilizados alguns inibidores de proteínas envolvidas em vias de sinalização celular a fim de avaliar um possível envolvimento destas vias no efeito antiproliferativo mediado pela indometacina na linhagem de glioma C6. O inibidor de caspases ZDEVD-CH_2F não alterou o efeito antiproliferativo da indometacina (Tabela 1, Capítulo I), confirmando assim o resultado obtido da ausência de ativação de caspases (Figura 4, Capítulo I). Por outro lado, o pré-tratamento das culturas com PD98059 (inibidor de MEK1) e PP2 (inibidor de c-Src) significativamente preveniu a inibição da proliferação celular mediada pela indometacina, indicando o envolvimento destas vias no efeito antiproliferativo da indometacina (Tabela 1, Capítulo I). Considerando que a via das MEK/ERK, pode afetar a proliferação celular e apoptose, foram realizados experimentos de *Western Blotting* para avaliar se a indometacina altera os níveis de fosforilação da ERK. Observou-se um aumento da fosforilação da ERK (p44 e p42, respectivamente) em ambas as linhagens quando as células tratadas foram comparadas aos controles, enquanto os níveis totais de ERK (fosforilada e não-fosforilada) não foram alterados com o tratamento (Figura 6, Anexos Capítulo I). Surpreendentemente, estes resultados nos sugerem que a ERK está apresentando um papel antiproliferativo em

resposta à indometacina. A ativação de ERK é classicamente associada com sobrevivência, sendo que quando fosforilada (ativa), resulta em aumento da proliferação celular (Mansour *et al*., 1994; Xia *et al*., 1995). Entretanto, recentes estudos têm indicado que nem sempre este é o mecanismo que ocorre (Elder *et al*., 2002). De acordo com Elder e cols. (2002) a sinalização da cascata da MEK/ERK medeia a indução da apoptose em células de carcinoma coloretal tratadas com NS-398. Por um outro lado, Pumglia e cols. (1997) mostraram que um sustentado aumento da atividade das MAPK pode levar a uma parada na progressão do ciclo celular por inibição da atividade das CDKs. Provavelmente, a resposta sinalizada pela ERK depende da natureza, intensidade e duração do estímulo, bem como do tipo celular (Stancium *et al*., 2000; Galve-Roperh *et al*., 2000; Pumiglia *et al*., 1997). Assim, acredita-se que uma ativação transitória pode contribuir para a progressão do ciclo celular, enquanto uma atividade elevada e sustentada pode resultar em parada na progressão do ciclo celular (Elder *et al*., 2002). Como a c-Src está "upstrean" da ERK em células de astrocitomas humanos (Gendron *et al*., 2003), a nossa proposta é um mecanismo em que a indometacina induz a ativação da c-Src, a qual leva à ativação da ERK e, assim, resulta em redução do número de células, embora não se possa excluir que estas duas vias sejam independentemente ativadas por indometacina. Além disso, a ativação da ERK poderia estar relacionada com a parada na progressão do ciclo celular observada nas células tratadas.

Recentemente Wink e cols. (2003) mostrou que várias linhagens celulares de gliomas apresentam um padrão alterado de hidrólise dos nucleotídeos extracelulares, quando comparado com astrócitos normais. Os gliomas possuem uma baixa velocidade de hidrólise dos nucleotídeos trifosfatados (ATP, GTP, CTP,

ITP e UTP), enquanto a atividade AMPásica é muito maior quando comparada com astrócitos. Assim, foi sugerido que este aumento nas concentrações de ATP e de Ado extracelulares atuariam na proliferação de gliomas, podendo fazer parte dos mecanismos que caracterizam esses tumores, como a alta capacidade de invasão e proliferação (Wink *et al.*, 2003). Considerando: 1) que a indometacina parece ter outros mecanismos além da inibição da COX; 2) que estudos prévios do nosso laboratório têm mostrado que células de glioma apresentam alterações na via de catabolismo dos nucleotídeos extracelulares; e 3) que estas alterações provavelmente estão associadas à patogenia dos gliomas, o objetivo geral do segundo capítulo foi investigar o efeito da indometacina sobre as enzimas ecto-nucleotidases.

As células tratadas com indometacina apresentaram um aumento significativo na atividade ATPásica (Figura 1, Capítulo II), o que pode ser uma explicação, pelo menos em parte, para o mecanismo antiproliferativo desse fármaco, uma vez que não haveria tanto ATP extracelular disponível para induzir a proliferação celular. Esse aumento de hidrólise do ATP foi observado de forma semelhante em ambas as linhagens celulares. O efeito da indometacina sobre a atividade das NTPDases parece ser um efeito não-genômico, uma vez que não foram observadas alterações nos níveis de mRNA dessas proteínas (Figura 1, Anexos Capítulo II). Ainda avaliando os perfis de hidrólise dos nucleotídeos pelos gliomas tratados com doses terapêuticas de indometacina, observamos que, ao contrário da atividade ATPásica, a atividade ADPásica não sofreu alteração (Figura 2, Anexos Capítulo II). Em conjunto, esses resultados sugerem que o tratamento com indometacina poderia ser considerado uma tentativa de devolver às células um perfil de hidrólise do ATP semelhante às células normais, os astrócitos.

