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FISIOLOGIA ANIMAL COMPARADA

MECANISMO DE AÇÃO DO ÁCIDO ACETILSALICÍLICO EM LINHAGENS CELULARES LEUCÊMICAS MDR E NÃO MDR

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Sumário

Lista de abreviaturas

AAS – ácido acetilsalicílico
ABC – "ATP binding cassette"
bcl-2 – gene responsável pela sobrevivência celular
Bcl-2 – proteína codificada pelo gene bcl-2
B-CLL – linhagem celular de leucemia linfocítica crônica
COX – enzima da via da ciclooxigenase
COX-1 – isoforma constitutiva
COX-2 – isoforma induzida
DOX – doxorubicina
EROs/ROS – espécies reativas de oxigênio
gpP – glicoproteína P
HCA-7 – linhagen celular de câncer colorretal humano
HCT116 - linhagen celular de câncer colorretal humano
HT29.FU - linhagen celular de câncer colorretal humano
K562 – linhagem celular de leucemia mielóide crônica
LMC – leucemia mielóide crônica
LRP – proteína associada a resistência em câncer de pulmão
Lucena – linhagem celular MDR
MDR – resistência a múltiplas drogas
MRP – proteína associada a resistência a múltiplas drogas
NF-κB – fator de transcrição nuclear
NSAIDs – drogas antiinflamatótias não-esteróides
p53 – gene supressor tumoral
P53 – proteína codificada pelo gene p53
SW480 - linhagen celular de câncer colorretal humano
VCR – vincristina

Resumo Geral

As estatísticas com relação ao câncer são impiedosas. Uma em cada cinco pessoas desenvolverá uma forma de câncer em determinado momento de sua vida e ainda é importante considerar que tumores malignos já foram constatados também em plantas e em outros animais. Além das ferramentas convencionais para o tratamento do câncer, que incluem radioterapia, quimioterapia e cirurgia, outras terapias alternativas têm sido propostas, como a terapia fotodinâmica. Uma outra tentativa promissora no combate ao câncer vem sendo demonstrada com o uso do ácido acetilsalicílico (AAS). O AAS, o salicilato mais importante da família de drogas antiinflamatórias não-esteróides (NSAIDs), adquiriu popularidade em 1899, quando foram reconhecidas suas propriedades antiinflamatórias. Dados experimentais sugerem que AAS e outros membros da família de NSAIDs inibem o crescimento de células cancerosas *in vitro* e *in vivo*. É atribuído às prostaglandinas o poder de iniciar e promover o câncer por causar a proliferação celular, inibição da apoptose (morte celular programada), estimulação da angiogênese ou supressão da resposta imune. Inibir a enzima Cox está relacionado com a inibição da produção de prostaglandinas, sugerindo assim a inibição do processo de cancerização. O AAS inibe irreversivelmente a enzima Cox em determinados tipos celulares, sendo que esta inibição é não-seletiva para ambas as isoformas da Cox; Cox-1 (isoforma constitutiva) e Cox-2 (isoforma induzida). Entretanto, estudos sugerem que o efeito antiproliferativo de AAS não está correlacionado exclusivamente com a ação inibitória da enzima Cox, já que existem relatos mostrando que NSAIDs podem induzir apoptose em células de câncer de cólon que não expressam a proteína Cox-2. Neste sentido, alguns autores demonstraram uma inibição no crescimento *in vitro* de células tumorais do endométrio humano pelo AAS, de uma maneira dose-dependente, sendo a apoptose um dos mecanismos envolvidos nesta resposta, mediada em parte pela "downregulation" do gene *bcl-2*. A redução no número de apoptoses contribui para o desenvolvimento do câncer sendo o gene *bcl-2* o primeiro membro de uma família de genes que regulam este processo. Foi demonstrado que a superexpressão do gene *bcl-2* aumenta a sobrevida das células tumorais protegendo-as da toxicidade causada pelos

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quimioterápicos, não permitindo que a apoptose ocorra. Por outro lado, a indução da apoptose é uma das ações centrais pela qual a proteína P53 exerce função na supressão do tumor. Esta proteína previne a transmissão da informação genética defeituosa para a geração das células seguintes, sendo denominada "a guardiã do genoma" e a perda desta função é um achado freqüente em câncer. A mutação do gene *p53* provavelmente inativa a função supressora da proteína P53, conferindo vantagem de crescimento celular, podendo contribuir para o desenvolvimento de tumores, dentre eles, as leucemias. Também a propriedade antioxidants das NSAIDs tem sido investigada, sendo que alguns autores também atribuem a isso os efeitos antitumorais do AAS. Também é de extrema relevância considerar a possibilidade de que determinadas células tumorais podem adquirir resistência a múltiplas drogas, caracterizando o fenótipo MDR. Atualmente, a procura de novas drogas capazes de vencer o mecanismo MDR e conduzir a morte de células tumorais é de extrema importância para a terapia do câncer. Assim, criar um modelo biológico que permita estudos comparativos entre uma linhagem tumoral MDR e uma não MDR é pertinente. Com base nas informações levantadas sobre a possível atividade antitumoral do AAS, objetivamos analisar como parâmetros de estudo sua citotoxicidade (em células tumorais e não tumorais); morte celular; atividade antioxidante e alterações de expressão nos genes *cox-2*, *bcl2* e *p53*, utilizando como modelos biológicos linhagens celulares normais e tumorais MDR e não MDR. AAS inibiu a proliferação celular ou induziu toxicidade nas linhagens celulares K562 e Lucena desconsiderando o fenótipo MDR. O tratamento com AAS provocou morte, nas células K562, principalmente por apoptose inicial e por necrose, nas células Lucena. Também AAS mostrou uma capacidade antioxidante em ambas linhagens. A expressão do gene *bcl-2* não apresentou diferenças significativas, considerando as células controle e tratadas com AAS, bem como as duas linhagens celulares. Para os genes *p53* e *cox-2*, a expressão foi concentração dependente para as células K562. Já para as células Lucena, a expressão de ambos os genes foi aumentada nas menores concentrações e, para o gene *p53*, diminuída na maior concentração quando comparadas as células controle. Como o perfil das expressões foi similar para os genes *p53* e *cox-2* foi possível sugerir

um fator de transcrição comum, justificando esta resposta. Por outro lado, os linfócitos normais tratados com as mesmas concentrações de AAS foram mais resistentes do que as linhagens tumorais. Os resultados deste trabalho mostraram que as duas linhagens celulares foram sensíveis ao tratamento com AAS, mas permitem sugerir que o mecanismo de ação foi diferenciado nas linhagens MDR e não MDR.

Introdução Geral

O corpo de um animal pode ser visto como uma sociedade ou um ecossistema cujos membros individuais são as células, que se reproduzem por divisão celular e são organizadas em conjuntos colaborativos ou tecidos. Assim, diferentemente das células de vida livre, tal como bactéria que compete para sobreviver, as células de um organismo multicelular são comprometidas para a colaboração. Mutação, competição e seleção natural operando dentro da população de células somáticas são os ingredientes básicos do câncer: uma doença na qual células individuais mutantes iniciam sua progressão a custa de células vizinhas, mas no final destrói toda a sociedade celular levando o organismo a morte (Alberts et al., 1997).

As estatísticas com relação ao câncer são impiedosas. Uma em cada cinco pessoas desenvolverá uma forma de câncer em determinado momento de sua vida. Em torno de seis milhões de óbitos por ano já são atribuídos ao câncer em todo mundo. Somente no Brasil ocorrem cerca de 270.000 novos casos por ano. O câncer é responsável por 13% a 15% dos óbitos ocorridos no país. É a segunda causa de morte em nosso meio (Sabbi, 2000).

Os tumores malignos já foram constatados em plantas e em outros animais (Sabbi, 2000). No caso das plantas, o câncer tem como um dos fatores oncoiniciadores a bactéria de solo *Agrobacterium tumefaciens*, que provoca na planta um incremento nos processos mitóticos que levam ao câncer (Matveeva et al., 2001). Também é proposto que fatores fito-hormonais, genéticos e nutricionais estão associados ao câncer em plantas (Sparrow et al., 1956). Em animais, temos os tumores de mama, por exemplo, que estão entre os mais comuns encontrados em cães, representando de 25% a 50% de todos neoplasmas nesta espécie, enquanto que em gatos, este tipo de câncer, não é muito comum (Madewell e Theilen, 1987; Carpenter et al. (1987), apud Millanta et al., 2005). Outro exemplo de tumores em animais é a fibropapilomatose que ocorre em tartarugas marinhas e é caracterizado por múltiplos tumores fibrovasculares cutâneos e viscerais (Herbst, 1994).

Os dados citados acima são bastante alarmantes, mas é sabido que o câncer é controlável em 75% dos casos e se descoberto precocemente, pode ser curado. Tratar esta patologia nas fases iniciais de evolução (iniciação e promoção), oferece maior possibilidade de êxito terapêutico; entretanto, quando atinge a fase de progressão e, mais especificamente, quando há formação de metástases, a probabilidade de cura é muito diminuída (Cotran et al., 2000).

Além das ferramentas convencionais para o tratamento do câncer, que incluem radioterapia, quimioterapia e cirurgia, outras terapias alternativas têm sido propostas, como por exemplo a terapia fotodinâmica, envolvendo uma substância fotossensível, radiação não ionizante de diferentes comprimentos de onda e oxigênio (Trindade et al., 2000; Burch et al., 2005). Uma outra tentativa promissora no combate ao câncer vem sendo demonstrada com o uso do ácido acetilsalicílico.

