UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

Investigação do efeito da doxazosina sobre linhagens de glioma humano (U-138MG) e de rato (C6)

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Orientadora: Dra. Christianne Gazzana Salbego

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do grau de **Mestre em Bioquímica.**

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DEDICO

Aos meus pais, minha irmã e meu esposo Eduardo.

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“Tenho a impressão de ter sido uma criança brincando à beira-mar, divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais bonita que as outras, enquanto o imenso oceano da verdade continua misterioso diante dos meus olhos.”

(Isaac Newton)

SUMÁRIO

RESUMO

Glioblastoma (GB) é o tumor cerebral maligno mais frequente. O prognóstico para os pacientes é ruim e a sobrevida média após o diagnóstico varia de 6 meses a 1 ano. Isso ocorre devido à ineficácia das estratégias terapêuticas dos tratamentos atuais, pois esses tipos de tumores são altamente invasivos. Deste modo, novas estratégias terapêuticas são necessárias. Neste contexto surge a doxazosina (2 - {4 - [(2,3-di-hidro-1,4-benzodioxano-2-il) carbonil] piperazina-1-il} -6,7-dimetoxiquinazolina-4-amina, um composto quinazolínico pertencente à classe farmacológica dos antagonistas dos receptores adrenérgicos α1, amplamente utilizada na clínica para o tratamento de pressão arterial elevada, assim como no tratamento de retenção urinária relacionado com a hiperplasia benigna da próstata (BPH). O presente estudo avaliou os efeitos do tratamento com doxazosina em modelos experimentais de gliomas humano (U-138MG) e de rato (C6). Observamos que a doxazosina foi capaz de inibir a viabilidade na linhagem celular de glioma de rato C6. Além disso, o fármaco permaneceu estável no meio de cultura após 48 horas de incubação e foi captado pelas células de glioma C6, não apresentando efeito tóxico sobre as células não tumorais (cultura organotípica e cultura primária de astrócitos) em concentrações inferiores a 250µM. Os resultados mostraram que doxazosina foi capaz de diminuir a densidade celular e induzir a morte celular em ambas as linhagens (U-138MG e C6). Além disso, o fármaco induziu a diminuição da fosforilação das proteínas Akt e GSK-3β em 24 e 48hs de tratamento. São vários os possíveis mecanismos que possam estar associados com a ação da doxasozina, dentre eles apoptose, necrose, senescência e/ou autofagia.

ABSTRACT

Glioblastoma (GB) is the most frequent and most malignant human brain tumor. The prognosis for the patients with GB remains dismal, as median survival after diagnosis varies from 6 month to 1 year. This is largely due to the inability of current treatment strategies to address the highly invasive nature of this disease. Thus, new therapeutic strategies are needed. Doxazosin (2-{4-[(2,3-dihydro-1,4-benzodioxin-2-yl)carbonyl]piperazin-1-yl}-6,7-dimethoxyquinazolin-4-amine, a quinazoline compound, is an selective α1-adrenoceptor antagonists, widely used for treatment of high blood pressure as well as in the treatment of urinary retention related with prostate benign hyperplasia (BPH). Doxazosin α1-adrenoceptor antagonists is a very promissing quinazoline drug, may represent chemical starting points to develop more potent death inducing agents free of α1-adrenoceptor antagonistic action and suitable for cancer treatment with minimal and well-tolerated side effects. Within this context, the present study was designed to evaluate the effects of doxazosin treatment in experimental models of gliomas. Doxazosin was able to viability inhibition of the C6 glioma cell line. Moreover, the drug seems to be stable in the culture medium after 48 hours of incubation and was taken up by C6 glioma cells and showed no toxic effect on non-tumor cells at concentrations below 250μM. The results showed that doxasozin was able to decrease cell density and induce cell death in both lineages. In addition, doxazosin induced the inactivation of Akt and of GSK-3β proteins after 24 and 48 hours. Taken together, our results show that doxazosin was able to significantly induce cells death of both, human and rat glioma lines (U-138MG and C6 respectively). The mechanisms associated with this effect involve apoptosis induction, necrosis, senescence or autophagy.

LISTA DE FIGURAS

TABELA

LISTA DE ABREVIATURAS

AA – Astrocitomas anaplásicos

AKT/PKB - Proteína cinase B (*Protein Kinase B*)

BHE - Barreira hemato-encefálica

CDK – Cinase dependente de ciclina (*Cyclin-Dependent kinase*)

CDKI - Inibidora de cinase dependente de ciclina (*Cyclin-Dependent kinase Inhibitor*)

CSCs - Células tronco tumorais

DNA – Ácido desoxirribonucleico

EGFR - Receptor de fator de crescimento endotelial (*Endothelial Growth Factor Receptor*)

ERK - Proteína cinase regulada por sinais extracelulares (*Extra cellular Signal-Regulated kinase*)

FasL- Ligante de Fas

GB - Glioblastoma

GBM - Glioblastoma multiforme

GSK-3β - Glicogênio sintase cinase-3 (*Glycogen Sinthase Kinase-3β*)

HIF - Fator induzível de hipóxia

INCA - Instituto Nacional do Câncer

LDL - Lipoproteína de baixa densidade

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

MTIC - 5-(3-metiltriazeno-1-il)imidazol-4-carboxamida

NFkB - Fator nuclear de kappa B

NIH – *National Institutes of Health*

OMS - Organização Mundial da Saúde

PDGFRA- Receptor do fator de crescimento derivado de plaquetas (*Platelet-derived growth factor receptors*)

PDK1 - Cinase dependente de Fosfoinositol (*Phosphoinositide-dependent kinase-1*)

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

PIP2 - Fosfatidilinositol-4,5-bifosfato

PIP3 – Fosfatidilinositol-3,4,5-trifosfato (*Phosphatidylinositol-3,4,5-trisphosphate*)

PTEN - Homólogo fosfatase e tensina deletado do cromossomo 10 (*Phosphatase and tensin homologue deleted from chromossome 10*)

Raf – *Rapidly Accelerated Fibrosarcoma protein*

Ras - *Rat sarcoma protein*

RB - Retinoblastoma

RNAi - Ácido ribonucleico de interferência

RTK - Receptores de tirosina cinases (*Receptor Tyrosine Kinase*)

rTNF - Receptores de fatores de necrose tumoral

SNC - Sistema Nervoso Central

Src - Fosforilação de resíduos de tirosina

TCGA – *Cancer Genome Atlas*

TIC - Células de Iniciação do Tumor

TMZ - Temozolomida

TNFa - Fator de necrose tumoral

TRAIL - Ligante indutor de apoptose relacionado ao TNF

1. INTRODUÇÃO

1.1. Gliomas (Classificação)

Gliomas são os tumores primários mais frequentes que acometem o Sistema Nervoso Central representando mais de 30% de todos os tumores primários e 80% dos tumores malignos do Sistema Nervoso Central (SNC). Cerca de 60% deles ocorrem nos quatro lóbulos do cérebro (Huse et al., 2011; CBTRUS, 2012).

São classificados, de acordo com a Organização Mundial da Saúde (OMS), de 2007, conforme três critérios principais: histopatológico, grau de malignidade (variando de I à IV – quanto maior o grau mais maligno) e conforme a localização do tumor no cérebro (Holland, 2000; Louis et al., 2007; Huse et al., 2012).

1.1.1. Classificação Conforme Critérios Histopatológicos

A análise histológica considera, basicamente, a presença de anaplasia tumoral (atipia nuclear, pleomorfismo celular, atividade mitótica, hiperplasia endotelial e necrose) (Holland, 2011; Huse et al., 2012), apresentando características de astrócitos (astrocitomas), oligodendrócitos (oligodendrogliomas), astrócitos e oligodendrócitos (mistos ou oligoastrocitomas) ou de células ependimais (ependimomas) (Kleihues e Ohgaki, 1999; Bredel, 2001). A distribuição histopatológica dos gliomas está ilustrada na Figura 1 (para revisão CBTRUS, 2012).

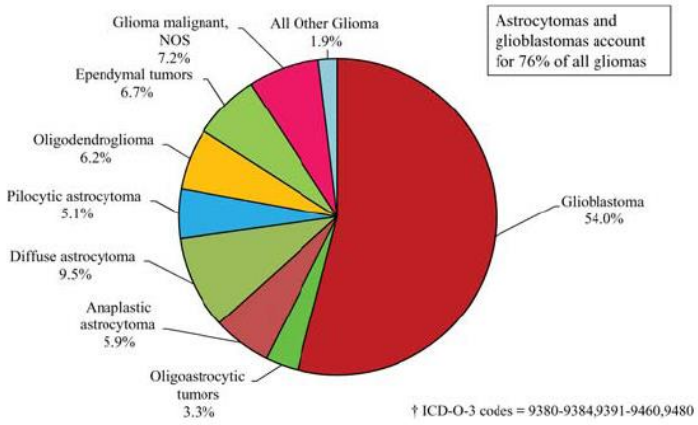

Figura 1: Classificação histológica dos tumores primários do SNC- Gliomas (N= 90,828). Obtido de: CBTRUS, 2012.

1.1.1.1. Astrocitomas

São sub-classificados em:

1.1.1.1.1. Astrocitomas de Baixo Grau

Astrocitomas caracterizam-se por apresentar recorrências tardias, motivo pelo qual os pacientes devem ser acompanhados por, pelo menos, 15 anos (Huse et al., 2012). A maioria destes pode evoluir para lesões mais anaplásicas e não são curados através de cirurgia e/ou radioterapia. As características favoráveis para o prognóstico incluem a idade (sendo que, quanto mais jovem, melhor) e o tamanho do tumor (<5cm) (http://atlasgeneticsoncology.org).

1.1.1.1.2. Astrocitomas Anaplásicos

Astrocitomas anaplásicos (AA) compreendem 5,9% de todas as neoplasias gliais. Atualmente, os únicos fatores que têm sido utilizados no prognóstico de

pacientes com AA são a idade e o índice de Karnofsky[1] (Huse et al., 2012; CBTRUS 2012).

1.1.1.1.3. Glioblastoma (GB)

Anteriormente chamado glioblastoma multiforme (GBM), representa o mais agressivo e maligno tumor primário cerebral (Huse et al., 2012). São altamente infiltrativos, difusos e multifocais (Kleihues et al., 1999; Jackson et al., 2001; Holland, 2012; Lima et al., 2012). Apresentam necrose endotelial, alta taxa prolifarativa e uma elevada densidade de células atípicas (Huse et al., 2012). Os pacientes com GB exibem uma sobrevida média de apenas 14,6 meses após o diagnóstico, mesmo depois de tratamentos multimodais (Lima et al., 2012). Constitui a segunda neoplasia maligna mais comum, representando 16% de todos os tumores primários cerebrais (CBTRUS, 2012). A célula de origem para formação de glioblastomas ainda não está esclarecida. A teoria mais aceita sustenta que tais tumores têm origem a partir de células progenitoras que sofrem vários eventos de transformação durante o desenvolvimento, de modo que estas células são as iniciadoras do tumor (Bao et al., 2006; Eramo et al., 2006; Visvader e Lindeman, 2008; Van Meiret et al., 2010; Schepers et al., 2012). O termo “multiforme” (GBM - gliomas grau IV) foi descrito assim por apresentar uma grande variabilidade histológica, bem como heterogeneidade molecular, levando a um diagnóstico e prognóstico ruins da doença (Wen et al., 2006).

Outros mecanismos, incluindo a desregulação de enzimas celulares e proteínas transportadoras de membranas, aberrações genômicas e alterações da

[1]Índice de Karnofsky: escala de resultados ou índice de Karnofsky classifica os pacientes de acordo com o grau de suas inaptidões ou deficiências funcionais. Pode ser utilizado para comparar a efetividade de diferentes terapias e para permitir o prognóstico de pacientes individuais. Quanto menor a classificação na escala, pior a expectativa de recuperação de enfermidades ou retorno às atividades normais (http://www.farmabrasilis.org.br).

susceptibilidade a apoptose, podem ser responsáveis pela alta incidência de quimio-resistência em pacientes com glioblastoma (GB) (Bredel, 2011; Yu et al., 2012).

Alterações genéticas são frequentes nos GBs, incluindo mutações de perda de função ou silenciamento das proteínas p53, p16, Rb e PTEN e mutações de ganho de funções como a amplificação do gene EGFR. Isso resulta em uma desregulação de vias de sinalização intracelulares que acabam levando a um aumento da proliferação, sobrevivência, invasão e angiogênese tecidual (Rasheed et al., 1999; Holland, 2000; Rao et al., 2000; Maher et al., 2001; Wechsler-Reya et al., 2001; Zhu et al., 2002; Ghosh et al., 2005).

A supressão espontânea da apoptose e desregulação da divisão celular constituem nas propriedades mais críticas que uma célula somática adquire no decurso da sua transformação em neoplásica.

Existem pelo menos quatro razões que contribuem para a resistência dos glioblastomas à terapia:

(1) o cérebro é um órgão diferenciado, com baixa ou ausente renovação celular, o que impede a aplicação de terapias direcionadas a essa via (Westphal e Lamszus, 2011);

(2) a massa tumoral muitas vezes pode estar localizada e invadir áreas cerebrais funcionais inacessíveis, impossibilitando a ressecção cirurgica (tumores infiltrativos), sem que sejam afetadas as atividades motoras e/ou os processos cognitivos, comprometendo de forma inequívoca a qualidade de vida do paciente (Sanai e Berger, 2008);

(3) a barreira hemato-encefálica (BHE) protege o sistema nervoso central e evita que muitos quimioterápicos administrados sistemicamente cheguem ao local do tumor. Por este motivo, muitas estratégias quimioterapêuticas que têm se

mostrado eficazes em outras malignidades não podem ser aplicáveis no tratamento de tumores cerebrais primários (Westphal e Lamszus, 2011). Assim, a investigação de fármacos inovadores no desenvolvimento de novas drogas com potencial terapêutico para o tratamento de tumores de sistema nervoso central se faz necessária;

(4) os glioblastomas são caracterizados por apresentarem uma variedade de anormalidades genéticas.

Esta heterogeneidade pode constituir um desafio terapêutico, porque as células que carregam anormalidades diferentes podem responder de forma diferente à terapia.

O uso generalizado da tecnologia de DNA *microarray* facilitou a identificação de subclasses moleculares em doenças aparentemente “uniformes”.

Estudos iniciais em gliomas malignos demonstraram que as assinaturas transcricionais conseguem distinguir de maneira eficaz as subpopulações de GB além de identificar vários tipos de genes cujos níveis de expressão podem ser correlacionados com o prognóstico (Fuller et al., 1999; Sallinen et al., 2000; Rickman et al., 2001; Fuller et al., 2002; Kim et al., 2002; Tanwar et al., 2002; Shai et al., 2003; Huse et al., 2012).

Um esforço inédito, patrocinado pelo National Institutes of Health (NIH), Rockville, foi desenvolvido para entender o câncer através da integração de dados de expressão e de perfis genéticos.

O Cancer Genome Atlas (TCGA) é o instrumento de integração de dados de expressão e de perfis gênicos, que cataloga os padrões moleculares e integra todo o espectro de anormalidades vistos em GB (The Cancer Genome Atlas Research Network, 2008; http://TCGA.cancer.gov; Huse et al., 2011; Huse et al., 2012). Esta

avaliação explora as bases moleculares das distintas subclasses em GB: além de considerar a sua importância na patogênese da doença e no desenvolvimento terapêutico, que poderiam ser melhores avaliados por testes pré-clínicos e adequadamente concebidos nos ensaios clínicos.

De acordo com essas análises, os glioblastomas se classificam em quatro subclasses moleculares: proneural, neural, clássica e mesenquimal (Huse et al., 2012).

Essas subclasses exibem correlações genômico definidas e de anormalidades epigenômicas.

1.1.1.2. Oligodendrogliomas

Sub-classificados em:

1.1.1.2.1. Mistos

De baixo grau / Oligodendrogliomas / Oligoastrocitomas. Representam 3,3% dos gliomas. Cerca da metade dos oligodendrogliomas mistos são caracterizados por perda de heterozigosidade nos cromossomas 1p e 19q, uma característica patogenômica do diagnóstico (CBTRUS, 2012; http://atlasgeneticsoncology.org; Huse et al., 2012).

1.1.1.2.2. Oligodendroglioma Anaplásico

Embora incomuns, os tumores oligodendrogliomas anaplásicos são reconhecidos por suas exclusivas características moleculares, histológicas e clínicas. A radioterapia é o procedimento normalmente prescrito, assim como a terapia pós-cirúrgica (http://atlasgeneticsoncology.org; Holland, 2012).