Um dado que nos pareceu extremamente relevante neste trabalho foi o aumento significativo na atividade da ecto-5'-NT (Figura 2, Capítulo II), estando este aumento relacionado com um aumento da expressão do mRNA desta proteína (Figura 3, Capítulo II). Inicialmente, esses resultados nos pareceram ser contraditórios, uma vez que os gliomas já apresentam uma atividade AMPásica muito maior quando comparados aos astrócitos (Wink *et al*., 2003). Além disso, esse aumento na atividade e na expressão da ecto-5'-NT resultaria em elevadas concentrações extracelulares de Ado, a qual já foi previamente descrita como agente proliferativo (Morrone *et al.,* 2003), o que não estaria de acordo com o efeito antiproliferativo da indometacina observado nos outros experimentos. Porém, existem referências na literatura indicando que os efeitos da Ado ainda são bastante contraditórios e antagônicos dependendo da circunstância. A Ado tem características únicas que diferencialmente modulam o crescimento de células normais e transformadas, dependendo da concentração extracelular, da expressão dos receptores de superfície e do estado fisiológico da célula alvo (Abbracchio *et al*., 1999). A estimulação da proliferação após incubação com Ado tem sido bem demonstrada em uma variedade de células normais numa faixa de concentrações micromolar (Fishman *et al*., 1998). Por outro lado, indução de apoptose em células normais e tumorais foi mostrada em elevadas concentrações de Ado (>100 μM) (Bajaj *et al*., 1983). O efeito bifásico mediado por agonistas A_3 tem sido também caracterizado em células astrocíticas (Jacobson *et al*., 1999). Assim, por exemplo, o receptor A_3 tem um papel chave no efeito inibitório ou estimulatório do crescimento celular mediado pela Ado (Abbracchio *et al*., 1999), sendo que essa molécula pode induzir morte celular por ativar os receptores específicos A_2 e A_3 (Ohana *et al*., 2001). Bajaj e cols*.* (1983) mostraram que a proliferação de linfócitos derivados de

pacientes com leucemia linfocítica crônica foi suprimida pela Ado. Nesse caso, a concentração extracelular da Ado pode ser um fator crucial na determinação das vias de sinalização ativadas, sendo que a inibição da proliferação se deve à ativação de vias que levam à apoptose ou um efeito citostático (Bajaj *et al*., 1983).

Considerando os dados da literatura e os resultados apresentados no presente estudo, nós hipotetizamos que o aumento da atividade da ecto-5'-NT estaria gerando concentrações aumentadas de Ado, a qual poderia estar envolvida no efeito antiproliferativo observado, provavelmente via receptor A_3. Confirmando a nossa proposta, observamos que o pré-tratamento com um antagonista do receptor A_3 (MRS1220) preveniu o efeito antiproliferativo da indometacina, indicando um provável envolvimento desse receptor no efeito mediado por este fármaco (Figura 4, Capítulo II). Os outros subtipos de receptores P1 provavelmente não estão envolvidos nesse efeito mediado pela indometacina pelo fato dos agonistas/antagonistas utilizados não terem sido capazes de alterar o efeito antiproliferativo (Figura 4, Capítulo II). Além disso, o α,β-metileno ADP (APCP), um inibidor da ecto-5'-NT, foi capaz de prevenir parcialmente a inibição da proliferação celular mediada pela indometacina, confirmando o papel da ecto-5'-NT na proliferação dos gliomas. Interessantemente, o tratamento com esse inibidor sozinho causou um efeito antiproliferativo, enquanto os outros agonistas/antagonistas sozinhos não apresentaram alterações significativas na proliferação celular (Figura 3, Anexos Capítulo II). Este efeito do tratamento com APCP sozinho está de acordo com os dados da literatura que sugerem um papel *per se* da ecto-5'-NT na sobrevivência celular (Turnay *et al*., 1989; Navarro *et al*., 1998; Zimmermann, 2002).

Assim, a análise de todos esses dados nos leva a supor que o aumento da atividade da ecto-5'-NT produz elevadas concentrações de Ado no meio extracelular e, quando em concentrações que ativem o receptor A_3, esta molécula poderá estar envolvida, pelo menos em parte, no efeito antiproliferativo da indometacina. Além disso, alguns trabalhos da literatura têm proposto que a ativação de receptores A_3 leva à inibição da proliferação celular por envolver a inibição da enzima telomerase e conseqüente parada na progressão do cilco celular em G0/G1, causando portanto um efeito citostático (Fishman *et al.,* 1998; Brambilla *et al.,* 2000; Merighi *et al*., 2002a). Dessa forma, os resultados apresentados no Capítulo I estão de acordo com essa proposta, uma vez que as células tratadas com indometacina apresentaram uma parada na progressão do ciclo celular em G0/G1 e G2/M. Assim, o conjunto dos resultados apresentados nos capítulos I e II nos sugerem que os AINEs apresentam outros mecanismos de ação além dos já estabelecidos como a inibição da COX. Esses mecanismos alternativos podem explicar os efeitos antiproliferativos destes fármacos observados em vários tipos celulares. Mais estudos com modelos *in vivo* de gliomas são necessários para que se tenha novas evidências da utilidade dos AINEs na prevenção e no tratamento quimioterápico desses tumores.

CONCLUSÕES

Capítulo I

1) Os AINEs (indometacina, acetaminofeno, sulfeto de sulindaco e NS-398) foram capazes de inibir significativamente a proliferação celular nas linhagens de glioma humano U138-MG e C6 de rato;
2) O efeito antiproliferativo foi observado em todas as concentrações testadas, tendo um perfil tempo e dose dependente;
3) A indometacina causou uma diminuição da viabilidade celular nas linhagens de glioma C6 e U138-MG;
4) Não houve diferença significativa na atividade das caspases 3/7, o que sugere que os AINEs induzem morte celular nos gliomas por uma via independente de caspases;
5) Doses terapêuticas de indometacina induziram morte celular com ausência de necrose em ambas as linhagens de glioma;
6) A indometacina induziu uma parada na progressão do ciclo celular nas fases G0/G1 e G2/M na linhagem U138-MG e na fase G0/G1 na linhagem C6;
7) A ativação de c-Src e ERK estão envolvidas no efeito antiproliferativo da indometacina nas células de glioma C6.