Corroborando com esta afirmativa, Weiss e colaboradores (2006) sugerem que o uso regular do ácido acetilsalicílico pode ser associado com uma modesta diminuição no risco de leucemia aguda em humanos. Shiff e colaboradores (2003) sugerem que, para a prevenção do câncer colorretal humano, o uso continuado de ácido acetilsalicílico, por um longo período de tempo, pode reduzir cânceres e pólipos.

Drogas antiinflamatórias não-esteróides (NSAIDs) são usadas clinicamente por suas propriedades antiinflamatórias, antipirética e analgésica. Como exemplo de NSAIDs estão os salicilatos que, possuem uma longa história que começa com o uso antipirético de extratos da casca do salgueiro (que contém salicina), documentado pela primeira vez em 1763. Os salicilatos são rapidamente absorvidos no estômago e na porção superior do intestino delgado, produzindo níveis plasmáticos máximos de salicilato dentro de 1-2 h. A aspirina (ácido acetilsalicílico, AAS), o salicilato mais importante, adquiriu popularidade em 1899, quando foram reconhecidas suas propriedades antiinflamatórias. O AAS é absorvido em sua forma inalterada, sendo rapidamente hidrolisado a ácido acético e salicilato por esterases presentes nos tecidos e no sangue (Katzung,

2002). Dados experimentais sugerem que AAS e outros membros da família de NSAIDs inibem o crescimento de células cancerosas *in vitro* e *in vivo* (Arango et al., 2001).

Em contraste com a maioria das outras NSAIDs, o AAS inibe irreversivelmente a enzima Cox em determinados tipos celulares, entre eles as plaquetas (Katzung, 2002). Esta enzima é chave na conversão do ácido araquidônico em prostaglandinas, prostaciclinas e tromboxanos (Dannenberg et al., 2001; Katzung, 2002) (Fig.1). Esta inibição feita por AAS é não-seletiva para ambas as isoformas da Cox; Cox-1 (isoforma constitutiva) e Cox-2 (isoforma induzida), embora alguns antiinflamatórios as inibam de forma seletiva (Bellosillo et al., 1998). As enzimas Cox-1 e Cox-2 diferem quanto a sua regulação na expressão e distribuição no tecido (Smith et al., 1996).

É atribuído às prostaglandinas o poder de iniciar e promover o câncer por causar a proliferação celular, inibição da apoptose (morte celular programada), estimulação da angiogêne se ou supressão da resposta imune (Subongkot et al., 2003), sendo que a angiogênese contribui para o desenvolvimento de lesões pré-neoplásicas invasivas, como adenomas colorretal, bem como carcinomas malignos (Tosetti et al., 2002). Desta forma é possível sugerir que quando a enzima Cox está inibida, também está inibida a produção de prostaglandinas, sugerindo a inibição do processo de cancerização.

Estudos epidemiológicos sugerem que o uso do AAS em longo prazo, em pequenas doses, está associado a uma menor incidência de câncer de cólon, possivelmente relacionada a seu efeito de inibição da Cox (Thun et al., 1991). Entretanto, estudos sugerem que o efeito antiproliferativo de AAS não está correlacionado exclusivamente com a ação inibitória do Cox-2, já que existem relatos mostrando que NSAIDs podem induzir apoptose em células de câncer de cólon que não expressam a proteína Cox-2 (Richter et al., 2001).

Corroborando com a idéia destes últimos autores, Bellosillo e colaboradores (1998), analisaram o efeito do AAS, salicilato e outras NSAIDs na viabilidade de células B-CLL (linhagem celular de leucemia linfocítica crônica) e demonstraram que o AAS induziu uma diminuição na viabilidade celular de uma maneira dose e tempo-dependente e sugeriram que a inibição de Cox não foi

suficiente para induzir a diminuição na viabilidade celular produzida por AAS nestas células. Tanto o AAS como o salicilato causaram fragmentação do DNA, demonstrando que ambos compostos induziram a apoptose em células B-CLL. Outro ponto importante deste último trabalho foi a comparação da sensibilidade ao AAS em células normais mononucleares do sangue periférico e em células tumorais, demonstrando que as células não tumorais são mais resistentes ao AAS do que as células leucêmicas B-CLL.
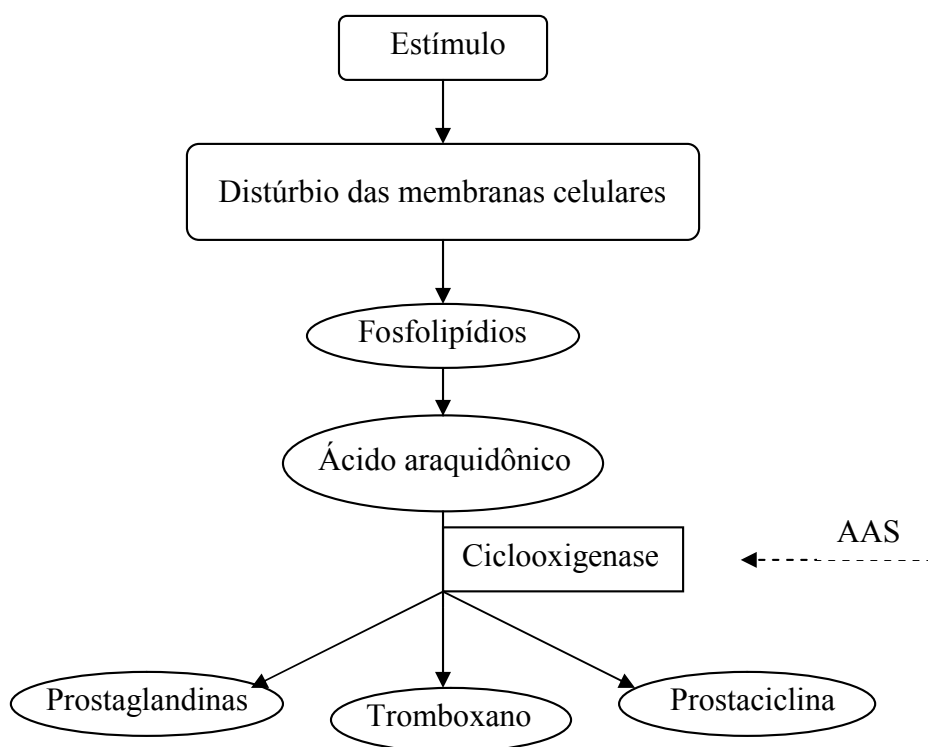

Fig. 1 – Esquema mostrando o local de ação do AAS. (Adaptado de Katzung, 2002)

Ainda avaliando a dependência ou não de Cox na indução da apoptose, Klampfer e colaboradores (1999) demonstraram que o salicilato de sódio, uma NSAID, induz apoptose em células de leucemia mielóide aguda através da ativação de caspases Cox independente.

Estas contradições, quanto a participação ou não da enzima Cox na indução da apoptose, estimulam a análise da expressão gênica de *cox-2* no nosso modelo experimental.

Um outro dado que também serviu como estímulo para este trabalho foi realizado com um outro tipo de NSAID, a indometacina, utilizando as células K562 (leucemia mielóide crônica), uma das

linhagens de estudo neste trabalho, e uma cultura primária de células da medula óssea proveniente de pacientes com leucemia mielóide crônica (LMC), onde foi demonstrada uma indução da apoptose e inibição na proliferação celular (Zhang et al., 2000).

Também Arango e colaboradores (2001) demonstraram uma inibição no crescimento *in vitro* de células tumorais do endométrio humano pelo AAS, de uma maneira dose-dependente, sendo a apoptose um dos mecanismos envolvidos nesta resposta, mediada em parte pela "downregulation" do gene *bcl-2*.

Por outro lado, Smith e colaboradores (2000) demonstraram que AAS inibiu a proliferação de células de câncer colorretal humano HT29.Fu, HCA-7, SW480 e HCT116, porém sem induzir apoptose. Entretanto, recentemente um grupo de pesquisadores mostraram resultados diferentes com relação a linhagem celular SW480 onde foi demonstrado que AAS inibiu a proliferação e promoveu apoptose (Yu et al., 2002).

A redução no número de apoptoses contribui para o começo da carcinogênese e desenvolvimento do câncer. O crescimento do tumor é definido pela diferença entre proliferação e apoptose (Reed, 2000).

O gene *bcl-2* é o primeiro membro de uma família de genes que regulam a apoptose. A família Bcl-2 consiste de membros anti-apoptóticos como a proteína Bcl-2 e de membros pró-apoptóticos como a proteína Bax, por exemplo (Reed, et al., 1998). A expressão regulada destes genes está relacionada à função fisiológica, ou seja, uma mutação em um destes genes leva a uma situação patológica. O câncer está relacionado a uma superexpressão do gene *bcl2*. Foi demonstrado que a superexpressão do gene *bcl-2* aumenta a sobrevida das células tumorais protegendo-as da toxicidade causada pelos quimioterápicos (Dole et al., 1994), não permitindo que a apoptose ocorra.

Por outro lado, a indução da apoptose é uma das ações centrais pela qual a proteína P53 exerce função na supressão do tumor (Moll et al., 2005). A proteína P53, tipo selvagem, é uma fosfoproteína nuclear que atua como um fator de transcrição para genes que controlam o

crescimento celular, sinalizando os pontos de reparo do DNA quando este sofre dano ou induzindo apoptose quando o dano é muito grande para sofrer reparo. Desta forma, a P53 previne a transmissão da informação genética defeituosa para a geração das células seguintes, sendo denominada "a guardiã do genoma" (Lane, 1992). A proteína P53 é codificada pelo gene *p53*, um gene com função supressora tumoral, e a perda desta função é um achado freqüente em câncer (Levine e Momand, 1991). A mutação do gene *p53* provavelmente inativa a função supressora da proteína P53, conferindo vantagem de crescimento celular, podendo contribuir para o desenvolvimento das leucemias (Felix et al., 1992).