1.1.1.3. Ependinomas de Baixo Grau / Ependinomas Anaplásicos

Constituem 6,7% dos gliomas. Ependinomas anaplásicos podem ocorrer em qualquer lugar do eixo da coluna vertebral, sendo comum o diagnóstico em crianças, na fossa posterior e medula espinhal. Tanto as lesões de baixo grau, como o anaplásico, podem difundir ao longo das superfícies nas meninges. Lesões de baixo grau na coluna vertebral são geralmente tratados apenas com cirurgia (CBTRUS, 2012).

1.1.2. Classificação conforme o Grau de Malignidade

Grau I - constituem lesões não-infiltrativas, sem atipias nucleares, mitoses, proliferação endotelial ou necrose. Atribui-se aos tumores mais circunscritos com potencial proliferativo lento (Westphal e Lamszus, 2011). As lesões de grau I são benignas e comuns em crianças (astrocitoma pilocítico).

Grau II - definem àqueles difusamente infiltrativos, com atipias nucleares e baixo índice mitótico, com uma taxa de crescimento lento e um alto grau de diferenciação celular sem proliferação celular endotelial ou necrose.

Grau III - são anaplásicos, infiltrativos, possuem uma variedade de atipias celulares, vasos sanguíneos e densidade celular, além de apresentarem atividade mitótica (Westphal e Lamszus, 2011). Compreendem os astrocitomas anaplásicos, oligodendrogliomas anaplásicos e oligoastrocitomas anaplásicos (Louis et al., 2007).

Grau IV - lesões citologicamente malignas, mitoticamente ativas, propensas à necrose. Apresentam rápida evolução pré e pós-operatória, levando a resultados fatais. Compreendem o glioblastoma (GB) e constituem os mais malignos e

agressivos tumores primários cerebrais (Lima et al., 2012; http://atlasgeneticsoncology.org).

1.1.3. Classificação conforme a Localização Tumoral

- Supratentoriais (acima do *tentorium*[2], no cérebro): correspondem 70% dos gliomas em adultos;

- Infratentorial (abaixo do *tentorium*[2], cerebelo): correspondem 70% dos gliomas em pacientes pediátricos.

1.2. Diagnóstico e Terapêutica

A avaliação inicial do paciente com tumor cerebral compreende exame clínico e neurológico detalhados, além de exames de neuroimagem. O estadiamento tumoral é diagnosticado minimamente por tomografia axial computadorizada contrastada, complementada por ressonância magnética e espectroscópica (http://www.rb.org.br).

O diagnóstico definitivo é firmado pelo estudo histopatológico de espécimes tumorais, obtido por biópsia estereotóxica ou a céu aberto, sendo essencial para o planejamento terapêutico (www.inca.gov.br).

Os sintomas clínicos podem incluir dores de cabeça progressivas, déficits neurológicos focais e convulsões (Tanwar et al., 2002).

A terapia inicial compreende a ressecção do tumor por meio de cirurgia. No entanto, o sucesso da mesma está comprometido pela alta capacidade de invasão das células tumorais no tecido normal circundante (Jackson et al., 2001). Dessa

[2]*Tentorium* denomina-se uma estrutura membranosa que separa o cérebro do cerebelo.

forma, o pós-operatório segue com radioterapia e tratamento concomitante com quimioterapia.

A quimioterapia para tumores cerebrais costuma apresentar os mesmos problemas que a quimioterapia para câncer sistêmico, incluindo falta de especificidade, a resistência intrínseca ou desenvolvida das células malignas, toxicidade sistêmica e intolerância do tecido não tumoral a toxicidade da droga. Além disso, o SNC é protegido por uma barreira hematoencefálica (BHE) que limita o acesso aos quimioterápicos. Regimes terapêuticos contendo nitrosuréias (carmustina ou lomustina), alquilantes do DNA (procarbazina, dacarbazina ou temozolomida) derivados da platina (cisplatina ou carboplatina), vincristina, teniposídeo, hidroxiuréia, cloroquina, bevacizumabe e irinotecano mostraram-se úteis no tratamento paliativo de gliomas cerebrais nos graus III ou IV, que, em geral, são administrados concomitantemente à radioterapia (Wick et al., 2011; Yu et al., 2012; http://portal.saude.gov.br).

A temozolomida pertence à classe dos quimioterápicos alquilantes do DNA, sendo um derivado da imidazotetrazina. Mesmo sendo um medicamento clássico para o tratamento de glioblastomas (TMZ, 75 mg/m^2), a sua ação terapêutica depende da habilidade de alquilar/metilar o DNA (Wick et al., 2011; Yu et al., 2012). Administrado por via oral, trata-se de um pró-fármaco, ou seja, apresenta atividade farmacológica quando hidrolisada *in vivo* para MTIC (5-(3-metiltriazeno-1-il)imidazol-4-carboxamida). O MTIC é quem possui a atividade citotóxica, agindo como agente alquilante (www.inca.gov.br; Najman e Gadelha, 2002).

A metilação, causada por esse agente, induz dano ao DNA e leva a célula à morte, porém a superexpressão da enzima de reparo nos glioblastomas (O-6-metilguanina-DNA-metiltransferase (MGMT) pode diminuir a eficácia terapêutica da

TMZ. Os pacientes, quando utilizando o quimioterápico, possuem uma sobrevida média de aproximadamente 15 meses (Riemenschneider et al., 2010; Yu et al., 2012).

1.3. Doxazosina

A doxazosina [4 - (4-amino-6,7-dimetoxiquinazolina-2-il)-piperazina] 1-il - (2,3-diidro-1,4-benzodioxina-3-il) metanona é um derivado quinazolínico que compreende a classe terapêutica dos alfa-bloqueadores adrenérgicos. São fármacos que bloqueiam seletivamente os receptores alfa1 adrenérgicos (Kirby, 1995; Hansson et al., 1998; Pool e Kirby, 2001; Ito et al., 2007).

É utilizada na clínica como Mesilato de doxazosina (Cardura®) para o tratamento de hipertensão, insuficiência cardíaca congestiva e hiperplasia benigna de próstata induzindo a apoptose de células prostáticas malignas através de um mecanismo alternativo ao relacionado com o adrenoceptor alfa-1 (Rasheed et al., 1999; Holland, 2000). A doxazosina também exerce efeitos pró-apoptóticos em células de câncer de mama e câncer de bexiga (Maher et al., 2001; Wechsler-Reya e Scott, 2001). Sua ação anti-hipertensiva está associada à sua capacidade de se ligar a receptores alfa-1adrenérgicos, mas o(s) mecanismo(s) de suas ações anti-proliferativas e pró-apoptóticas em células cancerosas ainda não são bem compreendidas (Jackisch, 2006).

Por apresentar em sua estrutura química o anel quinazolínico (figura 2), a doxazosina pode ser útil como ponto de partida para a síntese de compostos que tenham atividade biológica como inibidores do receptor de tirosina-quinases. Esta ação tem por base a ocupação de sítios de acoplamento do ATP por esses compostos, devido à semelhança na estrutura química, provocando a inibição da

atividade cinásica (Goldstein, et al., 2008). Nos últimos anos, os derivados de quinazolinas têm sido utilizados no combate de doenças proliferativas como câncer, devido a sua atividade inibidora de receptores tirosina cinase (Goldstein et al., 2008; Oliveira, 2009).

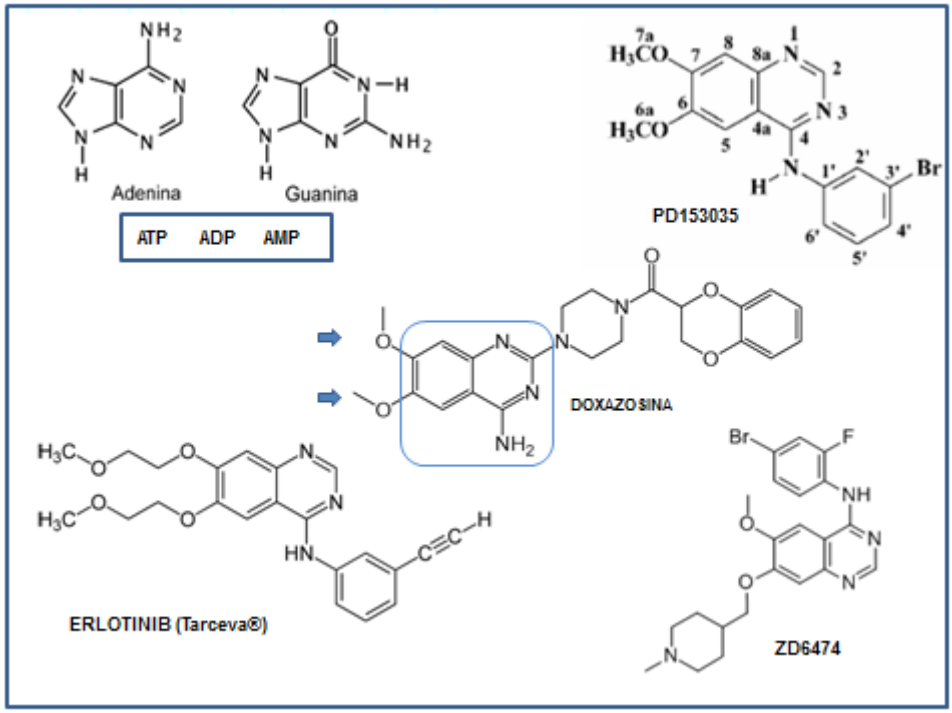

Figura 2. No centro, a estrutura química da doxazosina [4 - 4-amino-6 ,7-dimetoxiquinazolina-2-il)-piperazina] 1-il - (2,3-diidro-1,4-benzodioxina-3-il). A parte demarcada em azul corresponde ao anel quinazolínico e as setas correspondem aos grupos doadores de elétrons. As demais estruturas químicas correspondem a análogos parciais da doxazosina. PD153035: inibidor do receptor de tirosina cinase.

1.4. Vias de sinalização alteradas em glioblastomas

As alterações genéticas em glioblastoma ocorrem com frequência em três vias de sinalização celular: (a) via receptor tirosina-quinase (RTK), RAS, e fosfoinositol-3-quinase (PI3K), (b) via da p53, e (c) via Rb (retinoblastoma-supressor tumoral), conforme ilustrado na Figura 3 abaixo.

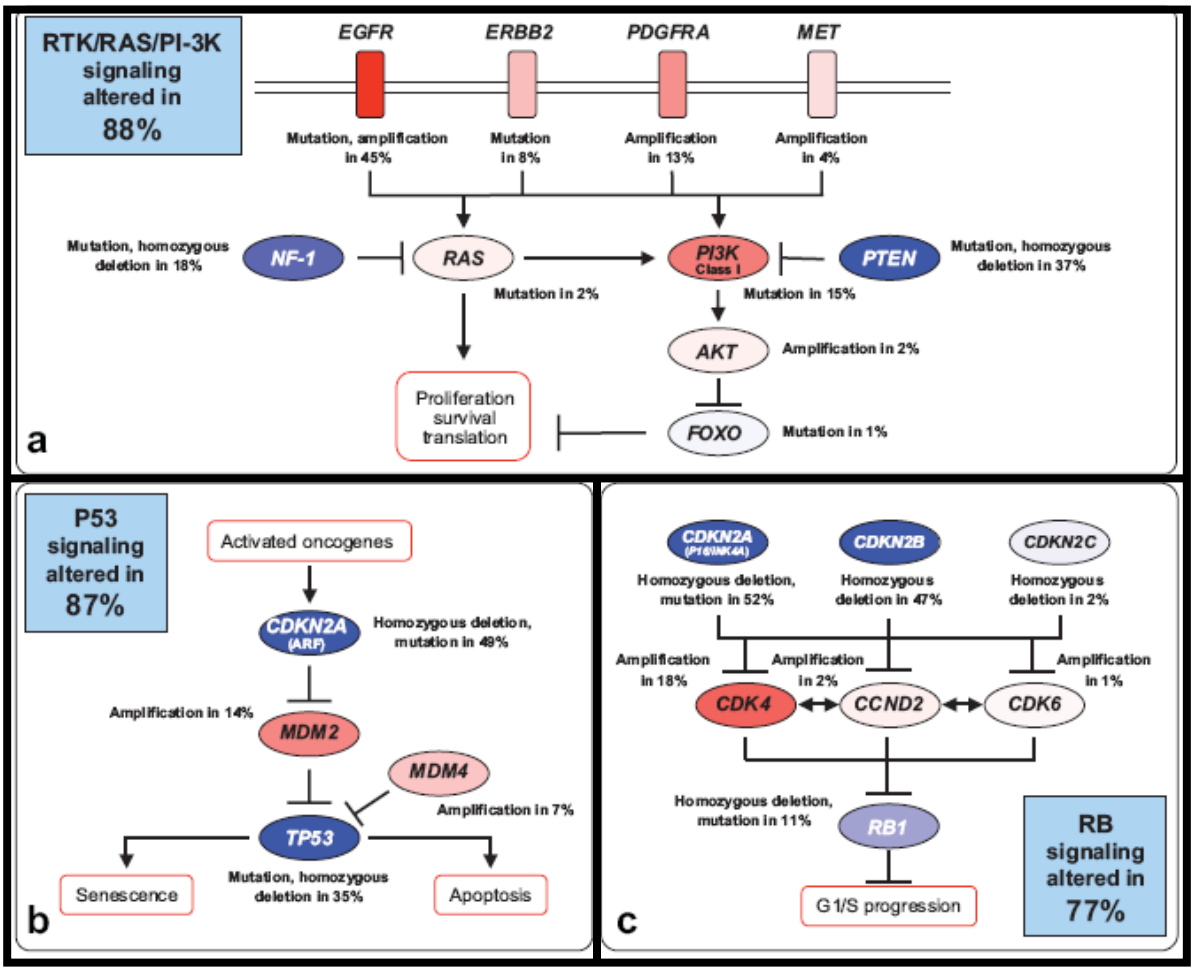

Figura 3. As alterações no DNA e as mudanças no número de cópias nas seguintes vias de sinalização estão indicadas em (a) receptor tirosina-quinase (RTK), RAS, e fosfoinositol-3-quinase (PI3K), (b) supressor de tumoral p53, e (c) supressor de tumores retinoblastoma (Rb). As alterações genéticas são mostradas em vermelho (*upregulation*) e as que conduzem a uma perda de função são indicadas em azul. Podemos observar que em cada via, mostra os componentes alterados, o tipo de alteração e a percentagem de tumores que transportam cada alteração. As caixas azuis contêm as percentagens totais de glioblastomas com alterações em pelo menos um gene conhecido da via designada. Obtido, com adaptações, de The Cancer Genome Atlas Research Network, 2008.

1.4.1. Receptor tirosina-quinase (RTK)

Entre os receptores tirosina cinases (RTK- Receptor TyrosineKinase) conhecidos, compreende a família ErbB, a qual inclui quatro receptores: EGFR / erbB-1/HER1, erbB-2 (HER2), erbB-3 (HER3), e erbB-4 (HER4) (Olayioye et al., 2000; Mastalerz et al., 2007; Chilin et al., 2010). A superexpressão (e/ou a co-expressão do EGFR e HER2) foi encontrada em alguns tipos de tumores, incluindo os de cólon, mama, de ovários, cabeça e pescoço.

A amplificação do EGFR ocorre em 40% dos glioblastomas primários, mas raramente em glioblastomas secundários (Rhenen et al., 2005; Smalley et al., 2012).

1.4.2. Via RTK/RAS e PI3K

EGFR torna-se ativado por meio da ligação de fatores de crescimento em seu domínio extracelular, através de homo ou heterodimerização com outros receptores levando a eventos de fosforilação. Sua ativação ativa principalmente duas vias de sinalização: via da Ras (Raf/MEK/ERK) e via da PI3K/Akt (PKB) (Kraskstad et al., 2010).

1.4.2.1. Via da RAS

A Ras é uma proteína G monomérica, pertencente à família das Proteínas Cinases ativadas por mitógenos (MAPK), cujas respostas desencadeadas pela via estão relacionadas com a progressão do ciclo celular, a transcrição de genes, a sobrevivência tumoral, a proliferação celular e a reorganização do citoesqueleto (Malumbres e Barbacid, 2003).