Capítulo II

1) A indometacina, em concentrações terapêuticas, causou um significativo aumento na atividade ATPásica na linhagem U138-MG;
2) O aumento da atividade ATPásica parece ser um efeito não-genônico, uma vez que não foram observadas alterações nos níveis de mRNA das NTPDases;
3) A indometacina não causou alterações significativas na atividade ADPásica em ambas as linhagens;
4) Um significativo aumento na atividade da ecto-5'-NT foi observado nas linhagens de gliomas tratadas com doses terapêuticas da indometacina;
5) O aumento da atividade AMPásica nos gliomas tratados com indometacina está relacionado com um aumento na expressão nos níveis de mRNA da ecto-5'-NT;
6) O pré-tratamento com um específico antagonista de receptor A_3 de Ado, foi capaz de prevenir o efeito antiproliferativo da indometacina nas linhagens de glioma, sugerindo um envolvimento deste receptor na ação deste fármaco;
7) O pré-tratamento com um inibidor da ecto-5'-NT preveniu o efeito antiproliferativo da indometacina em ambas as linhagens de glioma, confirmando o envolvimento da ecto-5'-NT na proliferação celular;
8) Não foram observadas alterações no efeito antiproliferativo da indometacina nos gliomas quando foram utilizados outros agonistas/antagonistas dos recepores P1 (A_1, A_{2A} e A_{2B}).

REFERÊNCIAS BIBLIOGRÁFICAS

ABBRACCHIO MP, CATTABENI F. Brain adenosine receptors as targets for therapeutic intervention in neurodegenerative diseases. *Annals New York Academy of Sciences* 890: 79-92, 1999.

AIRAS L, HELLMAN J, SALMI M, BONO P, PUURUNE T, SMITH DJ, JALKANEN S. CD73 is involved in lymphocyte binding to the endothelium: characterization of lymphocyte-vasculr adhesion protein 2 identifies it as CD73. *J. Exp. Med.* 182: 1603-1608, 1995.

BAEK JS, WILSON CL, LEE CH, ELING ET. Dual function of nonsteroidal anti-inflammatory drugs (NSAIDs): inhibition of cyclooxygenase and induction of NSAID-activated gene. *Pharmacol and Exp Terapeutics* 301: 1126-1131, 2002.

BAJAJ SL, INSEL J, QUAGLIATA R, HIRSCHHORN R, SILBER R. Adenosine and adenosine analogues are more toxic to chronic lymphocytic leukemia than to normal to normal lymphocytes. *Blood* 62: 75-80, 1983.

BALTUCH GH & YONG VW. Signal transduction for proliferation of glioma cells *in vitro* occurs predominantly through a protein kinase C-mediated pathway. *Brain Res*. 710: 143-149, 1996.

BAYLEI CC, SPOONER D. Brain tumors in children. In: Peckham M, Pinedo HM, Veronesi U (eds). Oxford Textbook of Oncology. New York: Oxford Univesrsity Press, p.2055-2063, 1995.

BEHIN A, HOANG-XUAN K, CARPENTIER AF, DELATTRE J. Primary brain tumors in adults. *Lancet*. 361: 323-331, 2003.

BIFFE WL, CARNAGGIO R, MOORE EE, CIESLA DJ, JOHNSON JL, SILLIMAN CC. Clinically relevant hypertonicity prevents stored blood- and lipid-mediated delayed

neutrophil apoptosis independent of p38 MAPK or caspase-3 activation. *Surgery.* 134: 86-91, 2003.

BOGLER O, NAGANE M, GILLIS J, HUANG HJS, CAVENEE WK. Maligmant transformation of p53-deficient astrocytes is modulated by environmental cues *in vitro. Cell Growth and Differ.* 10: 73-86, 1999.

BRAMBILLA R, CATTABENI F, CERUTI S, BARBIERI D, FRANCESCHI YC, KIM KA, JACOBSON KA, KLOTZ KN, LOHSE MJ, ABBRACHIO MP. Activation of the A_3 adenosine recptor affects cell cycle progression an cell growth. *Naunyn-Schmiedeberg's Arch Pharmacol*. 361: 225-234, 2000.

BRENDEL M, POLLACK IF. The role of protein kinase C (PKC) in the evolution and proliferation of maligmant gliomas, and the application of PKC inhibition as a novel approach to anti-glioma therapy. *Acta Neurochirurgia* 139: 1000-1013, 1997.

BRENDEL M, POLLACK IF. The p21-*Ras* signal transduction pathway and growth regulation in human high-grade gliomas. *Brain Res. Reviews* 29: 232-259, 1999.

CAMPBELL SL, KHOSRAVI-FAR R, ROSSMAN KL, CLARK GJ, DER CJ. Increasing complexity of Ras signaling. *Oncogene* 17: 1395-1413, 1999.

CARVALHO WA, CARVALHO RDS, RIOS-SANTOS F. Specific Cyclooxygenase-2 analgesics: therapeutical advances. *Rev Bras Anestesiol*. 54: 456-464, 2004.