Para proceder a análise do papel antitumoral do AAS, que corresponde ao objetivo central deste trabalho, levando em conta as alterações da expressão gênica, julgamos pertinente avaliar a expressão de genes envolvidos na maioria dos tumores, por exemplo os genes *bcl-2* e *p53*.

As propriedades antimutagênicas e antioxidantes de NSAIDs tem sido investigadas. Os efeitos supressivos e protetores do AAS como uma droga antitumoral também pode ser parcialmente atribuída a propriedades antioxidantes.

A utilização do oxigênio molecular por carreadores de elétrons mitocondriais e enzimas durante o metabolismo normal da fosforilação oxidativa, de células aeróbicas de mamíferos, produz espécies reativas de oxigênio (EROs) (Meewes et al., 2001). O oxigênio também é usado como um substrato por diversas outras enzimas. Por exemplo, Jones (1986, apud Storey, 1996) listou 30 enzimas em rim de mamífero, além da enzima citocromo oxidase que usam oxigênio com diferentes afinidades de substrato para oxigênio e envolvendo uma grande variedade de processos metabólicos incluindo o metabolismo biológico das amines, prostaglandinas, purinas, esteróides, aminoácidos e carnitina. Muitas dessas reações geram EROs como seus produtos. As EROs incluem radicais livres como o ânion superóxido (O_2^{-}), o radical hidroxila (OH^{-}) e o oxigênio singlete ($^1O_2$), assim como intermediários não-radicais, como o peróxido de hidrogênio (H_2O_2). Quando as EROs alcançam uma concentração crítica, se sobrepondo às defesas antioxidantes,

poderão ocorrer danos de macromoléculas, tais como, DNA, proteínas e lipídios, caracterizando o processo conhecido como estresse oxidativo (Meewes et al., 2001).

Por outro lado, segundo Darr e Fridovich (1994) para proteção contra o estresse oxidativo, as células aeróbicas possuem um complexo sistema de defesas antioxidantes, incluindo antioxidantes não-enzimáticos, como as vitaminas E e C, ácido úrico e glutationa e antioxidantes enzimáticos, como a superóxido dismutase, catalase e glutationa peroxidase.

Testando a propriedade antioxidante do AAS, Hsu e Li (2002) demonstraram que a inibição de estresse oxidativo por AAS foi concentração dependente em experimentos *in vitro*, e foi proposto que a atividade antioxidante do AAS pode contribuir para quimioproteção de cânceres humanos. Em um estudo mais recente, Antunes e colaboradores (2007) constataram que AAS age como um antioxidante, inibindo os danos induzidos por EROs geradas pela droga antineoplásica doxorubicina.

Finalmente, cabe ainda considerar a capacidade de algumas células tumorais adquirirem o fenótipo de resistência a múltiplas drogas (MDR). Este fenótipo é um fenômeno amplamente estudado, representando a principal causa do insucesso no tratamento quimioterápico do câncer, sendo um fenômeno no qual tumores inicialmente capazes de responder a certos agentes quimioterápicos adquirirem resistência, não somente aos agentes originalmente usados no tratamento, mas também resistência cruzada a outras drogas não relacionadas. As drogas relacionadas ao fenótipo MDR geralmente são alcalóides ou antibióticos originados de plantas ou fungos, assim como compostos citotóxicos, incluindo-se, entre outras, antraciclinas como a daunorubicina e doxorubicina (DOX), alcalóides da *Vinca* como vincristina (VCR) e vimblastina, epipodofilotoxinas (teniposídeo e etoposídeo), antibióticos (actinomicina D), anti-microtúbulos (colchicina e podofilotoxina) e inibidores de síntese proteica (Teeter, 1989; Chin et al., 1993; Pastan e Gottesman, 1991; Gottesman e Pastan, 1993).

São vários os fatores que podem levar ao fenótipo MDR, porém a superexpressão de uma proteína chamada glicoproteína P (gpP) é o mecanismo melhor estudado. Como já foi dito

anteriormente, as drogas para as quais as células que apresentam gpP são resistentes, possuem estruturas e mecanismos de ação bastante diversificados, mas em geral são alcalóides originados de plantas ou fungos, anfipáticos, preferencialmente solúveis em lipídios (Gottesman e Pastan, 1993).

É o gene *mdr1* que codifica a gpP, uma proteína de cerca de 170 KDa, da família das ATPases (super família ABC), que é expressa na membrana celular, e é responsável por um mecanismo de efluxo, dependente de energia, capaz de bombear agentes quimioterápicos para fora da célula (Uchiumi et al., 1993).

Resumindo, uma linhagem celular dita MDR apresenta características como resistência a drogas não relacionadas (Kartner e Ling, 1989; Tiirikainen e Krusius, 1991), expressão de glicoproteína P na superfície da membrana (Gottesman e Pastan, 1993), extrusão do corante rodamina (Neyfakh, 1988) e reversão da resistência pelos agentes reversores trifluoperazina, verapamil e ciclosporina A (Ford e Hait, 1990; Sikic, 1993).

Atualmente, a procura de novas drogas capazes de vencer os mecanismos de resistência e conduzir a morte de células tumorais é de extrema importância para a terapia do câncer (Fernandes et al., 2005). Assim, com o intuito de criar um modelo biológico que permitisse estudos comparativos entre uma linhagem tumoral MDR e uma não MDR, Rumjanek et al. (1994) estabeleceram um modelo *in vitro* utilizando VCR para induzir uma linhagem eritroleucêmica resistente. A essa linhagem MDR foi dado o nome K562-Lucena1 (Lucena) para distinguir de sua linhagem parental K562 e estas células apresentaram as características próprias de linhagem MDR (Maia et al., 1996a; 1996b; Marques-Silva 1996; Orind et al., 1997).

Com base nas informações levantadas sobre a possível atividade antitumoral do ácido acetilsalicílico, estudamos o mecanismo de ação utilizando como modelos biológicos linhagens celulares MDR e não MDR. Tivemos como parâmetros de estudo investigar a citotoxicidade (em células tumorais e não tumorais); morte celular; atividade antioxidante e alterações de expressão gênica a partir do tratamento com AAS.

Objetivos

Objetivo Geral

Estudar o possível efeito antitumoral do AAS em modelos celulares que expressam ou não o fenótipo de resistência a múltiplas drogas.

Objetivos Específicos

- Determinar a viabilidade das linhagens celulares, K562 e Lucena, a diferentes concentrações de AAS.

- Estudar a participação da gpP na resposta celular da linhagem MDR exposta ao AAS.

- Analisar a indução de morte celular por apoptose e/ou necrose nas linhagens K562 e Lucena incubadas com AAS.

- Avaliar a sensibilidade de célula não tumoral ao tratamento com AAS.

- Verificar a produção de espécies reativas de oxigênio nas linhagens K562 e Lucena tratadas com AAS.

- Comparar a expressão gênica de bcl-2 e p53 nas linhagens celulares expostas ao AAS.

- Avaliar a expressão gênica de cox-2 nas linhagens celulares expostas ao AAS.

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Acetylsalicylic acid action mechanism in MDR and non-MDR leukemia cell lines

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Abstract

Acetylsalicylic acid (ASA) is a non-steroidal anti-inflamatory drug (NSAID). ASA has gained attention as potential chemopreventive agent for several neoplasms. The aim of this study was to analyze its possible antitumoral effects in two erythroleukemic cell lines. The mechanism action of different concentrations of ASA were compared in K562 (non-MDR) and Lucena (MDR) cells analyzing cell viability, apoptosis and necrosis, intracellular ROS formation and *bcl-2*, *p53* and *cox-2* gene expression. ASA inhibited the cellular proliferation or induced toxicity in K562 and Lucena cell lines, irrespective of MDR phenotype. The ASA treatment provoked death majority by early apoptosis in K562 cells and by necrosis in Lucena cells. Also ASA showed antioxidant action in both cell lines. The regulation of *bcl-2*, *p53* and *cox-2* genes in both cell lines, treated with ASA seem to present different expressions. On the other hand, the normal lymphocytes treated with the same ASA concentrations were more resistant than tumoral cells. The results of this work showed that both cells were sensitive to ASA treatment but the action mechanisms were different in MDR and non-MDR cell lines.

Keywords: Acetylsalicylic acid; Leukemia; MDR; Death cell; Antioxidant; Lymphocytes; gene expression

Introduction

Acetylsalicylic acid (aspirin, ASA), is one non-steroidal anti-inflammatory drug (NSAID) which is used clinically by its anti-inflammatory, antipyretic and analgesic properties. ASA and other NSAIDs have gained attention as potential chemopreventive agents for several neoplasms, including colorectal [1], endometrial [2], ovarian [3], esophageal [4], lung and breast cancers [5].

Besides the conventional tools for cancer treatment, that includes radiotherapy, chemotherapy and surgery, other alternative therapies have been proposed, e.g., the photodynamic therapy, with use of a photosensitizer substance, different wavelengths non-ionizing radiation and oxygen [6,7]. Acetylsalicylic acid has been pointed out as another method to combat cancer.

ASA is known to inhibit the cyclooxygenases, Cox-1(constitutive isoform) and Cox-2 (induced isoform). These Cox enzymes have been implicated in carcinogenesis through of the production of prostaglandins and the decreased cancer risk may be attributable to analgesic-related inhibition of prostaglandin synthesis, enhancement of cellular immune response, or induction of apoptosis [8]. In such case it is possible to suggest that when the Cox enzyme is inhibited also the prostaglandin production is inhibited, suggesting an inhibition process of cancer formation.