A ativação via fosforilação da Ras inicia uma cascata de sinalização através das MAPKs e ERK-1 e 2, cinases que irão ativar alvos citoplasmáticos como p90RSK. Esta serina/treonina cinase transloca para o núcleo onde ativa os fatores de transcrição, como por exemplo o CREB, que regulam a sobrevivência e proliferação de células de glioma (Sturgill et al., 1988; Sozeri et al., 1992; Moodie et al., 1993; Krakstad e Chekenya, 2010).

1.4.2.2. Via PI3K

A PI3K é translocada para a membrana plasmática por meio da ligação de fosfotirosina a resíduos dos RTKS. A PI3K fosforila o PIP2 (fosfatidilinositol-4,5-bifosfato) à PIP3 (fosfatidilinositol-3,4,5-trifosfato), este, por sua vez, funciona como um importante sinalizador intracelular coordenando processos de crescimento celular, migração, metabolismo e regulação do ciclo celular (Feldkamp et al., 1999; Krakstad e Chekenya, 2010). O acúmulo de PIP3 recruta a PDK1 (*Phosphoinositide-Dependent Kinase 1*) e Akt/PKB (Protein Kinase B) à membrana plasmática. Akt é ativada através da fosforilação de pelo menos dois dos principais sítios regulatórios, Thr_{308} (por PDK1-(*3-Phosphoinositide-Dependent Kinase-1*) e Ser_{473} (por mTORC2-mTOR-Rictor KinaseComplex 2) (Guha et al., 1997; Feldkamp et al., 1999; Krakstad e Chekenya, 2010). A Akt é responsável pela fosforilação de vários substratos citosólicos e nucleares que regulam o metabolismo e o crescimento celular. Durante a sinalização da insulina, a Akt fosforila a Glicogênio Sintase Cinase 3β (GSK3β), fosfofrutoquinase e mTOR (*mammalian target of rapamycin*) para induzir glicogênese e síntese protéica. A Akt está relacionada com a fosforilação de proteínas que regulam a apoptose (Bad, caspase-9, MDM2, p21Waf1/Cip1, FOXO1 e GSK3β) e com a proliferação e sobrevivência celular (Gersbert et al., 2000). A GSK3β é um dos substratos da Akt. Está envolvida na regulação de várias funções celulares, incluindo diferenciação, crescimento, motilidade, proliferação, progressão do ciclo celular, apoptose e responsa à insulina. A sua atividade pode ser reduzida por fosforilação (Ser-9). Várias cinases são capazes de mediar essa modificação, incluindo p70S6, p90RSK, PKC e Akt (PKB). A desregulação da expressão da GSK3β conduz a muitos estados patplógicos: diabetes, doença de Alzeimer, Parkinson, distúrbios bipolares e câncer. A ativação da GSK3β (quando

defosforilada – Ser-9) promove parada no ciclo celular e encaminhamento para apoptose.

A sobrevida média de pacientes com GBM, que tem ativa a via da PI3K (n = 42/56) e Akt (37/56), é de 11 meses em comparação aos pacientes com níveis mais baixos de ativação de PI3K e Akt, que foi de 40 meses. Este fato é significativamente mais frequente em pacientes com GB do que outros tipos de câncer (Chakravarti et al., 2004; Krakstad e Chekenya, 2010).

1.4.2.3 Via da p53

O gene TP53 codifica uma proteína de 53kD, que desempenha um papel em vários processos celulares, incluindo regulação do ciclo celular, respostas à danos no DNA, morte celular, diferenciação celular e neovascularização. A proteína supressora tumoral p53 apresenta-se mutada em 87% dos glioblastomas. A p53 induz parada no ciclo celular, podendo recrutar as proteínas de reparo do DNA ou encaminhar à morte celular após uma injúria (dano ao DNA). Esse mecanismo é responsável pelo *turnover* das células presentes nos tecidos e órgãos do nosso corpo, ao passo que nos animais que perdem p53 ocorre o desenvolvimento de tumores ao longo da vida (Brenner e Mak, 2009; Guicciardi e Gores, 2009; Krakstad e Chekenya, 2010).

1.5. Apoptose e Tumorogênese

Os processos de morte celular podem ser classificados de acordo com suas características morfológicas e bioquímicas em: apoptose, autofagia, necrose, catástrofe mitótica e senescência. Segue em anexo tabela que caracteriza os tipos

de morte celular (Castedo et al., 2004; Okada et al., 2004; Dimri, 2005; Grivicich, Regner e Rocha, 2007; Huse et al., 2011).

Alterações na coordenação desses tipos de morte celular estão implicadas na tumorogênese (Holland, 2000).

Apoptose, ou morte celular programada, é um processo essencial para a manutenção do desenvolvimento dos seres vivos, sendo importante para eliminar células em desuso ou defeituosas. Durante a apoptose, a célula sofre alterações morfológicas, tais alterações incluem: a retração da célula, perda de aderência com a matriz extracelular e células vizinhas, condensação da cromatina, fragmentação internucleossômica do DNA e formação dos corpos apoptóticos (Locksley et al., 2001; Aggarwal, 2003; Krakstad e Chekenya, 2010).

Estas alterações são consequência de uma cascata de eventos moleculares e bioquímicos específicos e geneticamente regulados (Jackson et al., 2001).

Apoptose depende da ativação de diferentes vias de sinalização que são, muitas vezes, desreguladas em diversos tipos de câncer. Diversos são os fatores que podem desencadear a apoptose, entre eles: ligação de moléculas a receptores de membrana, agentes quimioterápicos, radiação ionizante, danos ao DNA, choque térmico, privação de fatores de crescimento, baixa quantidade de nutrientes e níveis aumentados de espécies reativas do oxigênio (Chen et al., 1997; Wechsler-Reya et al., 2001; Bellail et al., 2009; Krakstad e Chekenya, 2010).

Tabela 1. Comparação das alterações dos diferentes tipos de morte celular.

Caracterísitca	Apoptose	Necrose	Autofagia	Catástrofe Mitótica	Senescência
Quantidade de células afetadas	Uma célula ou um pequeno grupo	Em geral muitas células contíguas	Pode-se encontrar em muitas células, contíguas ou não (pela liberação de produtos da autodigestão)	Uma célula ou um pequeno grupo	Pode-se encontrar em muitas células, contíguas ou não.
Morfologia geral da célula	Encolhimento celular e convolução	Turgescência celular	Aumento da granulosidade celular, sem padrão de alteração no tamanho	Células gigantes com diversos núcleos	Aumento de tamanho celular
Alterações nucleares	Picnose e cariorexis	Cariólise, picnose e cariorexis	Sem condensação da cromatina	Micronucleação e multinucleação; arranjos cromossômicos aberrantes	Focos associados a heterocromatina
Alterações da membrana	Membrana celular intacta	Ruptura da membrana celular	Ruptura e fragmentação da membrana no final do processo	Membrana celular intacta inicialmente, podendo ou não haver fragmentação no final do processo	Membrana celular intacta
Alterações no citoplasma	Retenção do citoplasma em corpos apoptóticos	Vacuolização e degeneração organelar, seguido de liberação do citoplasma no meio extracelular	Maciça vácuolização do citoplasma (vesículas autofágicas com membrana dupla)	Sem alterações significativas no citoplasma e seus componentes	Achatamento celular, aumento da granulosidade e acidificação do citoplasma;
Resposta imune	Sem resposta inflamatória	Resposta inflamatória	Sem resposta inflamatória	Sem resposta inflamatória	Sem resposta inflamatória
Gasto energético	Com gasto de energia	Sem gasto de energia	Com gasto de energia	Sem gasto de energia	Sem gasto de energia

Obtido de: tese de doutorado de Lauren Lúcia Zamin (“Resveratrol e quersetina: avaliação da atividade antitumoral e dos mecanismos de ação em linhagens de glioma *in vitro* em um modelo de implante de gliomas *in vivo*”)

2. OBJETIVO

2.1. Objetivo geral:

Avaliar os efeitos da doxazosina sobre as linhagens de glioma humano (U-138MG) e de rato (C6).

2.2. Objetivos específicos:

2.2.1. Verificar, através de revisão bibliográfica, os possíveis efeitos biológicos da doxazosina por meio de uma avaliação de sua relação estrutura atividade (REA) com compostos já sintetizados e em fase de desenvolvimento, relacionando, assim, com suas possíveis ações, principalmente com seu potencial antitumoral e reconhecimento de prováveis sítios de ligação**.**

2.2.2. Investigar o efeito da doxazosina sobre a viabilidade celular em modelo de glioma de rato C6. E, desse modo, padronizar as diferentes concentrações do fármaco através de ensaio de viabilidade. Avaliar a estabilidade a doxazosina no meio de cultivo, bem como verificar se ocorre a captação do fármaco pelas células.

2.2.3. Avaliar a citotoxicidade da doxasozina frente a modelos não tumorais: em células (cultura primária de astrócitos) e de fatias de tecido cerebral de ratos (cultura organotípica).

2.2.4. Analisar o efeito antitumoral da doxasozina, já com as doses padronizadas, sobre das linhagens de glioma humano (U-138MG) e de rato (C6).

Avaliar a densidade celular, proliferação e morte celular, após tratamento de 48 horas em ambas as linhagens. Verificar a presença de mucinas ácidas e os possíveis efeitos na via de sinalização PI3K/Akt. Fazer a análise do imunoconteúdo das proteínas Akt e GSK-3β, após o tratamento de 30 minutos, 24 e 48 horas com doxazosina em modelo de glioma humano U-138MG.

3. RESULTADOS

Os resultados estão organizados em três artigos científicos a serem submetidos a periódicos internacionais.

3.1. Revisão bibliográfica e estudo da relação estrutura-atividade da doxazosina a ser submetido para publicação ao Biochemical Pharmacology.

Doxazosin, quinazoline α1-adrenoceptor antagonist: a powerful antitumoral drug.

Doxazosin, a quinazoline α1-adrenoceptor antagonist : a powerful antitumoral drug

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Abstract

Doxazosin α1-adrenoceptor antagonist is a very promising quinazoline drug, may represent chemical starting points to develop more potent apoptosis-inducing agents free of α1-adrenoceptor antagonistic action and is suitable for cancer treatment with minimal and well-tolerated side effects.

Keywords: apoptosis, doxazosin, antitumoral, quinazoline.

1. Introduction

The α1-adrenoceptor antagonist, doxazosin, terazosin and prazosin are one of the most frequently prescribed drugs, being the FDA (Food and Drugs Administration) approved drugs for benign prostatic hyperplasia (BPH) and elevated blood pressure [1-7]. These compounds bind alpha1-adrenoceptors on smooth muscle cells of the prostate and bladder tissues, reduce muscle tone, and relieve bladder obstruction. The therapeutic activities of these agents correlate with an α1-adrenoceptor occupancy in the range of 81-89% [4]. BPH or hypertension patients who were treated long-term with α1-adrenoceptor antagonists showed a significantly lower incidence of prostate cancer (a drop of 31.7%) [8].

Kaye et al. [9] investigated the metabolic fate of doxazosin in men, mice, rats and dogs using ^{14}C labelled compound. Following both oral and intravenous administration, the major route of elimination of drug related compounds was via faeces for all species studied. The drug is extensively metabolized, e.g. only about 5% of the dose was excreted unchanged in man.

Metabolism in man mainly involves 6- and 7-O-demethylation and 6’ and 7’-hydroxylation. These and some minor products were common to mice, rats, dogs and men (Fig.1). Plasma protein binding was high in all species studied, ranging from 95.3% in rats to 98.3% in human patients.

Doxazosin is more slowly eliminated than prazosin in men and its relatively long half-life provides the basis for once-daily dosing, a therapeutic advantage [10]. Studies in mice showed that doxazosin, an α1-adrenoceptor antagonist with a quinazoline based structure, induced apoptosis in murine prostatic stromal and

epitelial cells [11,12].

In other studies doxazosin induced apoptosis of normal and malignant prostate cells through an alternative mechanism unrelated to the α1-adrenoceptor [13-15]. The drug also exerted pro-apoptotic effects in breast cancer [16,17] by inhibiting EGFR (Epidermal Growth Factor Receptor) and activating the NFκB (nuclear factor kappa B) signaling pathway. Doxazosin was able to inhibit growth and tube formation of human vascular endothelium cells, and suppressed angiogenesis in mice [18,19]. Although their mechanisms of action are not fully understood, quinazoline based α1-adrenoceptor antagonists increase apoptosis in multiple cell types.

Hui et al. [16] investigated in MCF-7 and MDA-MB-231 (cell lines of breast cancer) the effect of doxazosin on proliferation and cell death. Doxazosin treatment inhibited HER positive and HER negative (epidermal receptor) breast cancer proliferation in a dose and time dependent manner. After 48h doxazosina treatment, MCF-7 proliferation was inhibited between 30% and 70% (20-30 μM), and proliferative rates were further inhibited 30-90% at 72 h.

In another study, Benning and Kyprianou [20] identified the precise molecular mechanism underlying this apoptosis induction. They examined whether doxazosin affected prostate growth via an α1-adrenoceptor regardless of this action. These findings provided the first evidence that the apoptotic activity of the quinazoline based α1-adrenoceptor antagonists against prostate cancer cells was independent of their capacity to antagonize α1-adrenoceptors and the hormone sensitivity status of the cells. This may have a potential therapeutic significance in the use of quinazoline based α1-adrenoceptor antagonists for the treatment of androgen-independent human prostate cancer via induction of apoptosis [21].

One of the most interesting aspects of this effect is the fact that apoptosis induction occurs independently of the α1-antagonistic properties of these drugs [13,14]. This concept emerges from evidence suggesting a significantly increased apoptotic index in prostatic cells exposed to doxazosin (quinazoline-based α1-antagonistic) but not following tamsulosin treatment (a sulfonamide-based α1-antagonistic). Furthermore, the irreversible α1-antagonist phenoxybenzamine had no effect on the anti-growth action of doxazosin [22]. Angelin et al. [13] also supported that the most interesting aspects of this effect is the fact that apoptosis induction occurred independently of the α1-antagonistic properties.

However, the mechanism of its anti-proliferative and pro-apoptotic actions in cancer cells are not well understood. Doxazosin could reduce EGFR phosphorylation by inhibiting MAP (Protein Activate for Mitogen) kinase signalling since doxazosin has a quinazoline ring structure, similar to the EGFR tyrosine kinase inhibitors, Iressa™ and Tarceva™. Liao hypothesized [1] a potential interaction of doxazosin with EGFR in breast cancer cells.

2. Quinazoline ring with mechanisms of action and tyrosine kinase inhibitors

The HER family of receptors are a group of transmembrane tyrosine kinase receptors (RTK) expressed in normal cells and responsible for regulating cellular processes [23, 24]. These receptors are overexpressed in cancer cells. RTK play vital roles in the processes controlling cellular proliferation, differentiation and evasion from apoptosis. Among the known RTKs, the ErbB family, particularly EGFR and HER2 have been extensively focused and clinically validated as rational targets for cancer therapies [25-27]. The ErbB family includes four receptors: epidermal growth factor receptor (EGFR/erbB-1/HER1), erbB-2 (HER2), erbB-3 (HER3), and erbB-4 (HER4). Each one can be activated through homo or heterodimerization with

other receptors leading to phosphorylation events and downstream signaling that could cause excessive growth by inducing cell proliferation and inhibiting apoptotic pathways [28-31].

Overexpression of the ErbB family allows tumor cell growth and survival and predicts poor clinical outcomes for cancer patients. Ligands bind to the receptors, which leads to dimerization, allowing an autophosphorylation reaction of tyrosine kinase receptors catalyzing a downstream cascade of reactions leading to cell-cycle progression, gene transcription, tumor cell survival, cell proliferation, and cytoskeletal organization. Several monoclonal antibodies and small tyrosine kinase inhibitors that target these receptors have been developed in the past decades [25, 26, 30, 31].

TKIs (Inhibitors of Tyrosine Kinase Receptors), in all series of synthesized and evaluated compounds, seem to be associated to substitute electron donating groups in position 6 or 7 of quinazolin. These groups are present in the chemical structure of doxasozin (Fig.2) [29].

TKIs are a class of chemotherapy medications that inhibit or block the enzyme tyrosine kinase. TKIs were created out of modern genetics, the understanding of DNA, cell cycle, and molecular signaling pathways, and thus represent a change from general to molecular methods of cancer treatment.