CARVALHO WA. Analgésicos, Antipiréticos e Antiinflamatórios. Farmacologia, 6 ed. Rio de Janeiro: Guanabara Koogan, 2002.

CERUTI S, FRANCESCHI C, BARBIERI D, MALORNI W, CAMURRI A, GIAMMARIOLI AM, AMBROSINI A, RACAGNI G, CATTABENI F, ABBRACCHIO MP. Apoptosis induced by 2-chloro-adenosine and 2-chloro-2- deoxy-adenosine

in a human astrocitoma cell line: differential mechanisms and possible clinical relevance. *J. Neurosci. Res.* 60: 388–400, 2000.

CHAMBERS TC, POHL J, RAYNOR RL, KUO JF. Identification of specific sites in human P-glycoprotein phosphorylated by protein kinase C. *J Biol. Chem.* 268: 4592-4595, 1993.

CHANDRASEKHARAN NV, DAI H, ROOS KL. COX-3, a cyclooxygenase-1 variant inhibited by acethaminophen and other analgesic/antipyretic drugs: cloning, structure and expression. *Proc Natl Acad Sci*. 99: 13926-13931, 2002.

CHERUKURI DP, NELSON MA. Glioma growth inhibition by selective COX-2 inhibitors via cyclooxygenase independent patways. *Cancer Biology & Therapy*. 1: 63-64, 2004.

CHOE G, HORVATH S, CLOUGHESY TF, CROSBY K, SELIGSON D, PALOTIE A, INGE L, SMITH BL, SAWYERS CL, MISCHEL PS. Analysis of the phosphatidilinositol 3-kinase signaling pathway in glioblastoma patients in vivo. *Cancer Res.* 63: 2742-2746, 2003.

CICCARELLI R, BALLERINI P, SABATINO G, RATHBONE MP, D'ONOFRIO M, CACIAGLI F, DI IORIO P. Involvement of astrocytes in purine-mediated reparative processes in the brain. *In. J. Dev. Neurosci*. 19: 395-414, 2001.

COULDWELL WT, UHM J, ANTEL JP, YONG VW. Enhanced protein kinase C activity correlates with the growth rate of malignant human gliomas. *Neurosurgery* 29: 880-887, 1991.

COULDWELL WT, HINTON DR, HE S, CHEN TC, SEBAT I, WEISS MH, LAW RE. Protein kinase C inhibitors induce apoptosis in human malignant glioma cell lines. *FEBS Lett.* 345: 43-46, 1994.

DEININGER MH, SCHLUESENER HJ. Cyclooxygenase-1 and –2 are differentially localizated to microglia and endothelium in rat EAE and gliomas. *J Neuroimmunol.* 95: 202-208, 1999.

DEWITT DL, SMITH WL. Primary structure of prostaglandin G/H sintase from ahep vesicular gland determined from the complementary DNA sequence. *Proc Natl Acad Sci*. 85: 1412-1416, 1988.

DING H, HAN C, ZHU J, CHEN CS, D'AMBROSIO SM. Celecoxib derivatives induce apoptosis via the disruption of mitochondrial membrane potential and activation of caspase 9. *Int J. Cancer* 5: 803-810, 2005.

DIRKS PB, JAMES MD, RUTKA JT. Current Concepts in Neuro-Oncology: The Cell Cycle – A Review. *Neurosurgery* 40: 1000-1015, 1997.

ELDER DJE, HALTON DE, PLAYLE LC, PARASKEVA C. The MEK/ERK pathway mediates COX-2-selective NSAID-induced apoptosis and induced COX-2 protein expression in colorectal carcinoma cells. *Int J Cancer* 99: 323-327, 2002.

EKSTRAND AJ, JAMES CD, CAVENEE WK, SELINGER B, PETTERSSON RF, COLLINS VP. Genes for epidermal growth factor receptor, transforming growth factor α, and epidermal growth factor and their expression in human gliomas *in vivo. Cancer Res*. 51: 2164-2172, 1991.

FISHMAN P, BAR-YEUDA S, WAGMAN L. Adenosine and other low molecular weight factors released by muscle cells inhibit tumor cell growth: possible explanation for the rarity metastases in muscle. *Cancer Res*. 58: 3181-3187, 1998.

FRAME MC. Src in cancer: deregulation and consequences for cell behaviour. *Biochim. Biophys. Acta* 1602: 114–130, 2002.

FREDHOLM BB, IJZERMAN AP, JACOBSON KA, KLOTZ KN, LINDEN J. International Union of Pharmacology: XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev.* 53: 527– 552, 2001.

FUCHS FD, WANNMACHER L. Farmacologia Clínica - Fundamentos da Terapêutica Racional. 2 ed. Rio de Janeiro: Guanabara Koogan, 1998.

FUJIWARA Y, TARNAWSKI A, FUJIWARA K, ARAKAWA T, KOBAYASSHI K. Inhibitory effects of indomethacin on growth and proliferation of gastric carcinoma cells KATO III. *J Physiol Pharmacol* 44: 147-153, 1993.

GALVE-ROPHERT I, SANCHES C, CORTES ML, GOMEZ Del Pulgar T, IZQUIERDO M, GUSMAN M. Anti-tumoral actino of canabinoids: involvement of sustainded ceramide accumulation and extracellular signal-regulated kinase activation. *Nat. Med* 6: 313-319, 2000.

GENDRON F, NEARY JT, THEISS PM, SUN GY, GONZALEZ FA, WEISMAN GA. Mechanisms of $P2X_7$ receptor-mediated ERK1/2 phosphorylation in human astrocytoma cells. *Am. J. Cell Physiol.* 284: 571-581, 2003.