The antimutagenic and antioxidant properties of NSAIDs have been investigated. The suppressive and protective effects of ASA as an antitumoral drug also might be partially ascribed to its antioxidant properties. ASA may have acted as an antioxidant and inhibited the chromosomal damage induced by the free radicals generated by doxorubicin [9]. Also Hsu and Li [10] demonstrated that the inhibition of oxidative stress by ASA was concentration-dependent *in vitro* assays, and it was proposed that the antioxidant activity of ASA may contribute for cancer chemoprotection in humans.

Cell death is an essential phenomenon in normal development and homeostasis, but also plays a crucial role in various pathologies. Apoptosis is genetically regulated and provides a vital protective mechanism against the development of neoplasms by removing cells with DNA damage. Thus, inhibition of apoptosis is conferred as a survival advantage on cells harboring genetic alterations

and may promote acquisition of further mutations that cause neoplastic progression and also contributes to the development of resistance to chemotherapy [11]. The *bcl-2* gene is the first member of a gene family that regulates apoptosis [12]. Overexpression of *bcl-2* gene increases tumoral cells survival, protecting them from chemotherapic toxicity [13] and preventing apoptosis occurrence. In contrast, apoptosis induction is one of the central actions by which P53 protein acts on tumor suppression [14]. The P53 protein, wild type, is a nuclear phosphoprotein that acts like a transcriptional factor for cellular growth genes, repairing DNA when it suffers damage or inducing apoptosis when the damage is too large for repair [15].

One of the major causes of chemotherapeutic failures in cancer treatment is the development of different kinds of resistance. Multidrug resistance (MDR) is a phenomenon by which tumors that initially respond to a determined chemotherapy, acquire resistance to chemically and non-chemically related drugs. The best understood mechanism of MDR is the one conferred by the membrane P-glycoprotein (Pgp), which acts by pumping several unrelated drugs out from the cells [16]. Despite the multifactorial nature of the resistance process, MDR phenotype exhibits some main characteristics: (1) resistance to non-related drugs [17,18], (2) expression of protein as the Pgp [16], (3) extrusion of rhodamine dye [19] and (4) reversion of the resistance induced by agents like trifluoperazine, verapamil and cyclosporin A [20,21]. So, with the intention to create a biological model that permits comparative studies between MDR tumoral cell lines and non-MDR tumoral cells, Rumjanek et al. [22] established an *in vitro* model utilizing vincristine (VCR) to induce a resistant erythroleukemic cell line. These MDR cells were named K562-Lucena1 (Lucena) to distinguish from its K562 parental cell line. The Lucena cells presented the MDR cells characteristics cited above [23-26].

Thus, the aim of the present study was to analyze whether the effect of ASA differs between non-MDR and MDR cells in the citotoxicity, in the induction apoptosis and/or necrosis, in the production of oxidative effects, in the capacity to alter the genes expression and besides to evaluate the normal lymphocyte cells response to different treatment with ASA.

Materials and methods

Cell line and culture conditions

The K562 and Lucena are human erythroleukemic cell lines. They were obtained from the Tumoral Immunology Laboratory at the Medical Biochemistry Department of the Rio de Janeiro Federal University, Brazil. The cells were grown at 37ºC in disposable plastic flasks containing RPMI 1640 (Gibco) medium supplemented with sodium bicarbonate (0.2 g/l) (Vetec), L-glutamine (0.3 g/l) (Vetec), Hepes (25 mM) (Acros), β-mercaptoethanol ($5x10^{-5}$ M) (Sigma), fetal bovine serum (FBS - 10%; Gibco), 1% of antibiotic (penicillin (100 U/ml) and streptomycin (100 mg/ml) Gibco) and antimicotic (0.25 mg/ml - Sigma). Lucena cells were grown under the same conditions as K562 cells, with the addition of 60 nM of VCR (SIGMA) in the culture medium.

Treatment of cells: ASA exposure

Acetylsalicylic acid (Vetec) was purified by recristalization methods [27] and stored in vacuum dessicator. Stock solutions of ASA (1.0 M) dissolved in absolute ethanol (Vetec) were freshly prepared for each experiment with the pH adjusted to 7.4. They were mixed with the culture medium free β-mercaptoethanol to achieve concentrations of 2.5, 5, 10 and 15 mM. For all the assays, untreated cells (control cells) received only absolute ethanol to achieve the maximum concentration of ethanol of the treated cells.

Cell viability assay

The cell viability of K562 and Lucena cell lines was assessed by trypan blue exclusion immediately, 24, 48 and 72 h after incubation with ASA. Cells were grown for 2 days (K562) and for three days (Lucena) before the experiments were performed [28]. Cells were then centrifuged, washed twice with PBS (Ca^{+2}-Mg^{+2}livre) and suspended in RPMI 1640 medium free of β-mercaptoethanol to $5x10^5$ cells/ml. VCR was removed from the medium before the experiments. The cells were treated with different concentrations of ASA (2.5; 5, 10 or 15 mM), or without ASA (control cells) in 24-

well culture plates. Each experiment was performed three times, and each sample was assayed in triplicate. The concentration of 15 mM was not employed in posterior tests because it enhanced citotoxicity (see Results).

Detection of apoptosis/necrosis by annexin-V/PI staining

Quantitative determination of apoptotic and/or necrotic cells was realized after incubation with 2.5, 5 and 10 mM of ASA by 48 hours through a reaction with Annexin V-FITC and Propidium Iodide (PI). Cells were washed twice with PBS ($2x10^5$ cells/well), suspended in 250 µl of binding buffer diluted 10x (0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$) plus 20 µL of Annexin V-FITC solution diluted in binding buffer (1:10). After 20 min of incubation in the dark PI was added (5 µL) and the acquisition by cells was detected by means of flow cytometer (FACSCalibur, BD Biosciences). The percentages of total cells that underwent apoptosis/necrosis were calculated with the Cell Quest Pro program. Annexin V-FITC+/PI- cells were counted as early apoptosis; Annexin V-FITC+/PI+ and Annexin V-FITC-/PI+ cells were counted as necrosis [29].

Sensibility of normal lymphocytes cells to ASA

The normal peripheral blood mononuclear cells (PBMC) were obtained by the separation of heparinized blood from healthy human volunteers on Ficoll-Histopaque (Sigma) density gradient centrifugation. After washing twice with PBS, the fraction of lymphocyte was suspended in RPMI 1640 medium with 5% FBS. The suspension ($1x10^6$ cells/well) was incubated in culture plates, and stimulated with phytohemagglutinin (PHA) lyophilized (2%) and incubated for 24 h at 37ºC. After 24 h, the cells were treated with concentrations of 2.5, 5 and 10 mM of ASA. The MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide] assay was used to monitor cell proliferation immediately, 24, 48 and 72 h after incubation with ASA, according protocol [28]. Briefly, lymphocyte cells, after incubation, were washed with PBS and 200 µL RPMI 1640 medium free b-mercaptoethanol and 20 µL of MTT (5 mg/mL) were added to each well. The plates were incubated for 4 h at 37 ºC. The medium was removed and formazan crystals were dissolved in 200 µL of

dimethylsulfoxide (DMSO, Sigma) with gentle shaking. The absorbance values at 490 nm were determined on a multiwell plate reader (ELX 800 Universal Microplate Reader, Bio-TEK).

Assessment of intracellular ROS formation

Suspensions of both cell lines ($5x10^5$ cell/ml) (control cells and cells treated with 2.5, 5 and 10 mM of ASA during 24 and 48 h) were washed twice with PBS and incubated for 30 min at 37°C with the fluorogenic compound 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA, 40 µM; Molecular Probes) [30]. H_2DCF-DA passively diffuses through cellular membranes and, once inside, the acetate is cleaved by intracellular esterases. Thereafter, the nonfluorescent compound H_2DCF is oxidized by reactive oxygen species (ROS) into a fluorescent compound (DCF). Once loaded with H_2DCF-DA, the cells were washed twice with PBS and resuspended in fresh PBS. Each treatment was performed in triplicate. Aliquots from 160 µL of each sample (three replicates) were placed into an ELISA plate and the fluorescence intensity determined during 90 min at 37°C, using a fluorometer (Victor 2, Perkin Elmer) with excitation and emission wavelengths of 485 and 520 nm, respectively. ROS levels were expressed in terms of fluorescence area and were obtained by integrating the fluorescence units (FU) over the measurement time (90 min) and expressed as FU min.

Evaluation of the gene expression by RT-PCR

The mRNA expression of *bcl-2*, *cox-2* and *p53* was evaluated by reverse transcriptase polymerase chain reaction (RT-PCR). After the incubation with different concentrations of ASA (2.5, 5 and 10 mM) for 48 h, total RNA from K562 and Lucena cells ($1x10^6$ cell/mL) was extracted using TRIzol reagent (Invitrogen, Brazil) according to the protocol suggested by the manufacturer. The RNA extracts were qualitatively evaluated by electrophoresis in 1% agarose gel, and quantified with the Qubit ^{TM} Fluorometer (Invitrogen, Brazil). The Quant-iT^{TM} RNA Assay Kit (Invitrogen, Brazil) was used, and calibration was performed using a two-point standard curve. The relationship between the