The quinazoline ring has frequently been used as a core scaffold to occupy the adenine ring region of the RTK's ATP binding site [29]. Goldstein et al. [30] studied the kinase interaction maps for five quinazoline compounds: AZD-1152HQPA, Erlotinib (Tarceva™; OSI/Genentech/Roche), Lapatinib (Tykerb; GlaxoSmithKline), MLN-518 and ZD-6474. They show that compounds based on the quinazoline scaffold can target a range of kinases with varying degrees of selectivity. A superposition of the binding conformation of these inhibitors reveals that the

quinazoline is in approximately the same position and forms a key hydrogen bond between the quinazoline N1 and the kinase hinge region. Lapatinib is the most selective compound in this set and only interacts with EGFR subfamily. Presumably this is due to the ability of lapatinib to recognize an inactive conformation of EGFR47 that is distinct from the classical active conformation recognized by the type I inhibitors erlotinib and ZD-6474.

However, they may be useful as starting points for the synthesis of more potent compounds. A recent pharmacological exploitation of the doxazosin structure led to two structural variants of doxazosin that exhibit greater apoptosis-inducing action at lower concentrations (~10-20 μM) than their parent compound [31, 32].

Quinazoline α1-antagonists might be responsible for additional α1-independent growth-suppressing mechanisms including the activation of anoikis (loss of cellular adhesion to the extracellular matrix) via death receptor-signaling pathway and prevention of tumor cell invasion and migration [33, 34].

Concluding remarks

Tumor malignancy is dependent upon the continued ability to grow and spread to new tissues**.** These properties necessitate anoikis avoidance and continued angiogenesis, as well as overexpression of tyrosine kinase receptors. Restoration or facilitation of anoikis, inhibition of angiogenesis and of tyrosine kinases are important targets for anticancer therapy. The effects of the quinazoline ring on these targets makes doxazosin a promising anticancer drug that may represent chemical starting points to develop more potent apoptosis-inducing agents free of α1-adrenoceptor antagonistic action and suitable for cancer treatment with minimal and well-tolerated side effects.

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3. Legends to the figures

Fig.1. The metabolism of doxazosin [9].

Fig.2. In the center, the chemical structure of doxazosin [4 - 4-amino-6 ,7-dimethoxyquinazoline-2-yl)-piperazine] yl-1 - (2,3-dihydro-1,4-benzodioxine-3-yl). The part marked in blue corresponds to the quinazoline ring and arrows correspond to electron donor groups. The other chemical structures correspond to partial analogs of doxazosin. PD153035: inhibitor of receptor tyrosine kinase.

Figures

Fig.1

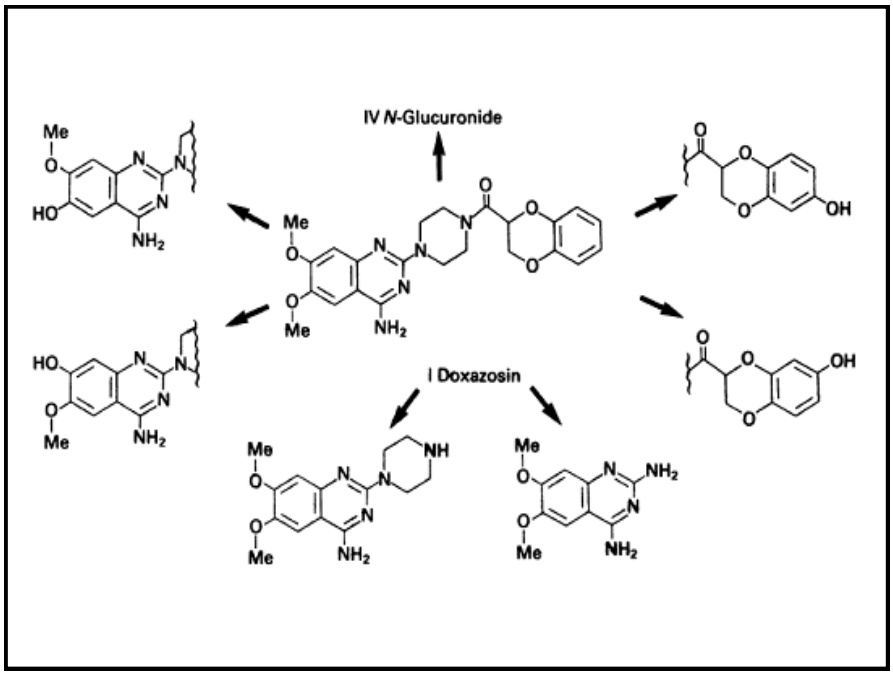
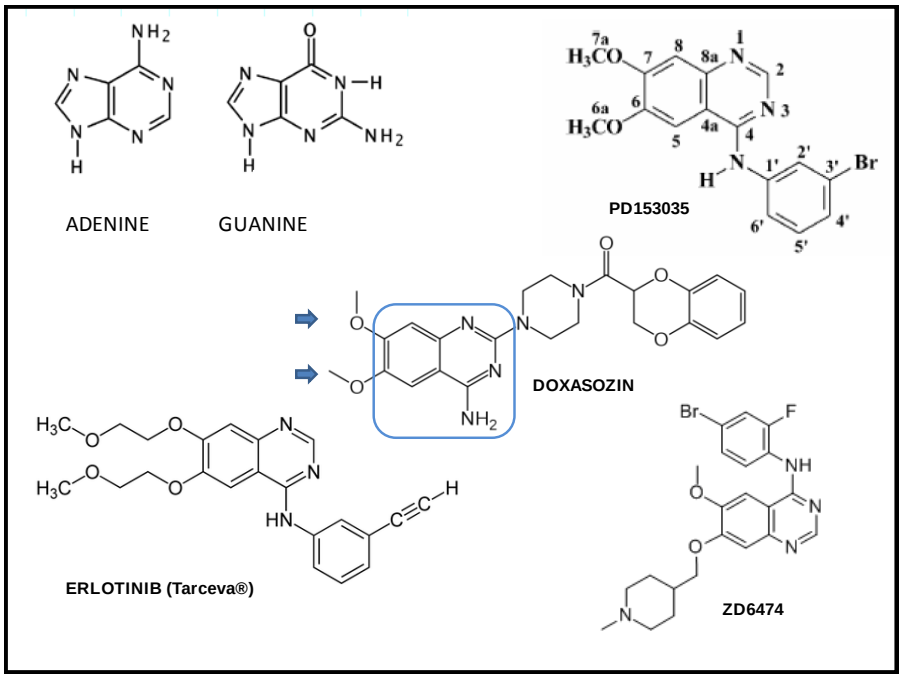


Fig.2



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Analysis and standardization of the possible toxic effect of doxazosin against C6 lineage of rat glioma

Analysis and standardization of the possible toxic effect of Doxazosin against C6 lineage of rat glioma

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Abstract

Glioblastoma (GB) is the most frequent and most malignant human brain tumor. The prognosis for the patients with GB remains dismal, as median survival after diagnosis varies from 6 month to 1 year. This is largely due to the inability of current treatment strategies to address the highly invasive nature of this disease. Thus, new therapeutic strategies are needed. Doxazosin, the quinazoline compound, with anticancer properties demonstrated in breast cancer, colon cancer and the urothelial cancer cells. In this context, the aim of this study was to evaluate the effect of doxazosin on C6 rat glioma cells. Also we analyzed the doxasozin uptake and its stability in the culture medium and cititoxicity on non-tumoral cells. Doxazosin uptake was evaluated by auto fluorescence of doxasozin into C6 glioma cells, the stability of the drug in the culture medium was verified by HPLC measurement. Toxicity assay, on non tumoral cells, was assessed by propidium iodide uptake on astrocyte culture as well as on organotypic hippocampal slice culture. Density cell assays was done on the primary astrocytes culture and was evaluated using Sulforhodamine B assay. Cell viability, was done with MTT assay. Our results showed a clear effect of the doxasozin on the cellular viability inhibition of the C6 glioma cell line. Moreover, the drug seems to be stable in the culture medium after 48 hours of incubation and was taken up by C6 glioma cells and showed no toxic effect on no-tumor cells at concentrations below 250µM. This work suggests the possibility of using doxasozin as a prototype drug for the development of a new therapeutic strategy for the treatment of brain tumors.

Keywords: glioma, doxazosin, antitumoral property

1. Introduction

Glioblastoma (GB) is the most frequent and most malignant human brain tumor. GB patients have a short life expectancy despite aggressive therapeutic approaches based on surgical resection followed by adjuvant radiotherapy and concomitant chemotherapy [1].

The World Health Organization (WHO) classified gliomas in four grades according to their malignancy degree and morphological features [2-5]. Low grade (I and II) gliomas cells are well differentiated; they bear histological similarity to astrocyte and oligodendrocyte. High Grade (III and IV) Gliomas (HGG) are more anaplasic, resembling immature astrocytes, or oligodendrocytes, or a mixture of both [2-4].

HGGs include Glioblastoma (GB) and Anaplastic Astrocytoma (AA); both are highly invasive and display high chemo resistance leading to tumor recurrence post surgery [5, 6].

The prognosis for the patients with GB remains dismal, since the survival of patients, after diagnosis, ranges from 6 months to 1 year. This is largely due to the inability of current treatment strategies to address the highly invasive nature of this disease [6].

Doxasozin (2-{4-[(2,3-Dihydro-1,4-benzodioxin-2-yl)carbonyl]piperazin-1-yl}-6,7-dimethoxyquinazolin-4-amine, a quinazoline compound, is an selective α1-adrenoceptor antagonists, widely used for treatment of high blood pressure as well as in the treatment of urinary retention related with prostate benign hyperplasia (BPH) [7-9].

It is an α1-adrenergic receptor blocker which inhibits the binding of norepinephrine (released from sympathetic nerve terminals) to the α1-receptors on the vascular smooth muscle cells membrane. The primary effect of this inhibition is relaxed vascular smooth muscle tone (vasodilation), which decreases peripheral vascular resistance, leading to decreased blood pressure [7-14].

The metabolism in humans mainly involves 6 - and 7 - O-demethylation and 6 'and 7'-hydroxylation [15]. Furthermore, due their physicochemical characteristics, Doxasozin can permeate the blood-brain barrier.

Early studies in mice showed that doxazosin, induced apoptosis in murine prostatic stromal and epitelial cells [12, 16]. This effect was confirmed by other studies on urothelial cancer [10], pituitary adenoma [17], breast cancer [18], colon cancer and HeLa cells [19].

In this context, we decided to evaluate the toxic effect of doxazosin on cell lineage C6 of rat glioma, to verify the uptake of the drug by gliomas cells as well as its stability in culture medium, and further analyzing the toxicity of doxasozin on non-tumor cells. For these purpouse we used primary astrocyte culture and organotypic hippocampal slice culture.

2. Materials and methods

2.1 Chemicals and materials

Cell culture media and fetal bovine serum (FBS) were obtained from Gibco-Invitrogen (Grand Island, NY, USA). Doxazosin and Propidium iodide (PI) were obtained from Sigma Chemical Co (St. Louis, MO, USA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) or Merck (Darmstadt, Germany). All other chemicals and solvents used were of analytical or pharmaceutical grade.

2.2 Cell culture

C6 rat glioma cell line we obtained from American Type Culture Collection (Rockville, Mariland, Md., USA). Cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco-Invitrogen, Grand Island, NY, USA) supplemented with 5% (v/v) fetal bovine serum (SFB; Gibco-Invitrogen, Grand Island, NY, USA), and containing 2.5 mg/mL de Fungizone® and 100 U/L de gentamicine (Shering do Brasil, São Paulo, SP, Brazil). Cells were kept at a temperature of 37ºC, a minimum relative humidity of a 5% CO_2 atmosphere. All the experiments throughout this study were conducted in serum supplemented DMEM.

2.3 Primary astrocyte culture

The primary astrocyte culture from Wistar rats were prepared as previously described [20]. Procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local authorities. Briefly, cerebral cortices of newborn Wistar rats (1-2 days old) were removed and mechanically dissociated in Ca^{2+} and Mg^{2+} free balanced salt solution, pH 7.4, containing (in mM): 137 NaCl; 5.36 KCl; 0.27 Na_2HPO_4; 1.1 KH_2PO_4 and 6.1 glucose. The cortices were cleaned of meninges and mechanically dissociated by sequential passage through a Pasteur pipette. After centrifugation at 1400 rpm for 5 min the pellet was resuspended in DMEM (pH 7.6) supplemented with 8.39 mM HEPES, 23.8 mM $NaHCO_3$, 0.1% amphotericin, 0.032% gentamicin and 10% Fetal Calf Serum (FCS). Cultures were maintained in DMEM containing 10% FCS in 5% CO_2/95% air at 37°C, allowed to grow to confluence, and used at 15 days *in vitro*.

2.4 Organoptypic hippocampal slice culture

Organptypic hippocampal slice cultures were prepared according to the method of Stoppini [21] with modifications [22]. All animal used procedures were approved by local Animal Care Committee were in acordance with the NIH Guide for the Care and Use of Laboratory Animals. Briefly, 400µM thick hoppocampal slices were prepared from 6-8-day-old male Wistar rats using a Mcllwain tissue chopper in ice-cold Hank's balanced salt solution (HBSS), pH 7.2. The slices were placed on Millicell® culture membranes and the inserts were transferred to a six-well culture plate. Each well contained 1mL of tissue culture medium consisting of the middle MEM with 25% of HBSS and 25% of horse serum supplemented with glucose 36 mM, HEPES 25 mM, $NaHCO_3$ 4 mM, Fungizone® 1% and gentamicine 0,1 mg/mL, pH 7,3. The cultures were kept in incubator 37ºC and 5% of CO_2 for 14 days.

3. Doxazosin treatment

C6 glioma cells were seeded at 5 x 10^3 cells per well in DMEM/ 5% FBS in 24 well plates and grown 24 hours. Doxazosin was dissolved in 20% ethanol/ milli-Q^{TM} water (vehicle). The cells were treated with the doxasozin for 24 or 48 hours with concentrations (µM) 5, 30, 50, 100, 150, 180, 200, 250, 280 or 300. The controls

groups were processed parallel without receiving treatment with the doxasozin. The medium was changed once a day with replacement of the drug.

For primary astrocyte culture and organotypic slice culture, they were treated with doxasozin for 48 hours at 30, 75, 100, 250 (μM). The controls groups were processed parallel without receiving treatment with the doxazosin. The positive control recieved Glutamate (1mM).

4. Sulforhodamine assay

The sulforhodamine B (SRB) assay is used for cell density determination, based on the measurement of cellular protein content [23, 24]. After 48 hours of treatment, the medium of the primary astrocyte culture was removed, the cells were washed three times with PBS and 500 μL PBS/FORMOL 4% was added. After 15 minutes, fixed cells were stained with sulforhodamine B. Subsequently, the wells were washed with deionized water to remove the unbound stain. The culture plates were air dried and protein-bound sulforhodamine was solubilizaed in 1% SDS. Absorbance was measured by an ELISA plate reader at 515 nm. This absorbance was linearly proportional to the number of cells.

5. MTT assay

Cellular viability was assessed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl) tetrazolium bromide, a yellow tetrazole, is reduced to purple formazan in living cells. Thus, MTT reduction assay, measures the redox activity of living cells [25]. The C6 glioma cell line were plated in a 96-well plate at 10^3 and grown 24 hours. This procedure was done after 24 and 48 hours treatment.

Following treatment, culture medium containing the drug was removed and the cells were washed twice with PBS. Then, 90μl of culture medium and 10μl of MTT stock solution (5 mg/ml in PBS) were added to each of the wells. The cells were incubated for 3h and the solution was then removed from the precipitate. The formazan product in cells was solubilized by adding DMSO and the level of 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.

6. Doxazosin uptake

C6 glioma cell lines were plated in a 24-well plate at 5.10^3 and treated with doxasozin for 48 hours. At the end of the treatment, the medium was removed, C6 cells were washed with phosphate-buffered saline (PBS). Added 100µL in 1% SDS and the plate was shakened for 10 minutes. Then, 150µM of serum free medium was added. Fluorescence was reader at 515nm [26]. Doxazosin was also measured in the culture medium (seen below).