GIARDIELLO FM, HAMILTON SR, KRUSH AJ, PIANTADOSI S, HYLIND LM, CELANO P, BOOKER SV, ROBINSON CR, OFFERHAUS GJ. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N. Eng. J. Med*. 328: 1313-1316, 1993.

GOEL A, CHANG DK, RICCIARDIELLO L, GASCHE C, BOLAND CR. A novel mechanism for aspirin-mediated growth inhibition of human colon cancer cells. *Clinical Cancer Research*. 9: 383-390, 2003.

GOODMAN & GILMAN. As Bases Farmacológicas da Terapêutica. 10 ed. Rio de Janeiro: Mc Graw Hill, 2003.

GROBBEN B, DE DEYN PP, SLEGERS H. Rat C6 clioma as experimental model system for the study of glioblastoma growth and invasion. *Cell Tissue Res.* 310: 257-270, 2002.

GROSCH S, TEGEDER I, NIEDERBERGER E, BRAUTIGAM L, GEISSLINGER G. COX-2 independent induction of cell cycle arrest and apoptosis in colon cancer cells by the selective COX-2 inhibitor celecoxib. *Faseb J*. 14: 2742-2744, 2001.

GRUBBS CJ, LUBET RA, KOKI AT, LEAHY KM, MASFERRER JL, STEELE VÊ, KELLOFF GJ, HILL DL, SEIBERT K. Celecoxib inhibits N-butyl-N-(4-hydroxybutyl)-nitrosamine-induced urinary bladder cancers in male B6D2F1 mice and female Fischer-344 rats. *Cancer Res*. 60: 5599-5602, 2000.

GUHA A, FELDKAMP MM, LAU N, BOSS G, PAWSON A. Proliferation of human malignant astrocytomas is dependent on Ras activation. *Oncogene* 15: 2755-2765, 1997.

HANDA M, GUIDOTTI G. Purification and cloning of ATP-diphosphohydrolase (apyrase) from potato tubers (solanum tuberosum). *Biochem. Biophys. Res.* Comm. 218: 916-923, 1996.

HANIF R, PITTAS A, FENG Y, KOUTSOS MI, QUIAO L, STAIANO-COICO L, SHIFF SI, RIGAS B. Effects of nosteroidal anti-inflamatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. *Biochem Pharmacol*. 52: 237-245, 1996.

HIAL V, DEMELLO MC, HORAKOVA Z, BEAVEN MA. Antiproliferative activity of antiinflamatory drugs in two mammalian cell culture lines. *J. Pharmacol. Exp. Ther*. 202: 446-454, 1997.

HOCHBERG FB, PRUITT A. Neoplasias do sistema nervoso central. In: harrison medicina Interna. 12ed. Rio de Janeiro: Guanabara Koogan, p.13-70, 1991.

HOLLAND EC. Gliomagenesis: genetic alterations and mouse models. *Nat. Rev. Genet.* 2: 120-129, 2001.

HOLLAND EC, CELESTINO J, DAI C, SCHAEFER L, SAWAYA RE, FULLER GN. Combined activation of Ras and Akt in neural progenitors induces glioblastoma formation in mice. *Nature Genetics* 25: 55-57, 2000.

HOSHINO R, CHATANI Y, YAMORI T, TSURUO T, OKA H, YOSHIDA O, SHIMADA Y, ARI-I S, WADA H, FUJIMOTO J, KOHNO M. Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. *Oncogene* 18: 813-822, 1999.

HOWE LR, SUBBARAMAIAH K, BROWN AMC, DANNENBERG AJ. Cyclooxigenase-2: a targed for the prevention and treatment of breast cancer. *Endocrine-related Cancer* 8: 97-114, 2001.

HWANG D, SCOLLARD D, BYRNE J, LEVINE E. Expression of cyclooxygenase-1 and cyclooxygenase-2 in human breast cancer. *J. Natl. Cancer Inst.* 90: 455-460, 1998.

IRBY RB & YEATMAN TJ. Role of Src expression and activation in human cancer. *Oncogene* 19: 5636–5642, 2000.

JACOBSON KA, HOFFMANN C, CATTABENI F, ABBRACCHIO P. Adenosine-induced cell death: evidence for receptor-mediated signalling. *Apoptosis* 4: 197-311, 1999.

JOKI T, HEESE O, NIKAS DC. Expression of cyclooxygenase-2 (COX-2) in human gliomas and in vitro inhibition by a specific COX-2 inhibitor, NS-398. *Cancer Res.* 60: 4926-4931, 2000.

JONES RJ, AVIZIENYTE E, WYKE AW, OWENS DW, BRUNTON VG, FRAME MC. Elevated c-Src is linked to altered cell matrix adhesion rather than proliferation in KM12C human colorectal cancer cells. *Br. J. Cancer* 87: 1128–1135, 2002.

KARDOSH A, BLUMENTHAL M, WANG WJ, CHEN TC, SCHONTHAL AH. Differential effects of selective COX-2 inhibitors on cell cycle regulation and proliferation of glioblastoma cell lines. *Cancer Biol Ther*. 1: 63-64, 2004.

KLEIHUES P, BURGER PC, SCHEITHAUER BW. The new WHO classification of brain tumours. *Brain pathol*, 3: 255-268, 1993.

KLEIHUES P & CAVENEE WK. Pathology and genetics of tumors of the nervous system. IARC Press, Lyon, 2000.