two standards and a curve-fitting algorithm was used to calculate the concentrations of the RNA samples. Total RNA from each pool was used as template for the RT-PCR with the AP primer (5'-GGCCACGCGTCGACTAGTAC(T)17-3', Invitrogen, Brazil). The complementary DNA (cDNA) synthesis was carried out using the enzyme RT SuperScript III (Invitrogen, Brazil) according to the protocol suggested by the manufacturer. The cDNA obtained was used as template for the gene amplification. Specific primers were used to *bcl-2* (bcl-2-F: 5'-GACTTCGCCGAGATGTCCAG-3'; bcl-2-R: 5'-CAGGTGCCGGTTCAGGTACT-3', giving an expected PCR product of 225 bp), *cox-2* (cox-2-F: 5'-TGAAACCCACTCCAAACACAG-3'; cox-2-R: 5'-TCATCAGGCACAGGAGGAAG-3', giving an expected PCR product of 232 bp), *p53* (p53-F: 5'-CTGAGGTTGGCTCTGACTGTACCACCATCC-3'; p53-R: 5'-CTCATTCAGCTCTCGGAACATCTCGAAGCG-3', giving an expected PCR product of 370 bp), glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (GAPDH-F: 5'-ATGGCACCGTCAAGGCTGAG -3'; GAPDH-R: 5'-GCAGTGATGGCATGGACTGT -3', giving an expected PCR product of 379 bp) gene expression. PCR was carried out in a 12.5 µL reaction volume containing 1.25 µL of 10X PCR buffer, 0.25 µM of each primer, 0.25 mM of each dNTP, 0.375 mM of MgCl2, 0.1 unit of Platinum Taq DNA polymerase (Invitrogen, Brazil) and 0.5 µL of cDNA solution. The reaction was incubated at 94 °C for 2 min followed by 25 (GAPDH (Lucena)), 28 (GAPDH (K562)), 35 (*p53*), 38 (*bcl-2* (Lucena) and *cox-2*) and 40 (*bcl-2* (K562)) cycles (denaturation at 94 °C for 30 s (GAPDH, *bcl-2* (Lucena) and *p53*), 15 s (*bcl-2* (K562) and *cox-2*), annealing at 56 °C for 30 s (GAPDH), 63 °C (*bcl-2* (Lucena) and *p53*), 65 °C (*bcl-2* (K562)), 55 °C for 45 s (*cox-2* (K562)) and 65 ºC (*cox-2* (Lucena)), and extension at 72 °C for 30 s) and with an additional (final) extension at 72ºC for 10 min. The PCR products were separated by electrophoresis on an 1.5% (W/V) agarose gel and stained with Sybr Safe™ (Invitrogen, Brazil) for densitometric analysis. Calculation of optical density (OD) was performed with the ONE-Dscan software (Scanalytics, Billeria, USA) for each gene and was normalized to corresponding GAPDH values.

Analysis of human bcl2, cox-2 and p53 promoters

For searching binding sites to transcription factors in the analyzed genes, sequences containing 2,000 bp of *bcl2*, *cox-2* and *p53* proximal promoters were identified at GenBank (http://www.ncbi.nlm.nih.gov). For human *bcl2*, *cox-2* and *p53*, the sequences used were **CCDS11981**, **CCDS1371** and **CCDS11118**, respectively. The potential transcription factor binding sites were localized using the MatInspector program [31], considering only core sequence with 100% of homology.

Statistical analysis.

At least three independent experiments were done using triplicates in each experiment. Data were expressed as mean (± standard error) and analyzed by ANOVA followed by Tukey multiple range test. ANOVA assumptions (normality and homogeneity of variances) were previously checked. Significance level was fixed at $P<0.05$.

Results

Cell viability

The final concentration of ethanol had no effect on the endpoints (data not shown).

Cell viability showed significant difference in the K562 and Lucena cells treated with different concentrations of ASA (2.5, 5, 10 and 15 mM) when compared with the control group ($P<0.05$). The effect of ASA treatment was concentration and time dependent, in both cell lines (Fig. 1A, 1B, 2A and 2B). The 2.5 mM concentration inhibited the cellular proliferation ($P<0,05$) only in 72h but did not shown toxic effect, to both cell lines. The 5 mM concentration inhibited the cellular proliferation from 48 h, in both cell lines, excepting Lucena cells in 72 h in which this concentration was citotoxic. The 10 mM ASA concentration inhibited the proliferation of K562 cells in 48 h. This concentration was citotoxic to Lucena cells at 48 h and to K562 cells at 72 h. The 15 mM

concentration, in Lucena cells, inhibited proliferation already in 24 h. From 48 h, this concentration was toxic to both cell lines.

Detection of apoptosis/necrosis by annexin-V/PI staining

As showed in Fig. 3, K562 and Lucena control cells, presented low staining with annexin V and PI. The same occurred with 2.5 and 5 mM of ASA treatment. However, after incubation with 10 mM K562 cells, was observed as early apoptosis (stained positive for annexin V) as well as necrosis (stained positive for PI and stained positive for annexin V plus PI) (Fig. 3A). On the other hand, the Lucena cells, only showed statistically significant difference ($P<0.05$) in staining to annexin V plus PI and only PI when treated with 10 mM, indicating death by necrosis (Fig. 3B).

ASA effects in normal peripheral blood mononuclear cells

The ASA effects in normal lymphocytes are presented in Figure 4. The ASA induced citotoxicity only in cells treated with 10 mM in 72 h when compared with the control cells ($P<0.05$). At this time, proliferation was inhibited with 5 mM when compared with its respective control ($P<0.05$).

Antioxidant effects of ASA

K562 cells treated with 10 mM of ASA, in 24 h, showed a significant decrease ($P<0.05$) in the amount of ROS. This was observed also in Lucena cells treated with 5 and 10 mM (Fig. 5A). Already in 48 h of incubation, K562 cells treated with 5 and 10 mM of ASA, showed a significant decrease ($P<0.05$) in the amount of ROS while that in Lucena cells, this result was gotten only with 10 mM of ASA (Fig. 5B).

Evaluation of the gene expression by RT-PCR

The ASA treatment did not provoke alteration in the expression of the *bcl-2* gene (Fig. 6A), although it induced apoptosis and/or necrosis in K562 and Lucena cells. Beside there was not difference in basal levels between this cell lines ($P>0.05$). The basal *p53* expression in Lucena cells was higher than in K562 cells (Fig. 6B). The same pattern was observed for *cox-2* expression (Fig 6C). K562 cells showed an increase in *p53* and *cox-2* expression concentration dependent. In contrast, the *p53* expression for Lucena cells was increased in 2.5 mM and decreased in 10 mM concentration of ASA. The increase of *cox-2* expression was observed just for 2.5 and 5 mM of ASA.

Analysis of human bcl2, cox-2 and p53 promoters

This analysis is shown in table 1. There were common transcriptional factors for three genes and only one for *cox-2* and *p53*.

Discussion

The ASA has showed the capacity of to prevent and even decrease some kind of cancer, including other leukemias as chronic lymphocytic leukemia and acute myeloid leukemia [32,33]. However the ASA action mechanism still requires study to validate its possible antitumoral properties. The present work aims at this issue. One of the major challenges in cancer treatment is to find a drug with antitumoral action in non-MDR and MDR cells. ASA showed this action by inhibiting the cellular proliferation or inducing toxicity in K562 and Lucena cells, although the Lucena cells present MDR phenotype [22,34]. Besides in some concentrations and times, the MDR cells were more sensitive than its parental line K562. Also, Trindade et al. [6] demonstrated similar results when these cells were exposed to photodynamic therapy with methylene blue and visible light. Other NSAID, as indomethacin, also inhibit proliferation in K562 cells [35].

In the last years, *in vitro* study showed that the citotoxic effects of several drugs in human tumoral cell lines were mediated by apoptosis [36,37]. Ishikawa cell line (human endometrial tumoral cells) for example, whose cells die from apoptosis when treated with concentrations at 3, 4 e 5 mM of ASA, in 96 h [2]. In agreement with these results, cells of patients with chronic lymphocytic leukemia, incubated with 1 to 10 mM of ASA, died for apoptosis in 48 h [32]. In the present work, K562 and Lucena cells died for apoptosis and/or necrosis, only when treated with the concentration of 10 mM in 48 h. This result suggest that K562 and Lucena cells are less sensitive by ASA treatment when compared with those studied by Bellosillo et al. [32]. Klampfer et al. [38] attributed the reduced sensitivity of K562 cells to sodium salicylate-induced apoptosis to the presence of the t(9;22) chromosomal translocation (Philadelphia chromosome) and the expression of the Bcr-Abl fusion protein in this cell line. The ultimate effect of *bcr-abl* product seems to be enhancing cell survival [39]. Also Yu et al. [40] demonstrated that the treatment, for 72 h, with 2.5 and 5 mM of ASA, induced apoptosis in colon tumoral cells (SW480). However at 10 mM of ASA, a secondary postapoptotic necrosis (delay apoptosis) was observed. In addition Zhang et al. [35] showed that K562 cells and fresh bone marrow cells from chronic myelogenous leukemia patients presented typical morphological changes of apoptosis after 48 h with 200 μM indomethacin (NSAID) treatment. On the other hand, there are evidences that Pgp, that is overexpressing in MDR cells induce resistance to programmed cell death [41]. A compatible result with Lucena cells was obtained in the present work.

The comparison between tumoral cells and normal lymphocytes indicates that the last are more resistant to ASA in all concentrations. Bellosillo et al. [32] also observed, that normal lymphocytes are more resistant to ASA than chronic lymphocytic leukemia cells (B-CLL), with doses up to 7.5 mM.

The toxicity induced by ASA in K562 and Lucena cells was not caused by the generation of ROS since ASA showed antioxidant action in both cell lines. This result is according with the expected, considering that prostaglandin induces ROS generation [42] and that ASA inhibits Cox-2 action,

reverting that process. Antunes et al. [9], found a inhibition in the total number of chromosomal aberrations and aberrant metaphases caused by ROS released by the doxorubicin in lymphocytes incubated for 24 h with ASA (25, 50 or 100 µg/ml) suggesting its action as an antioxidant agent. In another study, the preincubation with ASA (3-30 µM) protected bovine pulmonary artery endothelial cells from hydrogen peroxide-induced toxicity [43]. In copper-deficient rats that have their antioxidant defense system compromised, ASA reduces both lipid peroxidation and blood cholesterol [44].