7. Doxazosin identification

The experiments were carried out in a liquid chromatograph Shimadzu (Kyoto, Japan) equipped with the LC-10AD pump system, the SIL-10A manual injector, and the UV/Vis SPD-20AV system detector, controlled by the LC-10 program. A Shim-pack CLC-ODS (M) RP-18 column (5 µm, 250 x 4 mm i.d., Waters, Milford, USA) was employed [26]. The HPLC method for doxazosin quantification was described in the USP monograph: detection wavelength of 210 nm; injection volume of 20µL; flow rate of 0.8 mL/min; and column compartment temperature of 35°C. The mobile phase composed of acetonitrile and water (orthophosphoric acid) was eluted in gradient mode (Table 1), where Solvent A - 5 g of orthophosporic acid in 100 mL of water; Solvent B - acetonitrile; and Solvent C - water; and Solvent D - mixture of 100 mL of Solvent B and 2 g of orthophosporic acid. The samples were diluted in a mixture of Solvent C and Solvent D with a final proportion of 9:1 [27]. All samples evaluated were filtered in HVLP Millipore™ membrane (0.45 µm of pore diameter) (Bedford, USA) before HPLC injection (as shown in Table 1) [26].

8. Cell death assay

To identify cellular death, propidum iodide (PI) 5 µM was added to primary astrocyte culture and/or Organotypic hippocampal slice cultures for 1 hour before 48 hours of the treatment. The cultures were treated with the different concentrations of doxazosin for 48 hours. Propidum iodide fluorescence was excited at 515-560nm 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 MacBiophotonics ImageJ software, for the Organotypic hippocampal slice cultures, and the surfohodamine B assay for the primare astrocyte culture.

9. Statistical analysis

Data are expressed as means±SD. All results are representative of at least 4 independent experiments. Analysis of variance (ANOVA) was applied to the means to determine statistical differences between experimental groups. Post hoc comparisons were performed by Tukey test. Differences between mean values were considered significant when $p<0.05$.

10. Results

10.1 Standardized the concentration of the doxazosin

Considering that doxazosin have been reported to have antitumor proprieties [10, 12, 16-18], and there isn't any data about the effect of doxazosin on gliomas cells, firstly we decided to standardize the concentration to be used to analysis the effect on this tumor cell type. For this purposed, we studied the effect in the viability of C6 glioma cell line. This assay measures the mitochondrial activity, and, indirectly, the cell viability. The cells were treated with increasing concentrations of doxazosin for 24 and/or 48 hours. As show in figure 1, the cell viability decreased when treated with 250µM of doxasozin for 24 hours. However, after 48 hours of treatment, we observed a significant reduction on the percentage of viable cells with 150µM of doxasozin concentration, when compared of control group, as shown in figure 2.

The problem of current therapy is the lack of specificity and selectivity of certain drugs used for tumors treatment [28].

In order, to evaluate the toxicity of doxazosin on non-tumor tissue and monolayer cells, we exposed, organotypic hippocampal slice culture and primary astrocyte culture for 48 hours, using the same range of drug concentration and subsequently evaluate cell death by analysis of propidium iodide uptake.

According to the results, we observed that only at a concentration of 250µM, in both experiments, we could see an increase in PI uptake when compared of control, indicating cell death. Thus, our data demonstrate that doxasozin exerts a significant

toxic effect in glioma cells, but did not affect normal cells (healthy) in concentrations bellow 250µM (figures 3 and 4). The quantification of cell density, on primary astrocyte culture, shown in figure 5, was obtained with sulphorhodamine B assay (SRB) (figure 5) [25], where as the quantitation of cell death in organotypic cultures, observed on figure 6, was performed by analysis of propidium iodide uptake using MacBiophotonics ImageJ software.

10.2 Stability of doxazosin in the culture medium

The retention time, chromatographic peak shape and area there are physico-chemical characteristics of each molecule [29].

The chromatography analysis was performed to verify the stability of doxazosin in incubation medium, containing 5% and/or 10% fetal bovine serum (sample 1 and 2, respectively), after 48 hours. As shown in figure 7, it was detected a characteristic peak of doxazosin on samples 1 and 2. The medium used for control contains 10% of fetal bovine serum. Smaller peaks detected in sequence in control and sample (b, 1b, 2b and c, 1c, 2c) should probably be related to the presence of antifungal and antibiotic in the culture medium. These results showed that doxazosin is stable in culture medium.

10.3 Uptake of doxazosin

The presence of certain chemical groups in the chemical structure of molecules favors the process of radioactive fluorescent. These groups are referred to as chromophoric groups [30-34]. These groups are present on chemical structure of doxazosin, as we can observe on figure 8.

Based on this property of doxazosin molecule, it was possible to evaluate the amount of doxazosin into the cells of C6 glioma. Compared with the control, can be observed the presence of doxasozin within the cells over all the concentration range examined, which was from 30 to 100µM, and at the higher concentrations there is a decrease in uptake (figure 9).

10.4 Quantification of doxazosin on culture medium

In experiments performed to evaluate the amount of doxazosin within the cells, it was also evaluated doxazosin concentration in the culture medium, after 48 hours of incubation. In the figure 10, we showed that in lower concentrations (µM) 30 to 100, there was less drug quantity in the medium, but in higher concentrations (µM) 150, 180 observed a greater amount of drug in the culture medium.

11. Discussion

Glioblastomas have been described as rapidly growing tumors associated with necrosis and endothelial proliferation [35].

Therapeutic regimens containing nitrosoureas (carmustine or lomustine), alkylating DNA (procarbazine, dacarbazine and temozolomide), are used to treat such cancer cells. These drugs, often cause toxicity to others tissues and organs as a result of the adverse effects [38].

The main objective of this study was to investigate the effect of doxazosin on C6 cell line of glioma rat, as a possible prototype drug to add to the existing therapeutic strategies. The results obtained on the experiments with brain slices and astrocytes culture, suggested that doxazosin does not have toxic effect on non tumor cells to the same extent that affect cancer cells as observed by low incorporation of propidium iodide (PI) in both of cultures. These data are pointing that this drug has a selective toxicity for tumor cells, and stimulate us to develop in vivo models to investigate the effectiveness of this drug against the growth of gliomas.

Accordingly, our results showed that doxazosin induced a decrease on cell viability on the concentration of 250µM (to 24 hours) and 150µM (to 48 hours) inducing a significant decrease on the percentage of cells.

Interestingly, concentrations above 150µM (treated for 48 hours) with doxazosin, were not effective in causing a decrease of cell viability in a manner related to the concentration. Considering this, what can be inferred about this fact is the possibility of the presence of resistant cells.

Patients with glioblastoma (GB) may account for the high incidence of chemo-resistant are characterized for presenting a variety of genetic abnormalities.

This heterogeneity may occur because the cells that carry different abnormalities may respond differently to therapy [36]. Previous reports showed the

presence of the heterogeneous cells on glioma lines [28, 37], which could explain the presence of resistant cells on the culture on present work.

To verify the possibility of doxazosin degradation in others products which could interact with other components of the culture medium (antibiotics, cells metabolites, growth factors, anti-fungal), we performed a stability assay, once stability is a quality standard used in pharmaceutical drug development. The parameters of drug dosage form can be influenced by environmental conditions, as temozolamide, which is a pro-drug [37, 40].

The retention peak, retention times and shape were characteristic of the doxazosin (with compared of control) (figure 7). This test gives us a guarantee, that doxazosin is actually exerting action of inducing cell viability inhibition on C6 rat glioma line.

Fluorescence is a characteristic resulting from transitions between a π anti-ligand orbital and orbital π ligand or a transition between a π anti-ligand orbital and orbital no binder. The presence of certain groups in the chemical structure of molecules, for example, the grouping $–NH_2$ present in doxazosin (shown in figure 8) favors the process of radioactive fluorescence. Aromatic molecules, as the case, were rigid and the presence of imines groups (N=C), known as Schiff base, also exhibit this characteristic [30-34, 41-47]. This property was used to detect the uptake of doxazosin. As clearly shown in Figure 8, doxazosin is taken up by cells, particularly at lower concentrations. But when high concentrations were used we observed a decrease on this uptake, what may be occurring due to the toxic effect of the drug, many dead cells are released from the plate and washed with PBS buffer. On the other hand, when we measured the concentration of doxazosin in the culture medium, we observed that when higher concentrations were used, the amount of doxazosin medium was also high, suggesting that there may be less viable cells to capture the drug and thereby it remains higher concentration in the medium. What should also be considered is that the drug can be metabolized in the glial cell and thus not be detected in the assay quantitation [15]. The overall result obtained in these tests confirm that doxazosin is taken into the cells from the medium.

12. Conclusions

Our results showed for the first time, a clear effect of doxazosin on the cell viability on the C6 glioma cell line. Moreover, the drug was stable during the course of the incubation time of 48 hours was extracted by C6 glioma cells and showed no toxicity to healthy cells in both models examined, culture slices of brain and primary cultures of astrocytes.

The development of new effective therapeutic strategies for the treatment of brain tumors, in order to reduce the mortality and morbidity of the disease, is a huge challenge for scientists. The characterization of new drugs that may act as an adjuvant to other anti-tumor therapies can be a very useful strategy for treating gliomas. Here we demonstrate the effect of doxazosin on cell viability of C6 glioma cells that will guide us for the future investigation of the mechanism associated with drug action.

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Legends to the figures

Figure 1. Effect of doxazosin for 24 hours on cell viability inhibition assay of C6 glioma cell line. Data are means±SEM (n=4). ***p<0.001 compared to respective control, ANOVA followed by Tukey's test.

Figure 2. Effect of doxazosin for 48 hours on cell viability inhibition assay of C6 glioma cell line. Data are means±SEM (n=4). ***p<0.001 compared to respective control, ANOVA followed by Tukey's test.

Figure 3. Effect of doxasozin after treatment in the astrocyte primary culture for 48 hours. Photomicrographs (A), UV (B).

Figure 4. Effect of doxazosin after treatment in the organotipic slice cultures for 48 hours (A). (B) Glutamate 1mM (C) showns schematic picture of a slice hippocampal. UV, Photomicrographs. Magnification: 40X.

Figure 5. Quantification of cell density on primary astrocyte culture with doxazosin treatment of 48hours. Data are means±SD (n=3). #p<0.01 and ***p<0.001 compared to respective control, ANOVA followed by Tukey's test.

Figure 6. Quantification of PI staining after treatment with doxazosin on organotipic slice cultures for 48 hours. Data are means±SEM (n=6). #p<0.001 ***p<0.001 compared to respective control, ANOVA followed by Tukey's test.

Figure 7. Chromatography analysis of doxazosin incubating in the culture medium for 48 hours, with 10% (Sample 2) and 5% fetal bovine serum (Sample 1).
(a), (2a), (1a). Characteristics peaks of grow factors in the control medium (10%), sample 1, and 2 respectively.
(b,c), (2b,2c), (1b,1c). Characteristics peaks of garamicine and fungizone in the control medium (10%), sample 1 and 2 respectively.

Figure 8. Doxasozin fluorescence. Chemical structure of Doxasosine ((2-{4-[(2,3-Dihydro-1,4-benzodioxin-2-yl)carbonyl]piperazin-1-yl}-6,7-dimethoxyquinazolin-4-amine) (A) chromophore groups (arrows) and quinazolinic ring (B).

Figure 9. Uptake of doxasozin for 48 hours on C6 glioma cell line. The results are presented as average ±DP(n=6) ***p< 0.001.

Figure 10. Quantification of doxazosin on culture medium for 48 hours on C6 glioma cell line. The results are presented as average ±DP(n=4) ***p< 0.001.

Figures

Fig.1

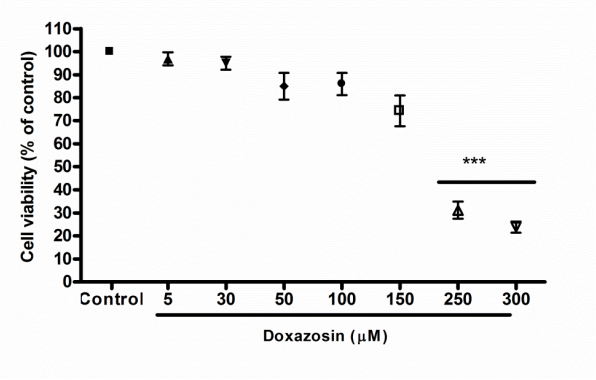


Fig.2

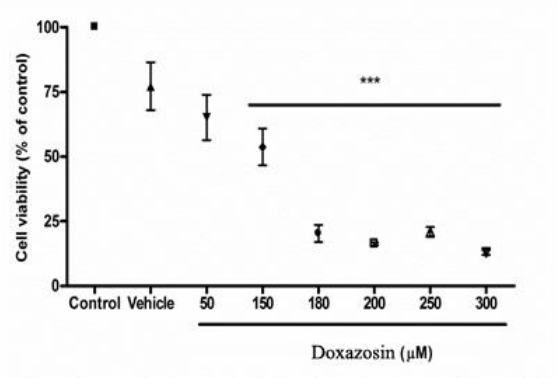


Fig.3

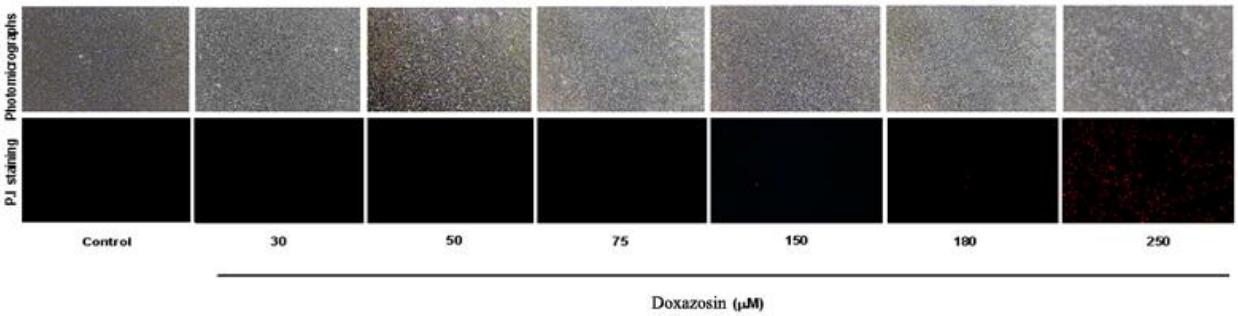


Fig.4

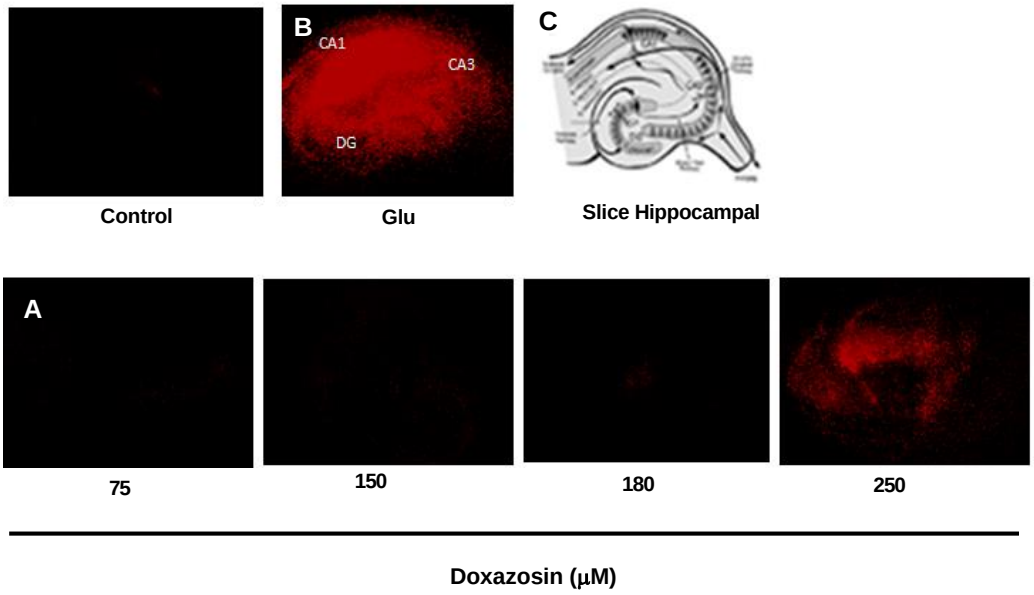

B
CA1
CA3
DG
C
Control
Glu
Slice Hippocampal
A
75
150
180
250
Doxazosin (μM)

Fig.5

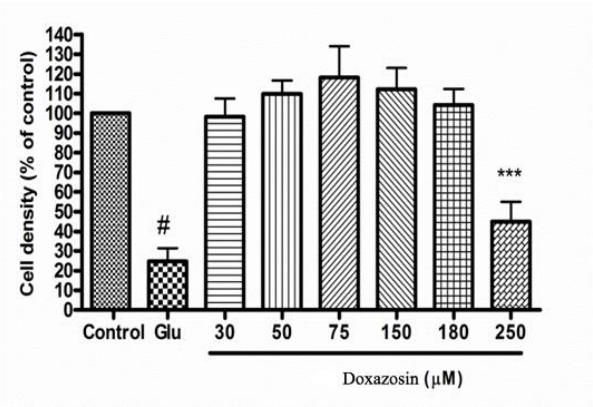

Cell density (% of control)
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
#
***
Control
Glu
30
50
75
150
180
250
Doxazosin (μM)