KULKARNI SK, JAIN NK, SINGH A. Cyclooxygenase isoenzymes and newer therapeutic potential for selective COX-2 inhibitors. *Methods Find Exp Clin Pharmacol*. 22: 291-298, 2000.

LIU Y, MARTINDALE JL, GOROSPE M, HOLBROOK NJ. Regulation of p21 WAF1/CIP1 expression through mitogen-activated protein kinase signaling pathway. *Cancer Res.* 56: 31-35, 1996.

LOH JK, HWANG SL, LINEU AS. The alteration of prostaglandin E2 levels in patients with brain tumors before and after tumor removal. *Journal of Neuro-Oncol*ogy 57: 147-150, 2002.

MAHER EA. Malignant glioma: genetics and biology of a grave matter. *Genes Dev.* 15: 1311-1333, 2001.

MAIENSCHEIN V, ZIMMERMANN H. Immunocytochemical localization of Ecto-5'-nucleotidase in cultures of cerebellar granule cells. *Neuroscience* 70: 429-438, 1996.

MANSOUR SJ, MATTEN WT, HERNANN AS, CANDIA JM, RONG S, FUKASAWA K, VANDEWOUDE GF, AHN NG. Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science* 265: 966-970, 1994.

MASTERS JRW. Human cancer cell lines: fact and fantasy. *Nature Ver Molec Cell Biol.* 99: 233-236, 2000.

MATSUO M, YANEMITSU N, ZAITSU M. Expression of prostaglandin H syntase-2 in human brain tumors. Acta Neuropathol. 102:181-187, 2001.

MAZERON JJ, KANTOR G. Radiotherapy in stereotactic conditions (radiosurgery) in maligmant brain tumors: clinical research. *Cancer Radiother*. 2: 215-217, 1998.

MERIGHI S, VARANI K, GENI S, CATTABRIGA V, LALLOTTA V, ULOUGLU C, LEUNG E, BOREA PA. Pharmacological and biochemical characterization of adenosine receptors in the human maligmant melanoma A375 cell line. *Br J. Pharmacol*. 34: 1215-1226, 2002a.

MERIGHI S, MIRANDOLA P. VARINI K, GESSI K, LEUNG E, BARALDI PG, TABRIZI MA, BOREA PA. A glance at adenosine receptors: novel target for antitumor therapy*. Pharmacol Ther*. 100: 31-48, 2003.

MORGAN DO. Priciples of CDK regulation. *Nature* 374: 131-134, 1995.

MORRONE FB, JACQUES-SILVA MC, HORN AP, BERNARDI A, SCHWARATSMANN G, RODNIGHT R, LENZ G. Extracellular nucleotides and nucleosides induce proliferation and increase nucleoside transport in human glioma cell lines. *J Neuro-Oncology* 64: 211-218, 2003.

MOUSSEAU M, CHAUVIN C, NISSOU MF, CHAFFANET N, PLANTAZ D, PASQUIER B, SCHAERER R, BENABID A. A study of tha expression of four chemoresistance-related genes in human primary and metastatic brain tumors. *Eu J Cancer* 29A: 753-759, 1993.

MULLER-DECKER K, REINERTH G, KRIEG P, ZIMMERMANN R, HEISE H, BAYERL C, MARKS F, FURSTENBERGER G. Prostaglandin-H-synthase isozyme expression in normal and neoplastic human skin. *Int J Cancer* 82: 648-656, 1999.

MURRAY AW, KIRSCHNER MW. Dominoes and clocks: The union of two views of the cell cycle. *Science* 246: 614-621, 1989.

MURPHY VJ, YANG Z, RORISON KA, BALDWIN GS, Cycloxygenase-2-selective antagonists do not inhibit growth of colorectal carcinoma cell lines. *Cancer Letters* 122: 25-30, 1998.

NAVARRO JM, OLMO N, TURNAY MT, LÓPEZ=CPNEJO, LIZRBE MA. Ecto-5'-Nucleotidase from a human colon adenocarcionoma cell line: correlation between enzyme activity and levels in intact cells. *Moll Cell Biochem*. 187: 121-131, 1998.

NEARY JT, RATHBONE MP, CATTABENI F, ABBRACCHIO MP, BURNSTOCK G. Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. *Trends Neurosci.* 19: 13-18, 1996.

NEARY JT, WHITTEMORE SR, ZHU Q, NORENBERG MD. Synergistic activation of DNA synthesis in astrocytes by fibroblast growth factor and extracellular ATP. *J. Neurochem.* 63: 490-494, 1994.

NGUYEN KD, LEE DA. *In vitro* avaluation of antiproliferative potential of topical cyclooxygenase inhibitors in human Tenon's fibroblasts. *Ext. Eye Res.* 57: 97-105, 1993.

OHANA G, BAR-YEHUDA S, BARER F, FISHMAN P. Differential effect of adenosine on tumor and normal cell growth: focus on the A3 adenosine receptor. *J. Cell Physiol.* 186: 19–23, 2001.

PAI R, SZAB IL, KAWANAKA H, SOREGHAN BA, JONES MK, TARNAWSK AS. Indomethacin inhibits endothelial cell proliferation by suppressing cell cycle proteins and PRB phosphorylation: a key to its antiangiogenic action. *Molecular Cell Biology Research Communications* 4: 111-116, 2000.

PARASHAR B, SHANKAR LS, O'GUIN K, BUTLER J, VIKRAN B, SHAFIT-ZAGARDO B. Inhibition of human neuroblastoma cell growth by CAY10404, a highly selective COX-2 inhibitor. *Journal of Neuro-Oncology* 71: 141-148, 2005.