It has been widely accepted that apoptosis is an active gene-directed cellular suicide mechanism and many genes contribute to its regulation, such as *p53* gene and *bcl-2* gene family. In the present study, the basal expression of *bcl-2* gene is similar in the controls of two cell lines. These results are according with study realized by Wagner-Souza et al. [45] that compared Bcl-2 expression in K562 and Lucena cell lines and demonstrated that no have different between both. Besides, the treatment with ASA for 48 h, demonstrated that the transcriptional expression of *bcl-2* also was not altered since this expression is similar to control cells in K562 and Lucena cells. It suggested that the mechanism of death cell induced by ASA was independent of *bcl-2*. Also analyzing the Bcl-2 protein expression, Klampfer et al. [38], demonstrated that treatment with sodium salicylate after 5 and 18 h, did not alter the expression of the Bcl-2 proteins in TF-1 acute myeloid leukemia cell line. On the other hand, several authors reported that protein Bcl-2 expression in some tumoral cells was enhanced by chemotherapeutic drugs [46,47]. In contrast, ASA inhibited the expression of Bcl-2 in colon cancer cells (SW480 cells) [40]. In relation to the biological model used in the present work, Zhang et al. [35] observed that indomethacin (NSAID) downregulated *bcl-2* gene expression in K562 cells. Among these contradictory results, the work of Subhashini et al. [48], prompts to suggest that the apoptosis in K562 cells, in this present study, occurred by poly (ADP-ribose) polymerase-1 (PARP-1) way. This suggestion is based in the fact that those authors demonstrated that the apoptosis mechanism for K562 cells treated with other NSAID drug occurred by *bcl-2* and PARP-1 way. These apoptosis ways also were described by Wang and Dubois [49]. Considering

that in the present study an alteration of *bcl-2* expression was not detected, it is possible suggest that apoptosis occurred by the PARP-1 way.

Several lines of evidence indicate that *p53* transcriptional activity does not always correlate to its apoptotic activity [50]. In the present work, a difference in the perfil of the *p53* expression between K562 and Lucena cells was observed: the first was concentration-dependent and the second was bell-shaped. The basal levels *p53* mRNA were significant difference between K562 and Lucena cells. In K562 control cells they were very low and this feature according with the work of Cavalcanti Junior et al. [51] could attributed to the mutation *p53* gene (stop codon) in K562 cell line, responsible by absence P53 protein expression. On the other hand, although the P53 protein is not present in the Lucena cells [51], its basal expression of mRNA in the present study was significantly higher. Several studies have shown increased levels of *p53* in tumor cells after treatment with a variety of DNA damaging agents [52,53]. This is generally observed in wild-type *p53*; however, mutant *p53* has been shown to induce transcription of the promoter of the *mdr1* gene, that express the Pgp protein, while wild-type *p53* does not induce transcription of this promoter [54]. Other studies have demonstrated that nuclear P53 accumulation is often associated with Pgp expression in primary cancer, and simultaneous expression of P53 and Pgp is associated with series of molecular events resulting in a more aggressive phenotype, drug resistance and poor prognosis [55,56]. Considering the premises above, a *p53* basal expression linked with Pgp expression reinforce the MDR phenotype in Lucena cell line. The cells also showed a difference when treated with ASA. The K562 cells showed a concentration-dependent increased in the *p53* expression. Since the increase of this expression can induce a mutant P53 protein, it can be concluded that the death by early apoptosis in these cells occurred by an independent P53 way. However for Lucena cells *p53* increased expression in the lower and decreased in the higher ASA concentration, when compared with control cells. The *p53* increased expression in the lower concentration in these cells can be associated to resistance by ASA treatment because this treatment not provoked viability

decrease in this cell line. On the other hand, the downregulation of *p53* observed for Lucena cells in the higher concentration can explain the fact of these cells died by necrosis.

Several mechanisms of growth inhibition by NSAIDs on cancer cell lines have been suggested. There is particular interest in to inhibit Cox-2 expression because its expression was associated with tumor progression [57] and NSAIDs as ASA was known to inhibit both cyclooxygenase enzymes [58]. It is relevant emphasize the *p53* and *cox-2* genes express similarity, considering basal as well as treated cells expression. This similarity can suggest an interaction between *cox-2* and *p53* expression. According to Choi et al. [59] Cox-2 regulates *p53* activity. On the other hand, Subbaramaiah et al. [60] demonstrated that levels of Cox-2 protein and mRNA were markedly suppressed by wild-type p53 but not by mutant p53, suggesting that interactions between *p53* and *cox-2* could be important to understand why levels of Cox-2 are undetectable in normal cells and increased in many tumors. In the present work, ASA induced the expression of *cox-2* in K562 and Lucena cells. The increase of the cox-2 expression could indicate that ASA really inhibited the Cox-2 protein and consequently increased the arachidonic acid concentration. This fact could have been the stimulus to the increase the *cox-2* transcription. Considering the above informations, it was proposed that the lack of Cox-2 active a transcription factor for *cox-2* and *p53* (Fig. 7). Perrotti et al. [61] reported that expression of CCAAT/enhancer-binding protein (C/EBPα) transcription factor is down-regulated by BCR/ABL in a dose-dependent manner. Considering that the cell lines utilized in the present work present the Philadelphia chromosome (*bcr/abl*), it also can be suggested that ASA induces some effect in BCR/ABL protein or on its gene. Calabretta and Perrotti [62] suggested that therapies targeting BCR/ABL can restore the C/EBP gene activity, lost during the leukemic process. The promoter analysis through the MatInspector program revealed that the only transcription factor binding site present in both p53 and cox-2 but not bcl-2 was C/EBP.

Also it is worth considering that in the Lucena cells treated with higher concentration of ASA, was observed a decrease *p53* and *cox-2* expression probably due to MDR phenotype.

Finally, this work suggests that ASA presents antitumoral properties in MDR as well as in non-MDR cells, while the mechanism of this action seems different in each cell line.

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Table 1 - Analysis of the P53, NFκB, C/EBP, CREB/ATF and AP-1 core sequences in human *bcl-2*, *p53* and *cox-2* proximal promoters.

Transcription factor	Position *bcl-2*	Position *p53*	Position *cox-2*
P53	-1997	-1679	-698
		-1237	-687
		-547	
		-457	
NFκB	-1902	-277	-583
	-682	-108	-358
	-173		
C/EBP	-	-36	-1744
			-676
			-269
CREB/ATF	-1337	-	-711
			-649
			-200
			-107
AP-1	-	-	-712

Captions to figures

Figure 1. (A) Number of viable cells ($\times 10^4$) and (B) Cell viability (%) of K562 cells treated with different concentrations of ASA at different times of exposure, according to trypan blue exclusion test. Data are expressed as mean $\pm$ standard error. * indicates $P<0.05$ when compared with control.

Figure 2. (A) Number of viable cells ($\times 10^4$) and (B) Cell viability (%) of Lucena cells treated with different concentrations of ASA at different times of exposure, according to trypan blue exclusion test. Data are expressed as mean $\pm$ standard error. * indicates $P<0.05$ when compared with control.

Figure 3. Induction of cell death (%) by early apoptosis and/or necrosis in (A) K562 and (B) Lucena cells treated with 2.5, 5 and 10 mM of ASA for 48 h. Data are expressed as mean $\pm$ standard error. * indicates $P<0.05$ when compared with control.

Figure 4. Sensitivity (optical of density) of lymphocytes treated with 2.5, 5 and 10 mM of ASA at different times, as measured by MTT assay. Data are expressed as mean $\pm$ standard error. * indicates $P<0.05$ when compared with control.

Figure 5. ROS production (fluorescence area) in K562 and Lucena cell lines treated with 2.5, 5 and 10 mM of ASA at (A) 24 and (B) 48 h. Data are expressed as mean $\pm$ standard error. * indicates $P<0.05$ when compared with control.

Figure 6. Analysis of gene expressions in K562 and Lucena cells treated with 2.5, 5 and 10 mM of ASA at 48 h. (A) Bcl-2 mRNA expression. (B) p53 mRNA expression. (C) Cox-2 mRNA expression. Data are expressed as mean $\pm$ standard error. * indicates $p<0.05$ when compared with control. Similar letters indicate absence of significant differences between controls ($P>0.05$).