Fig.6

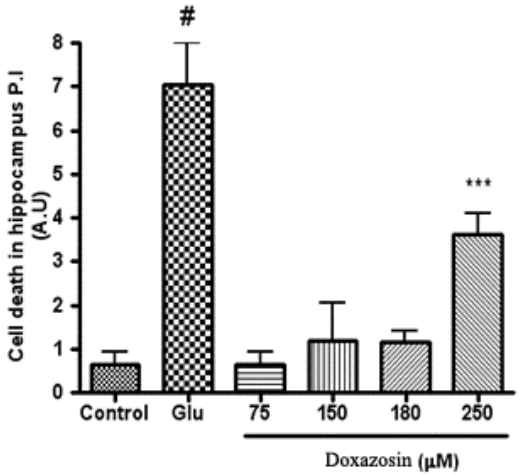

#
***
Cell death in hippocampus P.I (A.U)
0
1
2
3
4
5
6
7
8
Control
Glu
75
150
180
250
Doxazosin (µM)

Fig.7

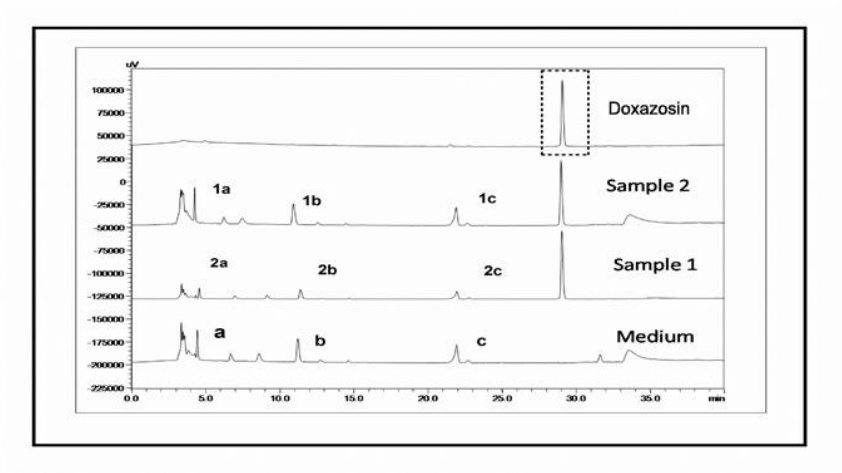
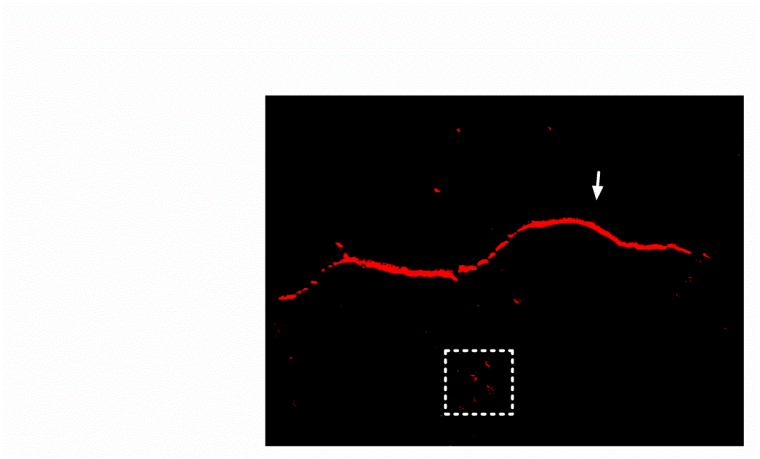

Doxazosin
Sample 2
Sample 1
Medium
1a
1b
1c
2a
2b
2c
a
b
c
min

Fig.8



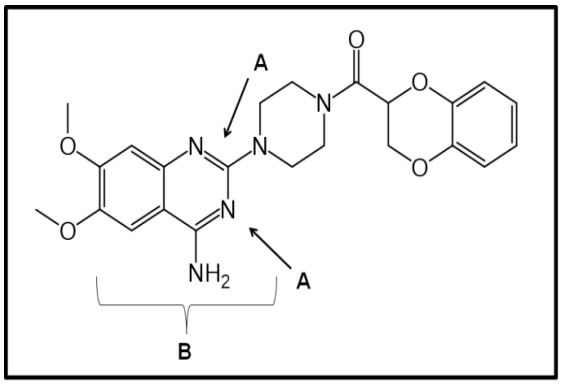

A
O
N
O
N
N
O
O
N
O
NH2
A
B

Fig. 9

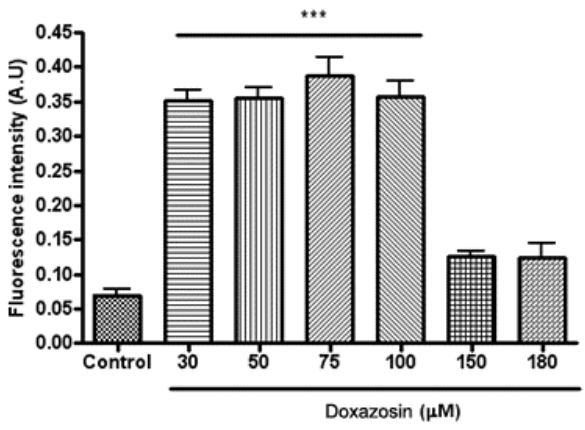
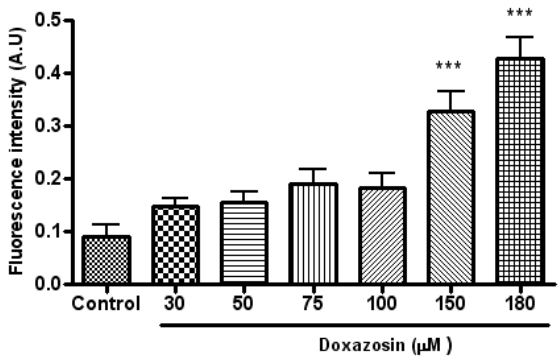

***
Fluorescence intensity (A.U)
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Control
30
50
75
100
150
180
Doxazosin (μM)

Fig.10


Fluorescence intensity (A.U)
0.5
0.4
0.3
0.2
0.1
0.0
***
***
Control
30
50
75
100
150
180
Doxazosin (μM)

Table

Table 1. Gradient elution system of the mobile phase for HPLC evaluation of doxazosin.

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)	Elution
0-10	20	10→22	70→58	Linear gradient
10-35	20	22→50	58→30	Linear gradient
35-40	20	50	30	Equilibration

3.3. Artigo a ser submetido para publicação ao periódico Cancer Letters.

ANTITUMORAL EFFECT OF DOXAZOSIN ON U-138 AND C6 CELL LINES

ANTITUMORAL EFFECT OF DOXAZOSIN ON U-138MG AND C6 CELL LINES

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Conflict of interest: The *authors declare* that there are no *conflicts of interest.*

Abstract

Glioblastoma (GB) is the most frequent and most malignant human brain tumor. GB patients have a short life expectancy despite aggressive therapeutic approaches based on surgical resection followed by adjuvant radiotherapy and concomitant chemotherapy. Doxazosin, a quinazoline compound, is an selective α1-adrenoceptor antagonist, widely used for treatment of high blood pressure as well as of urinary retention related with prostate benign hyperplasia (BPH). In this study we investigated the antitumoral effect of doxazosin to U-138MG and C6 glioma cell lines. Cell density was evaluated using Sulforhodamine B assay; cell death was assessed by LDH activity. To assess cell proliferation we used the measurement of mitotic index, cell signaling pathways was analyzed by immunoblotting of proteins Akt and GSK-3β. The identification of acid mucins was done by Alcian blue. The results showed that doxazosin was able to decrease cell density and induce cell death in both lines. In addition, doxazosin induced the inactivation of Akt and activation of GSK-3β proteins. The present findings may, at least in part, support the therapeutic effects of doxazosin for the development of a new adjuvant drug for glioma treatment.

Keywords: glioma, doxazosin, antitumoral property.

1. Introduction

Gliomas are the most common intrinsic brain tumors in adults. These tumors are graded from I to IV according to the World Health Organization (WHO) malignancy scale [1]. Grade IV tumors are the most malignant and also the most frequent gliomas and include glioblastoma and gliosarcoma [1-6].

Glioblastoma (GB) is the most frequent type of glioma, comprising almost 50% of all diagnosed gliomas cases [2, 6, 7]. Standard first line treatment for glioblastoma patients includes surgery followed by focal fractionated radiotherapy with concomitant adjuvant administration of the alkylating chemotherapy with temozolomide [1, 4]. These neoplasms are extremely resistant to treatment, and this is related to its cellular heterogeneity [1] and the exceptional migratory nature of these tumor cells, which are able to diffusely infiltrate normal brain tissue [4]. Therefore, patient median survival does not exceed 12–15 months [8]. Due to this poor prognosis and the absence of successful therapeutics for glioma patients, new therapeutic paradigms for the treatment of malignant gliomas need to be explored.

Doxazosin mesylate (4-amino-2-[4-(1,4-benzodiaxan-2-carbonyl)-piperazin-1-yl]-6,7-dimethoxyquinazoline mesylate), a quinazoline derivative, is effective and well known for treatment of hypertension and benign prostatic hyperplasia by selectively blocking α1-adrenoreceptor [9-11]. Early studies in mice showed that doxazosin, an α1-adrenoceptor antagonist with a quinazoline-based structure, induced apoptosis in murine prostatic stromal and epitelial cells [12], urothelial cancer cells [13], pituitary adenoma cells [14], breast cancer cells [15].

A well known objective for studying actions of doxazosin is the discovery of new adjuvant compound and/or using its quinazoline structure as the starting points

to develop more potent apoptosis inducers agents free of α1-adrenoceptor antagonistic action and suitable for cancer treatment with minimal and well-tolerated side effects.

In this context, we decided to evaluate the toxic effect of doxazosin on U-138MG and C6 glioma cell lines.

2. Materials and methods

2.1 Chemicals

Cell culture medium and fetal bovine serum (FBS) were obtained from Gibco-Invitrogen (Grand Island, NY, USA). Doxazosin and Propidium iodide (PI) were obtained from Sigma Chemical Co (St. Louis, MO, USA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

2.2 Cell culture

The U-138MG human glioma and C6 rat glioma cell lines were obtained from American Type Culture Collection (Rockville, Mariland, Md., USA). Cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM,Gibco-Invitrogen, Grand Island, NY, USA) supplemented with 10% and 5% (v/v), respectively, fetal bovine serum (SFB; Gibco-Invitrogen, Grand Island, NY, USA), and containing 2.5 mg/mL de Fungizone and 100 U/L de gentamicine (Shering do Brasil, São Paulo, SP, Brazil). Cells were kept at a temperature of 37ºC, a minimum relative humidity of

5% CO_2 atmosphere. All the experiments throughout this study were conducted in serum supplemented DMEM.

2.3 Cell treatment

The cells were seeded in 24 or 6 well plates and maintained in medium supplemented with FBS, and were allowed to grow for 24h. The doxazosin was dissolved in 20% ethanol/ milli-Q^{TM} water (vehicle) and added to the culture medium at concentrations of 5, 10, 15, 30, 50, 75 and 100 µM for 24 or 48h for strain U-138MG. For the C6 cell line was used the concentrations of 30, 50, 75, 100, 150 and 180 µM for 48h. Cells not treated were used as controls. The concentrations used were diferent because the drug is species specific.

2.4 Sulforhodamine B assay

The sulforhodamine B (SRB) assay was used for cell density determination, based on the measurement of cellular protein content [16, 17]. After 48h of treatment, the medium of the C6 and U-138MG cells line was removed, the cells were washed three times with PBS and 500 µL PBS/FORMOL 4% was added. After 15 minutes, fixed cells were stained with surfohodamine B. Subsequently, wells were washed with deionized water to remove the unbound stain. The culture plates were air dried and protein-bound sulfohodamine was solubilizaed in 1% SDS. Absorbance was measured by an ELISA plate reader at 515 nm. This absorbance was linearly proportional to the number of cells. Images were captured using confocal microscope.

2.5 Mitotic index

After 48h of treatment, U-138MG and C6 were fixed with the mixture of ethanol/acetic acid (70:30). Following fixation, cells were stained with DAPI, then analysed by fluorescence microscopy. At least 100 cells were counted for each determination and the result shown is representative of those observed in four experiments. Mitotic cells were judged with chromosome condensation and nuclear membrane destruction [18]. Images were captured using a digital camera connected to the microscope and analyzed using confocal.

2.6 Western blot assay

After treatment for 30 minutes, 24 and 48h with doxasozin, cells were homogenized in lysis buffer (4% sodium dodecyl sulfate (SDS), 2 mM EDTA, 50 mM Tris). Aliquots were taken for protein determination, and β-Mercaptoethanol (Sigma Chemical) was added to a final concentration of 5%. Proteins were resolved (75 µg per lane) on 10 or 12% SDS-PAGE (Sigma Chemical). After electrophoresis, proteins were electro-transferred to nitrocellulose membranes (Hybond™ ECL™ nitrocellulose membrane, Amersham Biosciences™, Freiburg, Germany) using a semi-dry transfer apparatus (Bio- Rad™, Trans-Blot SD, Hercules™, CA, USA). Membranes were incubated for 60 minutes at 4°C in blocking solution (Tris-buffered saline containing 5% powdered milk and 0.1% Tween-20, pH 7.4). After blocking, membranes were incubated overnight with specific antibodies. Primary antibodies (purchased from Cell Signaling Technology™, Beverly, MA, USA) against the following proteins were used: p-Akt_{Ser473} and Akt (1:1000), p-GSK-$3\beta_{Ser9}$ and GSK3β (1:1000). The

membranes were then incubated with horseradish peroxidase conjugated anti-rabbit antibody (1:1000; Amersham Pharmacia Biotech, Piscataway, NJ, USA). Chemioluminescence (ECL, Amersham Pharmacia Biotech™) was detected using X-ray films (Kodak X-Omat™, Rochester, NY, USA).

2.7 LDH

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture medium upon damage of the plasma membrane. Cell death was evaluated by measuring the activity of lactate dehydrogenase (LDH, E.C.1.1.1.27). Briefly, after 48h treatment with doxazosin, the U-138MG and C6 cell culture medium was collected and incubated by enzymatic colorimetric reaction (Cytotoxity Detection Kit – LDH, Roche Applied Science) Absorption was measured at 490nm.

2.8 Alcian blue staining

Alcian blue stains acid muco substances and acetic mucins [19]. After 48h of treatment, the medium of the U-138MG human glioma cell line was removed, the cells were washed three times with PBS, and fixed with ethanol 70% [20]. The cells were stained with Alcian blue over night. Subsequently, wells were washed with deionized water to remove unbound stain. After, images were captured using a digital camera connected to the microscope.

2.9 Statistical analysis

Data are expressed as means±SD. All results are representative of at independent experiments. Analysis of variance (ANOVA) was applied to the means to determine statistical differences between experimental groups. Post hoc comparisons were performed by Tukey test. Differences between mean values were considered significant when $p<0.05$.

3. Results

3.1 Doxazosin decrease cell density on U-138MG and C6 lines

Cells were quantified with Sulphorhodamine B assay (SRB). SRB assay is widely used for cell density determination, based on the measurement of cellular protein content [16, 17]. In the figure 1A, we can be observed that the concentrations (µM) 150 and 180 induced a decrease on cell density in C6 lineage cells as compared to the control. We observed a similar decrease in cell density in the line U-138MG with concentrations of 50 and 75µM of doxazosin (figure 1B).

Once SRB is a molecule that emits fluorescence, we evaluate cell morphology after doxazosin exposure. In figures 2a and 2b we can observe that cells treated with doxazosin were more rounded, probably due to a decrease in adhesion proteins. This effect was more pronounced for U-138MG cells.

3.2 No mitotic activity after exposure to doxazosin

Mitotic activity is one of the parameters used for prognostic and diagnostic of gliomas. We performed the Mitotic Index assay using the concentration of 30, 50 and 75µM for the U-138MG and 100, 150 and 180µM, for the C6 glioma cells. As shown in figure 3, no significant difference was observed.