PEYOT ML, GADEAU AP, DANDRE F, BELLOC I, DUPUCH F, DESGRANGES C. Extracellular adenosine induces apoptosis of human arterial smooth muscle cells via A(2b)-purinoceptor. *Circ. Res.* 86: 76-85, 2000.

PIAZZA GA, ALBERTS DS, HIXSON LI, PARANKA NS, LI H, FINN T, BORGET C, GUILLEN JM, BRENDEL K, GROSS PH. Sulindac sulfone inhibits azoxymethane-induced colon carcinogenesis in rats without reducing prostaglandin levels. *Cancer Res.* 57: 2909-2915, 1997.

PRAYSON RA, CASTILHA EA, VOGELBAUM MA. Cyclooxygenase-2 (COX-2) expression by imunohistochemistry in glioblastoma multiforme. *Ann Diag Pathol.* 6:148-153, 2002.

PUMGLIA KM & DECKER SJ. Cell cycle arrest mediated by the MEK/Mitogen-activated-protein kinase phatway. *Proc. Natl. Acad. Sci USA* 94: 448-452, 1997.

RALEVIC V & BURNSTOCK G. Receptors for purines and pyrimidines. *Pharmacol. Rev.* 50: 413-492, 1998.

RANG HP, DALE M M. Farmacologia , Guanabara Koogan, 4 ed., 2001.

RATHBONE MP, MIDDLEMISS PJ, KIM JK, GYSBERS JW, DEFORGE SP, SMITH RW, HUGHES DW. Adenosine and nucleotides stimulate proliferation of chick astrocytes and human astrocytoma cells. *Neurosci. Res.* 13: 1-17, 1992.

RATHBONE MP, MIDDLEMISS PJ, GYSBERS JW, ANDREW C, HERMAN MA, REED JK, CICCARELLI R, DI IORIO P, CACIAGLI F. Trophic effects of purines in neurons and glial cells. *Prog. Neurobiol*. 59: 663-690, 1999.

ROCHE S, FUMAGALLI S, COURTNEIDGE SA. Requirement for Src family protein tyrosine kinases in G2 for fibroblast cell division. *Science* 269: 1567–1569, 1995.

SAMUELSSON B, GRANSTROM E, GREEN K. Prostaglandins. *Ann Rev Biochem.* 44: 669-694, 1975.

SANO T, LIN H, CHEN X, LANGFORD LA, KOUL D, BONDY ML, HESS KR, MYERS JN, HONG YK, YUNG WK, STECK PA. Differential expression of MMAC/PTEN in glioblastoma multiforme: relationship to localization and prognosis. *Cancer Res.* 59: 1820-1824, 1999.

SCHELEGEL, J., PIONTEK, G., BUDDE, B., NEFF, F., KRAUS, A. The Akt/PKB-dependent anti-apoptotic pathway and the MAPK cascade are alternatively activated in human glioblastoma multiforme. *Cancer Letters* 158: 103-108, 2000.

SCHOEN SW, GRAEBER MB, TÓTH L, KREUTZBERG GW. 5'-Nucleotidase in posntatal ontogeny of rat cerebellum: a marker for migrating nerve cells? *Dev. Brain Res.* 39: 125-136, 1988.

SEGER R, KREBS EG. The MAPK signaling cascade. FASEB J 9: 726-735, 1995.

SHENG H, SHAO J, IRKLAND SC, ISAKSON P, COFFEY RJ, MORROW J, BEAUCHAMO RD, DU BOIS RN. Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2*. J. Clin. Invest*. 99: 2254-2259,1997.

SHERR CJ. G1 phase progression: cycling on cue*. Cell* 79: 551-555, 1994.

SHIRAHAMA T. Cyclooxygenase-2 expression is up-regulated in transitional cell carcinoma and its preneoplastic lesions in the human urinary bladder. *Clin Cancer Res* 6: 2424-2430, 2000.

SHONO T, TOFILON J, BRUNER M, OWOLABI O, LANG F. Cyclooxigenase-2 expression in human gliomas: prognostic significance and molecular correlations. *Cancer Res.* 61: 4375-4381, 2001.

SMALLEY W, DUBOIS RN. Colorectal cancer and nonsteroidal anti-inflamatory drugs. *Adv Pharmacol*. 39: 1-20, 1997.

SMITH ML, HAWCROFT G, HULL MA. The effect of nonsteroidal anti-inflamatory drugs on human colorectal cancer cells: evidence of different mechanims of action. *Eur. J. Cancer* 36: 664-674, 2000.

SPYCHALA J. Tumor-promoting functions of adenosine. *Pharmacol Ther.* 87: 161-173, 2000.

STANCIUM M, WANG Y, KENTOR R, BURKE N, WATKINS S, KRESS G, REYNOLDS I, KLANN E, ANGIOLOERI MR, JOHNSON JW, DEFRANCO DB. Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. *J Biol Chen.* 6: 12200-12206, 2000.

TAKAHASHI JA, SUZUKI H, YASUDA Y, ITO N, OHTA M, JAYE M, FUKUMOTO M, ODA Y, KIKUCHI H, HATANAKA M. Gene expresion of fibroblast growth factor receptors in the tissues of human gliomas and meningiomas. *Biophys. Res. Commun.* 177: 1-7, 1991.

TEGEDER I, PFEILSCHIFTER J, GEISSLINGER G.Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *FASEB J*. 15: 2057-2072, 2001.