Figure 7. Mechanism suggested for expression similar of *p53* and *cox-2* genes in K562 and Lucena cell lines. A common transcriptional factor is suggested activate both *p53* and *cox-2* genes when Cox-2 protein in the cytoplasm is low, due to action of ASA.
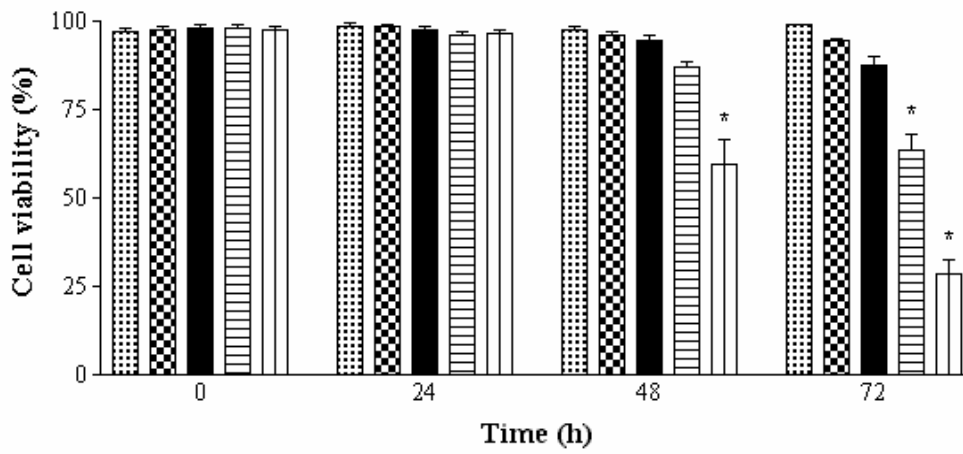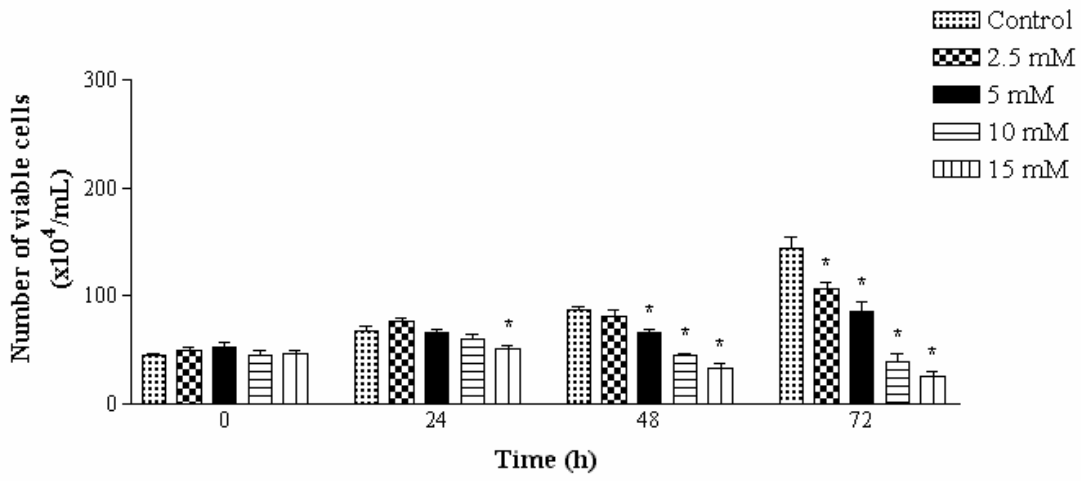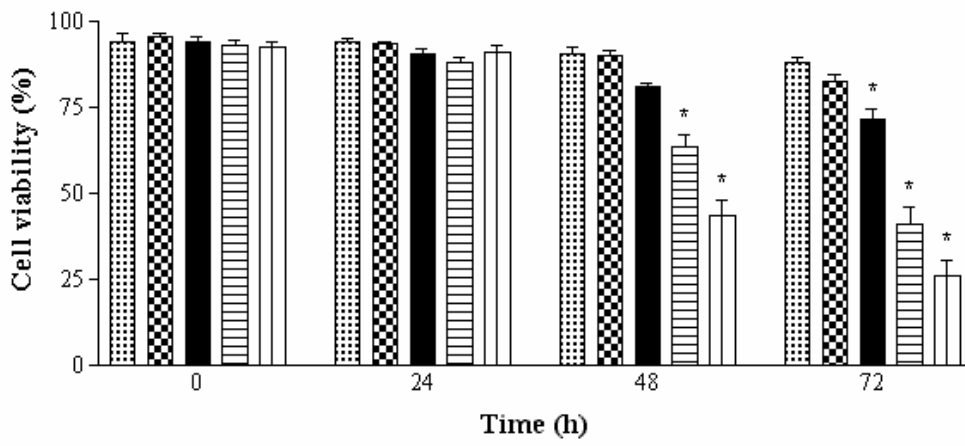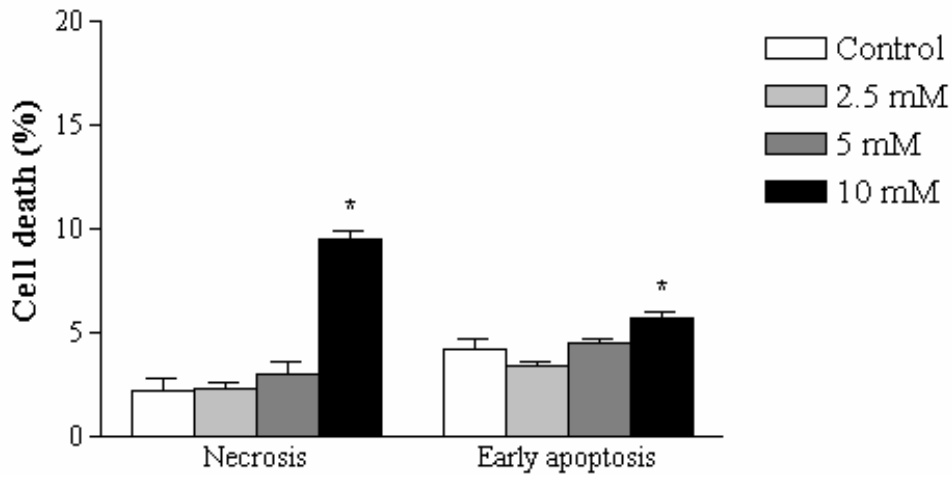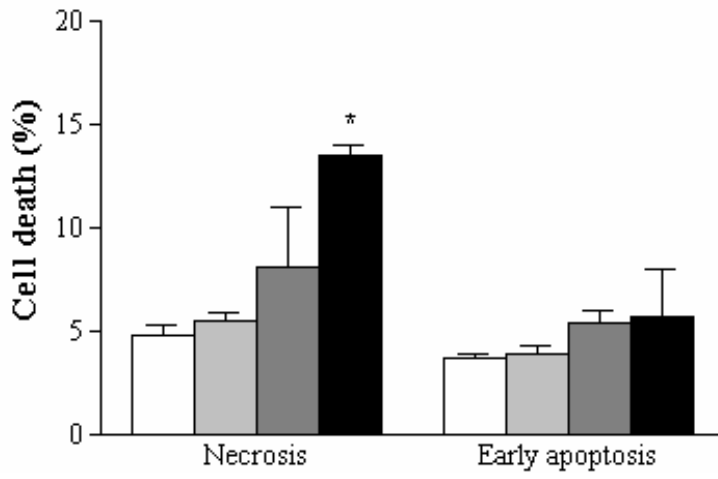
Figure 1

(A)
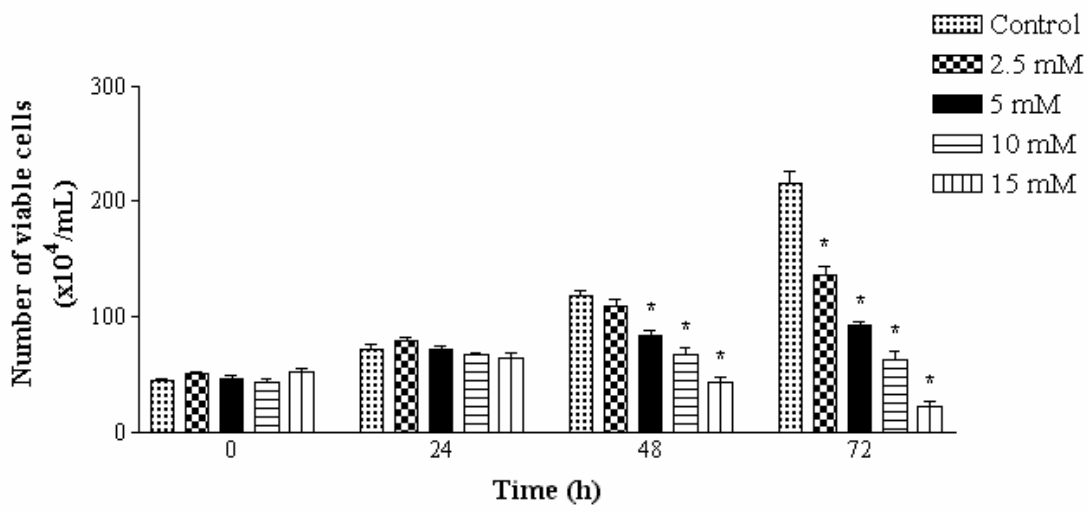
(B)

Figure 2

(A)

(B)

Figure 3

(A)

(B)