DAPI, the stainning used to the proliferation assay, form fluorescent complexes with natural double-stranded DNA, and by this way it was possible observe the chromossome condention, as can not be seen in Figure 4.

3.3 Doxazosin cells leads to death

The assessment of necrotic cell death can be evaluated by plasma membrane integrity and one of the methods used for this purpose is the quantification of cytoplasmic enzymes activity released into the culture medium. To verify cell death, we treated the U-138MG and C6 glioma cell with the concentration from 15 µM to 75µM, and 30 to 180µM of doxazosin and vehicle for 48h, respectively. As shown in figure 5, the decrease of U-138MG and C6 cells was clearly diminished.

3.4 Alterations in mucin expression on U-138MG cell line

Alterations in the mucin expression or glycosylation, accompanying the process of cancer development and influence cellular growth, differentiation, transformation, adhesion, invasion and immune surveillance and adhesion of tumor cells. Mucins are used as screening diagnostic markers of cancer cells [21]. In figure 6, is shown that the concentration 50(µM) decreases the staining blue on the U-138MG which means a decrease of tomorogenicity.

3.5 Doxazosin effect on cell signaling molecules

As the doxazosin has a chemical structure similar to inhibitors of receptor tyrosine kinase and these drugs cause inhibition of activation of the MAPKs pathway, we decided to analyze the mechanisms involved in cellular signaling [22, 23]. So, we assessed the phosphorylation status of proteins involved in the phosphatidylinostitol-3-kinase (PI3K)/Akt by immunoblotting in U-138MG cell. These signaling pathways play fundamental roles on the regulation of numerous cellular processes such as cell growth, proliferation, apoptosis and survival [24-26].

Thus, the cells were cultured in presence of 30, 50 e 75 (μM) doxazosin for 30 minutes, 24 or 48h and the immunocontent of Akt, pAkt, GSK3β and pGSK3β was analyzed by western blotting as described in materials and methods section. Doxazosin was added for 30 minutes, 24 and 48h. As shown in figure 7A, Akt immunocontent decreased, as well as p-Akt after 48h of treatment tested concentrations (30, 50, 75 μM).

Additionally, we extended our observations to evaluate whether Akt downstream target molecule GSK-3β was affected. As shown in figure 7B, GSK-3β levels decreased at 24 and 48hs of treatment.

4. Discussion

Through analysis of death and cell density for evaluation of (U-138MG) human and rat (C6) glioma lines, we found that doxazosin was effective in inducing death in both lines at concentrations of 50μM and 75, 150 and 180μM, respectively. The main

routes of control of proliferation are regulated by extracellular signals that targets the RTKs, among which we highlight the growth factor receptors such as EGFR [24].

Through this signaling, one of the pathways that can be activated is PI3K/Akt. When active, this means operative to promote activation or inhibition of a several of effectors involved in signals that regulate cell death, angiogenesis, proliferation, differentiation, migration and survival. According to the results obtained by evaluating the phosphorytated form of Akt protein (pAkt) after treatment 30 minutes, 24 and 48h in the human line (U-138MG), it can be seen that within the first 24h, a decrease in the pAkt immunocontent, and this was maintained after 48h of treatment at the concentrations 30, 50 and 75μM. Also it is observed a decrease on the immunocontent of Akt at concentration of 75μM only after 48h of treatment. Regarding the protein GSK3β, which downstream of Akt, we observed a decrease on the phosphorylated form (pGSK3β-immunocontent) at 24h (75μM) and 48h of treatment at tested concentrations (30, 50, 75 μM).

The decrease on the immunocontent of Akt and its phosphorylated form, in non tumor cells, is linked with an decrease activity of the GSK-3β protein. We can speculate that doxazosin could be acting by increase activity of GSK-3β protein which induce a decrease on the phosphorylation of pGSK-3β$_{Ser9}$ after 24h of treatment. The decrease of GSK-3β phosphorylation increase its enzyme activity. Based on this fact, we can infer that GSK-3β promotes apoptosis by inhibing prosurvival transcription factors, such as CREB and heat shock factor-1, and facilitating proapoptotic transcription factors such as p53.The decrease of the phosphorylation / activation of Akt protein leads to a decrease in phosphorylation of the MDM2 protein inhibitor of constitutive p53, which, when phosphorylated, becomes less active, leaving free p53 to exert its actions.

Thus, levels of p53 increase and protein migrates to the nucleus, acting as a transcription factor. These genes, when transcribed, have their action involved in cell cycle arrest, and some cell death pathways (mainly apoptotic pathway). The Akt immunocontent diminished, can regulate pro-apoptotics proteins, for example, Bax and XIAP, which would add to this effect programmed cell death induced by the activation of p53.

Another mechanism of action of Akt protein is decreases the phosphorylation/activation of protein PRAS40 (on Thr_{236}). This protein is associated with the resistance mechanisms in cancer cells.

Conversely, decreased expression and/or of Akt leads to a decrease of mTOR protein phosphorylation (onSer_{2448}). Additionally, mTOR participates in the inactivation of 4E-BP1, eIF4E inhibitor (initiation factor of the eukaryotic translation 4E). These events result in activation of the translation of specific subsets of mRNAs in cell proliferation, as this complex is less active, when Akt protein is diminished, results in decreased cell proliferation. The inhibition of Akt and activation GSK3β, lead the activation of proteins that are responsible for cell cycle control. This inhibitory action of both leads to a synergistic effect, both in apoptosis, such as antiproliferative effect [24-28].

Some authors include the SRB assay as a test of cell proliferation [29]. Thus, Our results of Akt immunocontent and SRB, suggest that doxazosin decreases proliferation in both cell lines. The presence of necrotic cells in gliomas cell cultures treated with therapeutic doses of doxazosin was detected by the measurement of the release LDH activity. In confocal microscopy images of cultures exposed to doxasozin through the SRB technique, we observed the retraction of the cytoskeleton and the decrease of the extensions ("blebs") cell, confirming the results obtained by

analyzing the immunocontent and Alcian Blue. These mechanisms of cell death may occur concurrently, and one does not exclude other through the exposure.

4.1 Conclusion

Taken together, our results show that doxazosin was able to significantly induce cells death of both, human and rat glioma lines (U-138MG and C6 respectively). The mechanisms associated with this effect involve apoptosis induction.

The results of this study point to the importance of doxazosin as a promising drug for the development of new drugs. By optimizing the chemical structure of the molecule synthesis standard, the doxasozin can be used in the adjuvant treatment of glioblastomas.

5. Acknowledgements

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7. Legends

Figure 1. Effect of doxazosin for 48 hours on cell density assay (SRB) of C6 (A) and U-138 (B) glioma cell lines. Data are means±SEM (n=4). ***p<0.001 compared to respective control, ANOVA followed by Tukey's test.

Figure 2a. Confocal microscopy. SRB assay of C6 (A)(D) Control group (B)(E)100μM (C)(F) 180μM of doxazosin after treatment for 48hours (Panels at the top are correspondent SRB staining and down photomicrographs). Magnification: 10X + 2,5. 2b. Confocal microscopy. SRB assay of U-138MG (A)(C) Control group (B)(D) 50μM of doxasozin after treatment for 48hours (Panels at the top are correspomdent SRB staining and down photomicrographs). Magnification: 10X + 2,5.

Figure 3. Mitotic index of C6 (a) and U-138MG (b) cells after 48hs of treatment with the indicated concentration of doxazosin. No significant difference.

Figure 4. DAPI staining microscopy. Effect of doxazosin on mitotic index in C6 (A) control group (B,D) 100μM (C) 180μM and U-138MG cells (E) control group (F,H) 50μM (G,I) 75μM , as assessed by DAPI staining. Cells were treated with indicated concentration for 48hs. Magnification: 10X+2,5.

Figure 5. Effect of doxazosin for 48 hours on cell death (LDH assay) of C6 (a) glioma and U-138 (b) cell line Data are means±SEM (n=4). ***p<0.001 compared to respective control, ANOVA followed by Tukey's test.

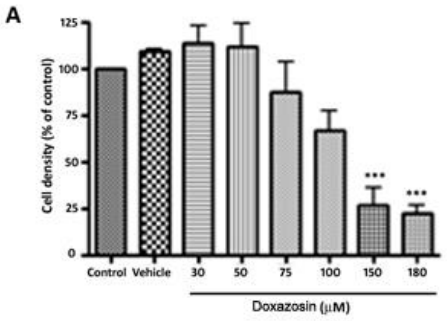
Figure 6. Alcian Blue staining microscopy. Effect of doxazosin on mucines in U-138MG cells. Cells were treated with 50μM for 48hs. (A), (E) control group (B), (D) 50μM of doxasozin. Magnification: 10X+2,5.

Figure 7. Effect of doxazosin on pAKTSer473, AKT(A) and pGSK-3ßSer9, GSK-3ß (B). Cells were exposed to 30, 50, 75 μM doxasozin for 30min, 24, 48hs (Akt) and 24 and 48hs (GSK-3ß).Were assessed by western blotting using the respective specific antibody(n=3). ß-actin was used as an internal control.

Figure 8. Photomicroscopy. U-138MG (A) after treatment for 48hours. Presence of apoptotic (arrow) and senescent (arrow*) cells. Magnification: 10X .

Figures

Fig.1



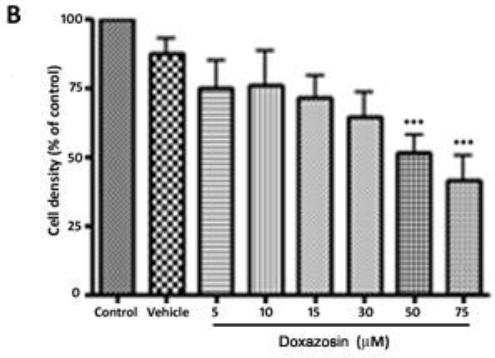
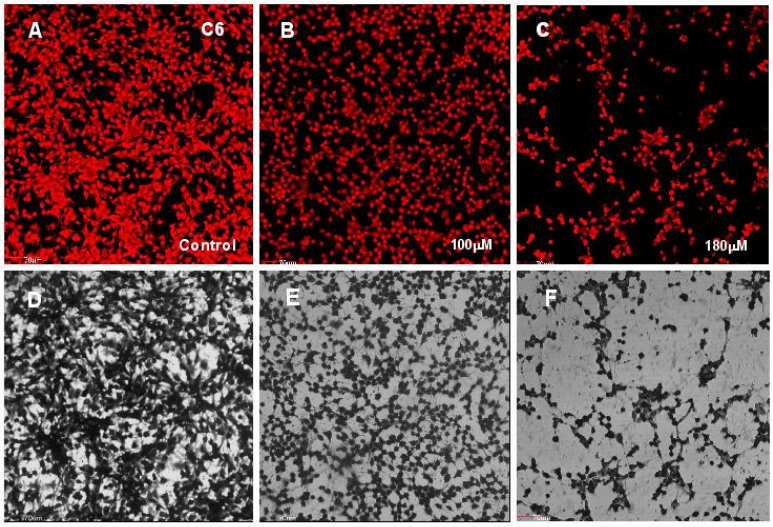


Fig.2

a



b

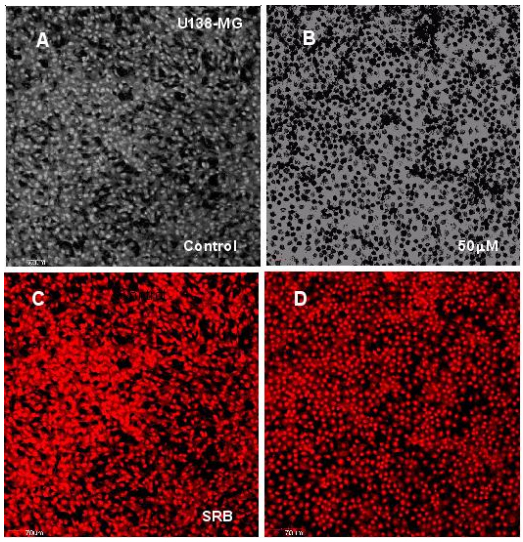
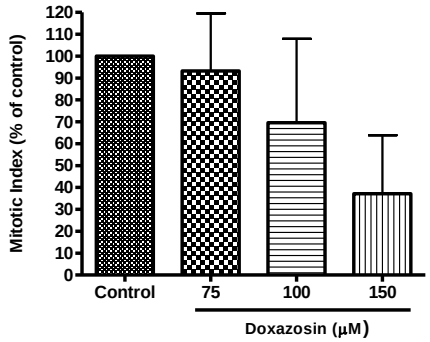

A
U138-MG
Control
B
50μM
C
SRB
D

Fig.3

a


Mitotic Index (% of control)
120
110
100
90
80
70
60
50
40
30
20
10
0
Control
75
100
150
Doxazosin (μM)

b

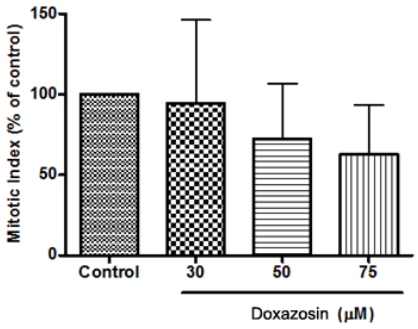

Mitotic Index (% of control)
150
100
50
0
Control
30
50
75
Doxazosin (μM)

Fig.4

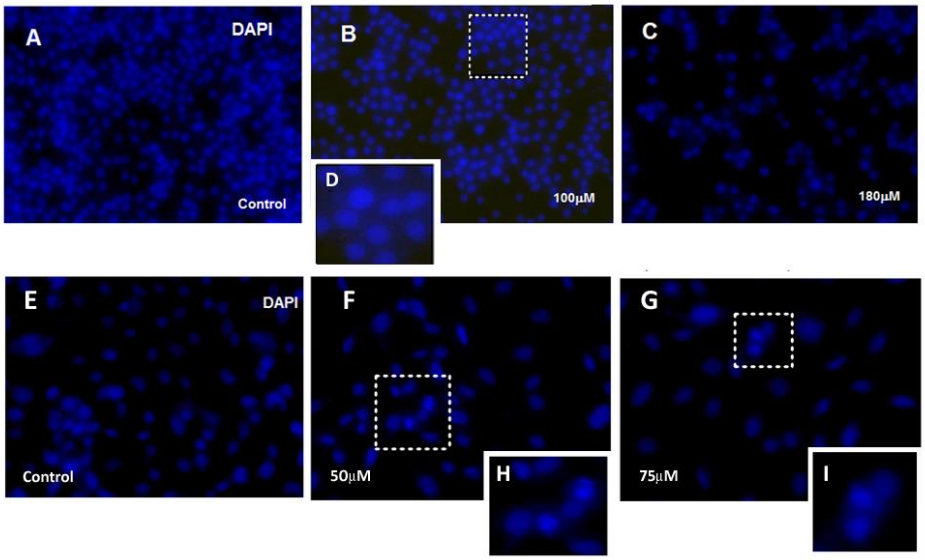
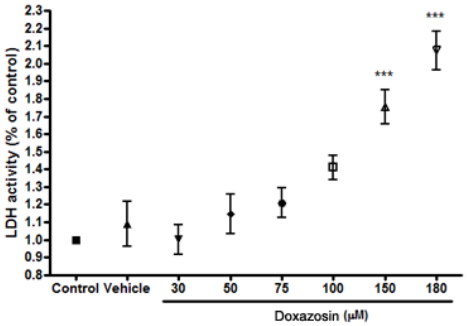

A
DAPI
Control
B
D
100μM
C
180μM
E
DAPI
Control
F
50μM
H
G
75μM
I

Fig.5

a


LDH activity (% of control)
2.3
2.2
2.1
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
***
***
Control Vehicle
30
50
75
100
150
180
Doxazosin (μM)

b

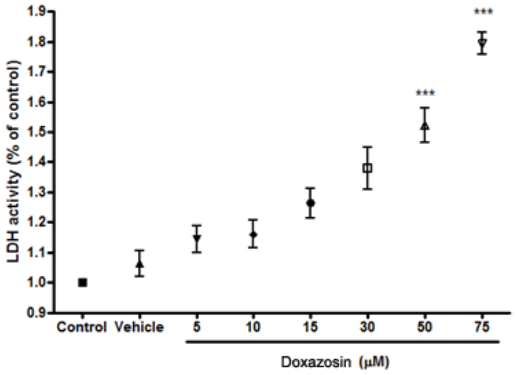

LDH activity (% of control)
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
***
***
Control
Vehicle
5
10
15
30
50
75
Doxazosin (μM)