THUN MJ, NAMBOODIRI MM, HEATH CW. Aspirin use and reduced risk of aftal colon cancer. *N Eng J Med*. 325: 1593-1596, 1991.

THUN MJ, HENLEY SJ, PATRONO C. Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic, and clinical issues. *J Natl Cancer Inst.* 94: 252-266, 2002.

TRIFAN OC, SMITH RM, THOMPSON BD, HLA T. Overexpression of cyclooxygenase-2 induces cell cycle arrest: evidence for a prostaglandin-independent mechanism. *J Biol Chem* 274: 34141-34147, 1999.

TRONICK SR, ARONSON AS. Growth factors and signal transduction. In: Mendelsohn J, Howlwy PM, Israel MA, Liotta LA (eds). The Molecular Basis of Cancer. Philadelphia: Saunders, p.117-140, 1995.

TSUJII M, DU BOIS RN. Alterations in celular adhesion and apoptosis in epihelial cells overexpressing prostaglandin endoperoxidase synthase 2. *Cell* 83: 493-501, 1995.

TSUJII M, KAWANO S, TSUJI S, SAWAOKA H, HORI M, DU BOIS RN. Cyclooxigenase regulates angiogenesis induced by colon cancer cells. *Cell* 93: 705-716, 1998.

TURNAY J, OLMO N, REENCE G, VON DER MARK, LIZARBE MA. 5'-Nucleotidase actrivity in cultured cell lines: effect of different assay conditions and correlation with cell proliferation. *In Vitro Cell Dev. Biol.* 25: 1055-1061, 1989.

UEDA Y, HIRAI S, OSADA S, SUZUKI A, MIZUNO K, OHNO S. Protein kinase C activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf. *J. Biol. Chem.* 271: 23512-23519, 1996.

UHM JH, DOOLEY NP, VILLEMURE JG, YONG VW. Glioma invasion *in vitro*: regulation by matrix metalloprotease-2 and protein kinase C. *Clin. Exp. Metastasis* 14: 421-433, 1996.

UJHAZY P, BERLETH ES, PIETKEWICZ JM, KITANO H, SKAAR JR, EHRKE MJ, MIHICH E. Evidence for the involvement of ecto-5'-nucleotidase (CD73) in drug resistance. *Int. J. Cancer* 68: 493-500, 1996.

VANE JR, BOTTINGG RM. New insights into the mode of action of anti-inflamatory drugs. *Inflamm Res*. 1-10, 1995.

VASCONCELOS EG, FERREIRA ST, CARVALHO TMU, ET AL. Partial purification and immunohistochemical localization of ATP Diphosphohydrolase from *Schistosoma mansoni. J. Biol. Chem.* 271: 22139-22145, 1996.

WECHTER WJ, MURRAY ED, KANTOCI D, QUIGGLE DD, LEIPOLD DD, GIBSON KM, MCCRACKEN JD. Treatment and survival study in the C57BL/6J-APC(Min)/+(Min) mouse with R-flurbiprofen*. Life Sciences* 66: 745-753, 2000.

WEINBERG RA. The retinoblastoma protein and cell cycle control. *Cell* 81: 323-330, 1995.

WEINSTEIN IB, CAROTHERS AM, SANTELLA RM. Molecular mechanisms of mutagenesis and multistage carcinogenesis. In: Mendelsohn J, Howlwy PM, Israel MA, Liotta LA (eds). The Molecular Basis of Cancer. Philadelphia: Saunders, p. 59-85, 1995.

WILLIAMS CS, WATSON AJM, SHENG H, HELOU R, SHAO J, DUBOIS RN. Celecoxib prevents tumor growth in vivo without toxicity to normal gut: lack of correlation between in vitro and in vivo models. *Cancer Res.* 60: 6045-6051, 2000.

WINK MR, LENZ G, BRAGANHOL E, TAMAJUSUKU ASK, SCHWARTSMANN G, SARKIS JJF, BATTASTINI AMO. Altered extracellular ATP, ADP and AMP catabolism in glioma cell lines. *Cancer Lett.* 198: 211-218, 2003.

WONG AJ, RUPPERT JM, BIGNER SH, GRZESCHIK CH, HUMPHREY PA, BIGNER DS, VOGELSTEIN B. Structural alterations of the epidermal growth

factor receptor gene in human gliomas. *Proc. Natl. Acad Sci USA* 89: 2965-2969, 1992.

YAMAMOTO Y, YIN MJ, LIN KM, GAYNOR RB. Sulindac inhibits activation of the NF-κB pathway. *J Biol Chem* 274: 27307-27314, 1999.

XIA Z, DICKENS M, RAINGEAUD J, DAVIS RJ, GREENBERG ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 270: 326-331, 1995.

XU M, ZHANG GY. Effect of indomethacin on cell cycle proteins in colon cancer cell lines. *World Journal Gastroenterology* 11911: 1692-1696, 2005.

ZHANG W, YAMADA H, SAKAI N, NIKAWA S, NOZAWA Y. Enhancement of radio-sensitivity by tamoxifen in C6 glioma cells. *Neurosurgery* 31: 125-130, 1992.

ZIMMERMANN H. 5'-Nucleotidase: molecular structure and functional aspects. *Biochem J.* 285: 345-365, 1992.

ZIMMERMANN H. Ectonucleotidases: some recent developments and a note on nomenclature. *Drug Dev. Res.* 52: 44-56, 2001.

ZIMMERMANN H. 5'-Nucleotidase: Molecular structure and functional aspects. *Biochem. J.* 285: 345-354, 2002.