Figure 4
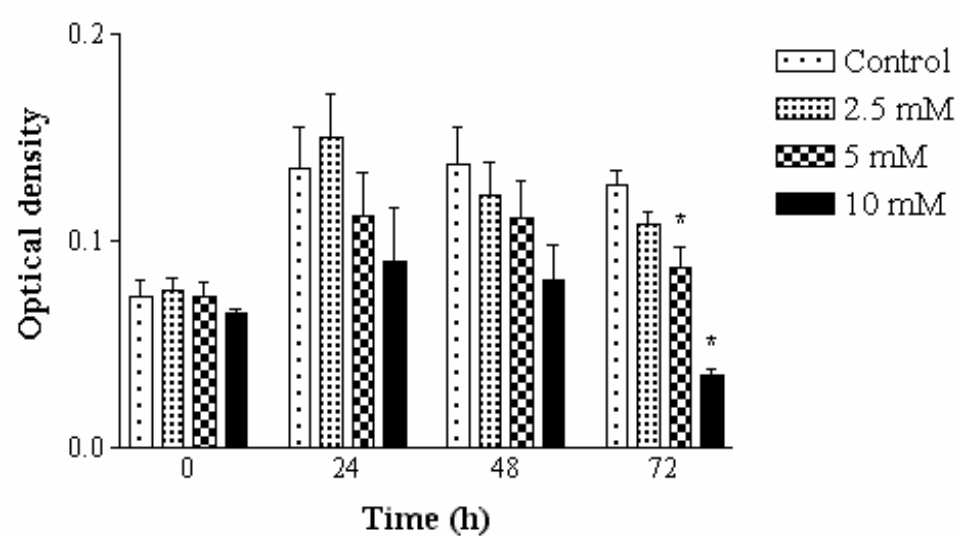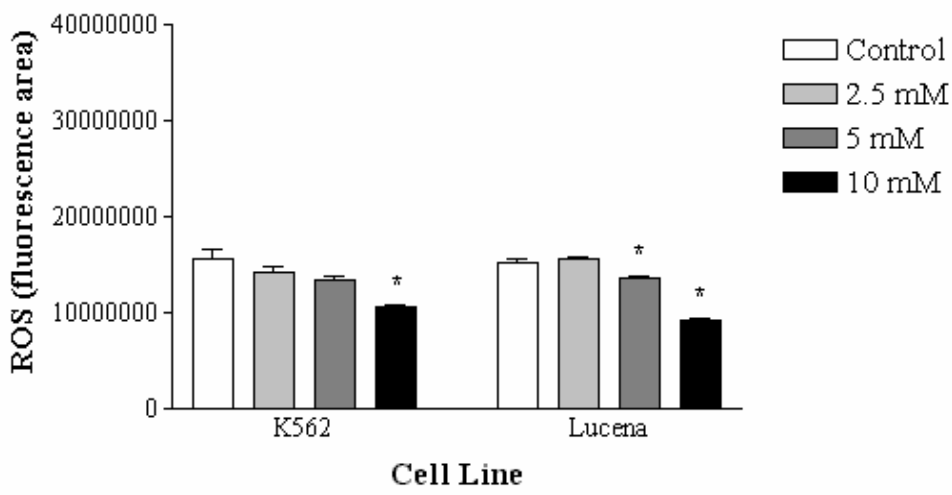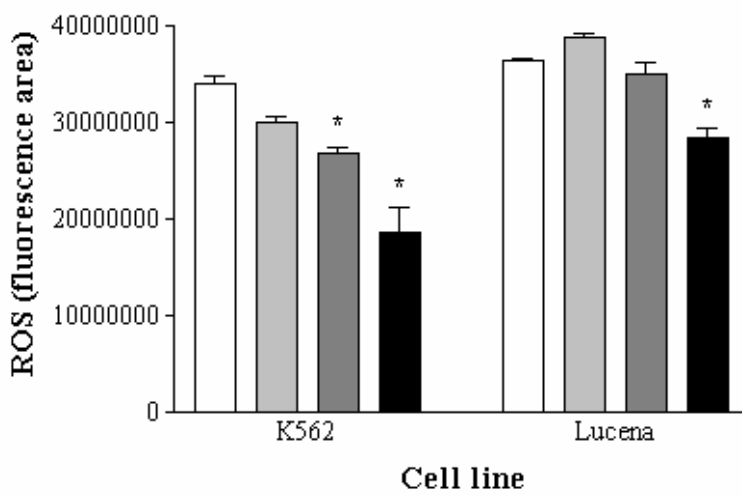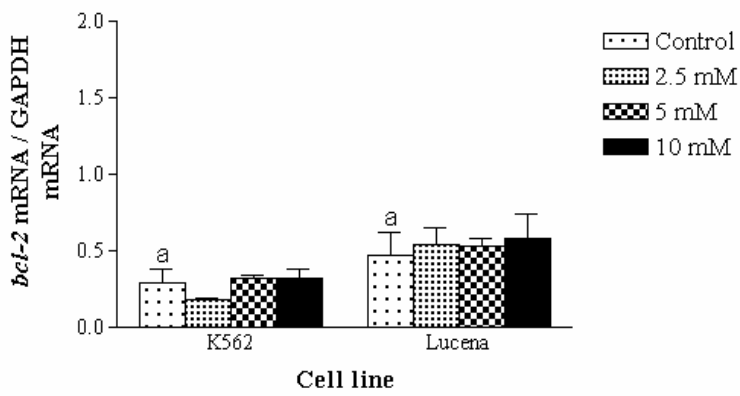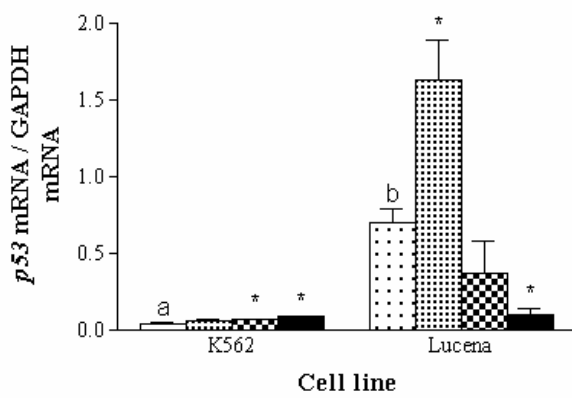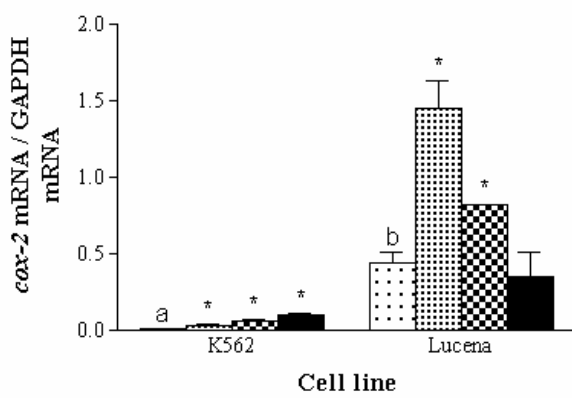
Figure 5

(A)

(B)

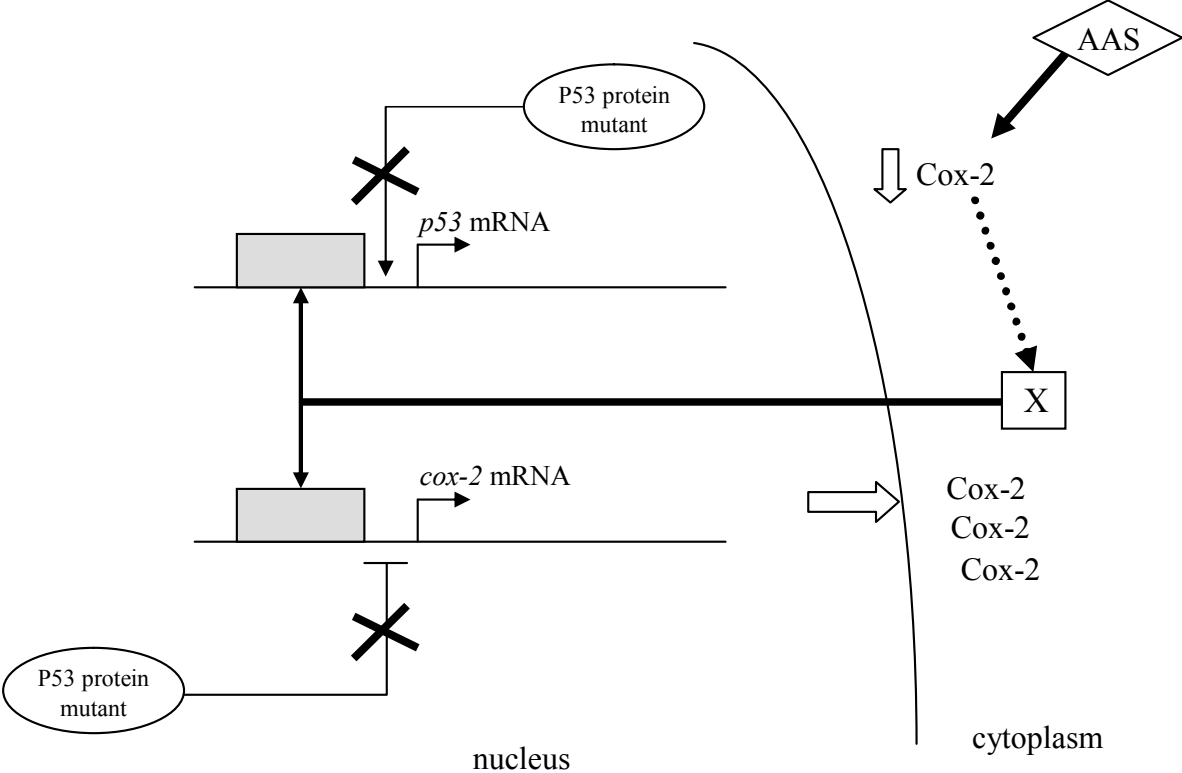Figure 6

(A)

(B)

(C)

Figure 7

Conclusões Gerais

- AAS inibiu a proliferação celular e induziu toxicidade nas linhagens celulares K562 (não MDR) e Lucena (MDR).

- O tratamento com AAS provocou morte, nas células K562, principalmente por apoptose, e por necrose, nas células Lucena.

- AAS mostrou uma capacidade antioxidante nas duas linhagens celulares.

- AAS não alterou a expressão do gene *bcl-2*, considerando tanto as células controle e tratadas com AAS, como a expressão basal entre as duas linhagens celulares.

- O tratamento com AAS nas células K562 induziu a expressão dos genes *p53* e *cox-2*, de uma forma concentração dependente.

- Nas células Lucena, o tratamento com AAS aumentou a expressão dos genes *p53* e *cox-2* nas menores concentrações e, para o gene *p53*, diminuiu na maior concentração quando comparadas as células controle.

- Devido à semelhança na indução dos genes *p53* e *cox-2*, em cada linhagem celular, foi sugerido que o tratamento com AAS, indiretamente afeta um fator de transcrição comum para os dois genes.

- As células normais, linfócitos, foram menos sensíveis ao tratamento com AAS.

- Os resultados obtidos demonstram que as células tumorais são mais sensíveis ao tratamento com AAS do que as normais e permitem sugerir que o mecanismo de ação nas duas linhagens tumorais é diferenciado.

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