Fig.6

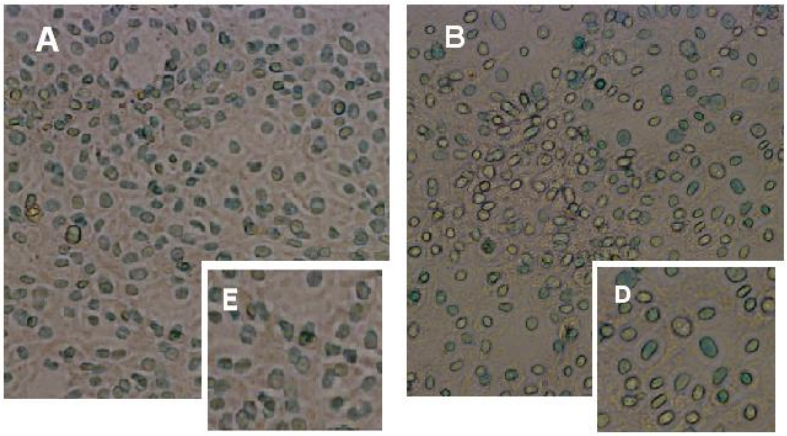

A
B
E
D

Fig.7

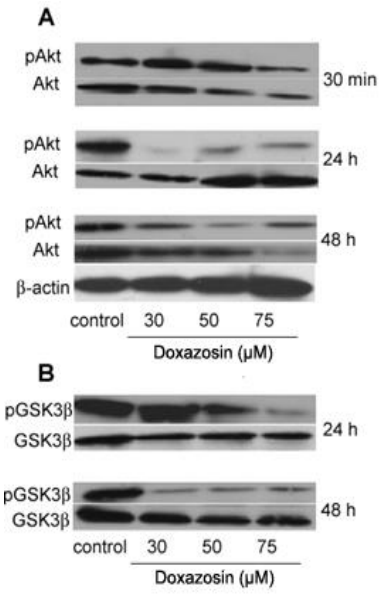
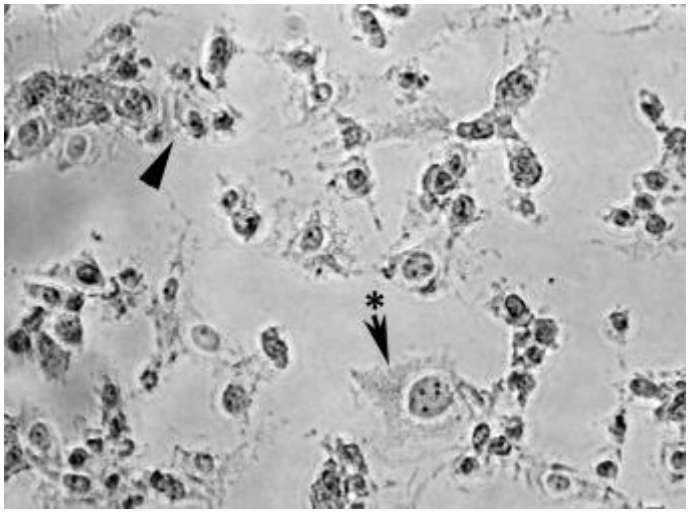

A
pAkt
Akt
30 min
pAkt
Akt
24 h
pAkt
Akt
48 h
β-actin
control 30 50 75
Doxazosin (μM)
B
pGSK3β
GSK3β
24 h
pGSK3β
GSK3β
48 h
control 30 50 75
Doxazosin (μM)

Fig.8



IV - DISCUSSÃO

Glioblastoma representa o mais comum e maligno tumor primário cerebral (Holland, 2000; Wechsler-Reya e Scott, 2001; Zhu et al., 2002; Ghosh et al., 2005; Huse et al., 2012). Os tumores cerebrais são responsáveis por 2% dos cânceres de adultos, apresentando a terceira menor sobrevida e uma incidência de 5-8/100000 habitantes. Apresentam alta taxa proliferativa e são muito invasivos, sendo resistentes aos tratamentos padrões utilizados na clínica. Pacientes com esse tipo de tumor possuem uma alta taxa de recidiva.

Os fármacos utilizados para essa malignidade ainda se mostram ineficazes ao tratamento desses tipos de câncer, pois apresentam muitos efeitos adversos, além de apresentarem dificuldade de ultrapassar a barreira hematoencefálica, sendo que muitas vezes a necessidade de altas doses terapêuticas provoca toxicidade.

Alterações gênicas são frequentes nos glioblastomas, sendo que essas alterações resultam em desregulação de vias de sinalização intracelulares que acabam levando a um aumento da proliferação, sobrevivência, invasão, resistência à morte celular programada e angiogênese tecidual (Rasheed et al., 1999; Maher et al., 2001; Wechsler-Reya e Scott, 2001; Ghosh et al., 2005).

A doxazosina, apesar de ser um anti-hipertensivo utilizado na clínica, possui características na sua estrutura química que a torna semelhante a vários fármacos antitumorais já em uso, bem como a outros que ainda estão em desenvolvimento. O anel quinazolínico, pertencente à estrutura química da molécula, pode fazer parte do sítio de ação da doxazosina responsável pelo efeito antitumoral. Os grupamentos doadores de elétrons presentes na posição 6 e 7 (na figura 2) são importantes para o efeito inibitório do fármaco.

Outro fator relevante é a similaridade com inibidor do receptor tirosina cinase (PD1535), sendo seu provável mecanismo de ação antitumoral associado a essa analogia (Oliveira, 2009).

Desta forma, baseado nos resultados obtidos desta dissertação, a doxazosina mostrou-se um fármaco promissor no tratamento dos gliomas.

Características físico-químicas presentes em sua estrutura possibilitam a passagem do fármaco pela barreira hematoencefálica, bem como as doses que foram utilizadas nos ensaios desenvolvidos neste estudo correspondem a doses relevantes para uso na clínica.

O primeiro passo, objetivando avaliar o uso do fármaco na cultura *in vitro*, foi o de certificar que realmente é a doxazosina que exerce ação neste ambiente.

Através da avaliação físico-química (HPLC) e do ensaio de captação, podemos afirmar que a doxazosina é estável no ambiente de cultivo, durante 48 horas. O fármaco não apresentou degradação em subprodutos através da interação com os constituintes do meio e metabólitos da própria célula, que poderiam estar exercendo efeito tóxico durante o tratamento, sendo atribuído a esses o efeito antitumoral. A emissão da fluorescência pela molécula, devido à presença de grupos cromóforos (mostrados na fig.8 do artigo II) em sua estrutura química, possibilitou a avaliação da captação do fármaco pelas células.

Mediante o ensaio de MTT na linhagem (C6), fizemos um *"screening"* inicial para avaliarmos o efeito inibitório da doxazosina sobre a viabilidade celular. Demonstramos que o fármaco é capaz de reduzir a viabilidade celular após 24 horas e 48 horas de tratamento.

É interesante observar que nas concentrações em que o fármaco exerce efeito antitumoral, visto no artigo II, não foi observado citotoxicidade em modelos de

célula/tecido não tumoral (cultura primária de astrócitos e em cultura organotípica) (figuras 3 e 4). Estes resultados sugerem que o mecanismo da ação da doxazosina possui certa especificidade ao tecido canceroso, viabilizando uma possível avaliação *in vivo* do fármaco. A toxicidade é um problema associado a vários fármacos, principalmente quimioterápicos.

É descrito na literatura que os glioblastomas caracterizam-se por apresentarem uma variedade de anormalidades gênicas, incluindo a desregulação de enzimas celulares e proteínas transportadoras de membranas, aberrações genômicas e alterações da susceptibilidade a apoptose (Wechsler-Reya e Scott, 2001; Ghosh et al., 2005).

Diante dessas considerações, é interessante observar que quando foram utilizadas altas concentrações de doxazosina (artigo II- capítulo II, figura 1), ainda são observadas células remanescentes, sugerindo que possa haver células resistentes ao tratamento. Como os glioblastomas apresentam uma heterogeneidade genética e molecular, as células que carregam anormalidades diferentes podem responder de forma diferente à terapia. As células remanescentes merecem um estudo mais aprofundado de caracterização molecular (Wen et al., 2006).

Através da análise da morte e avaliação da densidade celular para as linhagens de glioma humano (U-138MG) e de rato (C6), vimos que a doxazosina foi eficaz em induzir morte em ambas as linhagens nas concentrações de 50 e 75µM; 150 e 180µM, respectivamente.

As principais vias de controle da proliferação são reguladas por sinais extracelulares que tem como alvo os RTKs, entre os quais se destacam os

receptores de fatores de crescimento como o EGFR (Goldstein et al., 2008; Chilin et al., 2010).

Através dessa sinalização, uma das vias que pode ser ativada é a via da Akt. Quando ativa, esta via funciona promovendo a ativação ou inibição de uma série de efetores envolvidos em sinalizações que regulam a morte celular, angiogênese, proliferação, diferenciação, migração e sobrevivência.

De acordo com os resultados obtidos através da avaliação do imunoconteúdo de proteínas da via Akt (artigo III- capítulo III), após o tratamento de 30 minutos, 24 e 48 horas na linhagem humana (U-138MG), pode-se perceber que já nas primeiras 24 horas ocorre a diminuição no imunoconteúdo; mantendo-se diminuído no tratamento de 48 horas nas concentrações 30, 50 e 75µM de doxazosina. Percebe-se que houve diminuição do imunoconteúdo da Akt total na concentração de 75µM em 48 horas de tratamento. Em relação à proteína pGSK-3β$_{Ser9}$, que se localiza abaixo (*"downstream"*) da Akt, também foi observado diminuição do imunoconteúdo, mas somente após o tratamento de 24hs, na concentração de 75µM, e de 48 horas, em todas as concentrações (30, 50, 75µM) que as células foram expostas.

O aumento da ativação da proteína pGSK-3β$_{Ser9}$ (diminuição da fosforilação) como foi demonstrado no artigo III, pode estar associado a inibição da transcrição de fatores pró-sobrevivência e proliferativos.

A diminuição da fosforilação (ativação) da proteína Akt leva a uma diminuição na fosforilação da proteína MDM2, inibidor constitutivo da p53, que, quando fosforilada, torna-se menos ativa, deixando a p53 livre para exercer suas ações.

Dessa maneira, os níveis de p53 aumentam e a proteína migra para o núcleo, agindo como fator de transcrição. Esses genes, quando transcritos, têm sua ação envolvida na parada do ciclo celular, e algumas vias de morte celular (principalmente

a via apoptótica). A ativação da p53 está associada a outro mecanismo de morte celular, que é a senescência, como mostrada na fig.8, do artigo III – capítulo III, morfologia celular característica de morte por senescência (Huse, Holland e DeAngelis, 2012).

Akt também é responsável pela regulação de proteínas pró-apópoticas, como, por exemplo, a Bax e XIAP, o que poderia resultar em um efeito adicional de morte celular programada, induzida pela ativação da p53 (Gesbert, 2000).

Alguns autores incluem o teste de SRB como sendo teste de proliferação celular (Voight, 2005). Assim, baseando-se neste teste, ocorreu a diminuição da proliferação celular em ambas as linhagens.

Observando as imagens de microscopia confocal das culturas expostas à doxasozina, através da técnica de SRB, observou-se a retração do citoesqueleto e a diminuição dos prolongamentos (“*blebs”*) celulares.

O ensaio de morte celular, através da liberação da enzima LDH, confirma a morte celular necrótica e o extravasamento de enzimas e proteínas no meio intracelular por permeabilidade/lesão da membrana plasmática.

Os resultados do presente estudo apontam para a importância da doxazosina como uma droga promissora para o desenvolvimento de novos fármacos. Enfim, a doxazosina pode ser utilizada no tratamento coadjuvante de glioblastomas, bem como sofrer modificações em sua estrutura química visando otimização nos seus mecanismos de ação. Segue em anexo a fig. 2, que demostra o possível mecanismo de ação da doxazosina e seu envolvimento na via da Akt.

V - CONCLUSÕES

- ✓ A doxazosina foi capaz de reduzir significativamente a quantidade de células tumorais após o tratamento nas linhagens de glioma humano (U-138MG) e de rato (C6).
- ✓ Provavelmente os mecanismos relacionados com a morte celular envolvem a via de sinalização da PI3K/Akt, não excluindo a participação de outras vias.
- ✓ A doxazosina induz morte celular, podendo estar associada a apoptose, senescência e/ou autofagia, merecendo investigação mais detalhada para caracterização das mesmas.
- ✓ O tratamento nas concentrações efetivas de indução de morte celular, nas células tumorais, não exerceu efeito citotóxico na cultura primária de astrócitos, nem mesmo para o tecido cerebral não tumoral de ratos (cultura organotípica de hipocampo de ratos).
- ✓ O fármaco induziu morte por necrose em ambas as linhagens, conforme visto no ensaio de LDH.
- ✓ O fármaco é estável em meio de cultivo por 48 horas, é captado pelas células (C6) e possui autofluorescência.
- ✓ A doxazosina pode ser utilizada no tratamento coadjuvante de glioblastomas, bem como sofre modificações em sua estrutura química visando otimização nos seus mecanismos de ação.

VI - PERSPECTIVAS

- ✓ Avaliar os mecanismos de morte celular com marcadores específicos para cada tipo de morte: LC3 (autofágica), ensaio de caspases 3, 10, anexina V (apoptose), senescência (Rb, p21, β-galactosidase).
- ✓ Verificar proliferação celular em citometria de fluxo com marcadores (Ki67, Brdu), bem como avaliar ciclo celular.
- ✓ Analisar a expressão da proteína PI3K, a fim de relacionar a ativação do receptor tirosina quinase com o mecanismo de ação da doxazosina.
- ✓ Caracterizar o possível mecanismo de morte celular em tumores de rato.
- ✓ Verificar se a doxazosina apresenta efeito sinérgico com outras drogas quimioterápicas já utilizadas na clínica (por exemplo, temozolamida), numa tentativa de otimização da terapêutica através de estudo *in vitro*.
- ✓ Caracterizar as subpopulações resistentes através de biomarcadores (CD133, nestina, OCT-4) por imunocitoquímica.

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ANEXO

1. Figura

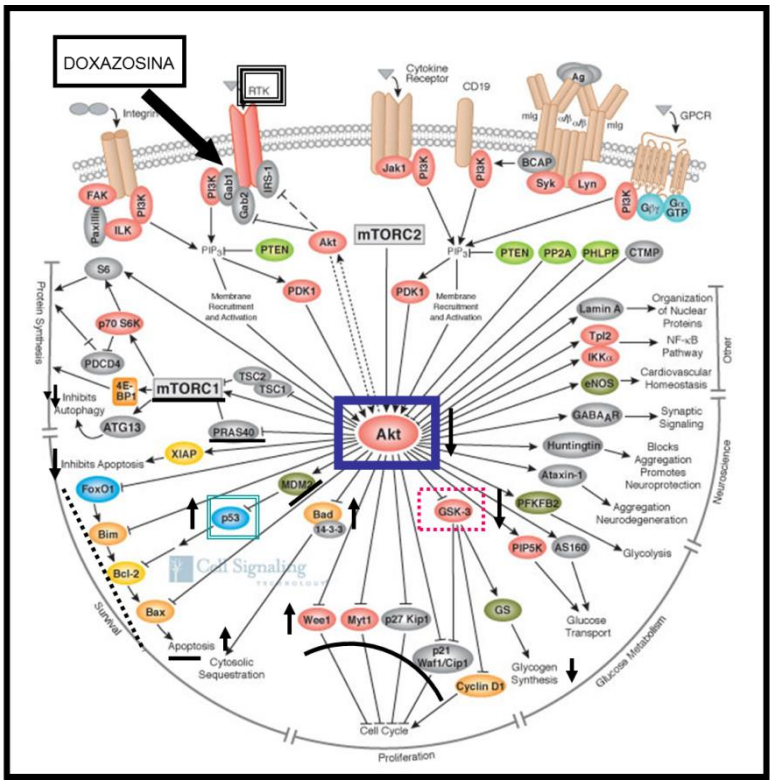

Figura 1. Via da PI3K/Akt e possíveis mecanismos de ação da doxazosina. As setas indicam ativação, enquanto os traços indicam inibição. Obtido de: http://www.cellsignal.com/pathway/akt-signaling.jsp