UNIVERSIDADE FEDERAL DE PERNAMBUCO

CENTRO DE BIOCIÊNCIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

MARIA DO DESTERRO RODRIGUES

AVALIAÇÃO DA ATIVIDADE CITOTÓXICA, ANTIMICROBIANA E GENOTÓXICA DE TIAZOLIDINA-2,4-DIONAS

RECIFE

2017

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AVALIAÇÃO DA ATIVIDADE CITOTÓXICA, ANTIMICROBIANA E GENOTÓXICA DE TIAZOLIDINA-2,4-DIONAS

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco como pré-requisito para obtenção do título de Doutora em Ciências Biológicas. Área de concentração – Biologia Química para a Saúde.

Orientadora: Profª. Dra. Silene Carneiro do Nascimento

Co-orientadora: Profª. Dra. Gardenia Carmen Gadelha Militão

RECIFE

2017

Catalogação na fonte
Elaine Barroso
CRB 1728

Rodrigues, Maria do Desterro
Avaliação da atividade citotóxica, antimicrobiana e genotóxica de tiazolidina-2,4-dionas. / Maria do Desterro Rodrigues - Recife: O Autor, 2017.

147 folhas: il., fig., tab.
Orientadora: Silene Carneiro do Nascimento
Coorientadora: Gardênia Carmem Gadelha Militão
Tese (doutorado) – Universidade Federal de Pernambuco.
Centro de Biociências. Ciências Biológicas, 2017.
Inclui referências e anexos

1. Tiazois 2. Câncer 3. Testes de toxicidade I. Nascimento, Silene Carneiro do (orient.) II. Militão, Gardênia Carmem Gadelha (coorient.) III. Título

547.594 CDD (22.ed.) UFPE/CB-2017-641

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Aprovada em: 21/02/2017

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É impossível avaliar a força que possuímos sem medir o tamanho do obstáculo que ela pode vencer, nem o valor de uma ação sem sabermos o sacrifício que ela comporta. ”

(Autor desconhecido)

Aos meus pais (In memórian),

Maria do Socorro Rodrigues e Luiz Rodrigues do Nascimento

Exemplos de força, caráter, dignidade e fé.

AGRADECIMENTOS

Agradeço a Deus por todas as bênçãos recebidas, por iluminar os meus caminhos e guiar os meus passos me tornando mais forte e determinada a cada dia na conquista dos meus objetivos como mãe, estudande e profissional;

A minha orientadora, Profa. Dra. Silene Carneiro do Nascimento obrigada pela oportunidade, por todos os ensinamentos, por sempre acreditar em mim, por todo apoio nos momentos mais difíceis, por todos os anos de amizade. Serei eternamente grata!

A minha co-orientadora Profa. Dra. Gardenia Carmen Gadelha Millitao por todos os ensinamentos, por toda contribuição que foi fundamental para o desenvolvimento deste trabalho;

À profa. Dra. Teresinha Gonçalves da Silva obrigada pelo apoio, pelos ensinamentos e pela colaboração para que este trabalho se realizasse;

Á profa. Dra. Julianna F. C. de Albuquerque obrigada pelos ensinamentos, por nos ceder as moléculas e pela disponibilidade em me receber no seu laboratório;

Agradeço também à Dra. Valéria Rêgo Alves Pereira por todo apoio e torcida e por me receber tão bem no seu laboratório;

Á Professora Kêsia Xisto da Fonseca Ribeiro de Sena obrigada pela colaboração para a realização dos testes microbiológicos;

À Dra, Mônica Adam obrigada pelos ensinamentos e por disponibilizar o seu laboratório para a realização destas pesquisas;

Agradeço (In memória) aos meus pais Maria do Socorro Rodrigues e Luiz Rodrigues do Nascimento pelo amor e pela educação recebida e principalmente pelo exemplo de pessoas generosas, de princípios éticos e morais. E por me fazerem acreditar, desde muito pequena, que estudar era o melhor caminho a se trilhar.

Aos meus filhos amados que são a minha razão de viver João Vítor, João Paulo e João Guilherme. Muito obrigada por vocês existirem!

Agradeço aos meus queridos irmãos e irmãs Rodrigues, Sávio, Gill, Gó e Meg, que mesmo distantes me dão amor e apoio incondicional;

Ao meu companheiro de jornada Ricardo Leite, um anjo que Deus colocou no meu caminho no momento em que mais precisei para me fazer acreditar com seu amor, apoio e paciência, que tudo iria dar certo. Muito obrigada, meu amor!

Obrigada a todos que fazem o departamento de Antibióticos em especial as secretárias Sueli, Betânia e Romênia pela ajuda e amizade, agradeço também as técnicas do Laboratório de Cultura e Coleção de Micro-organismos, Fátima e Marcela pelo apoio e pela amizade, a todos os colegas e técnicos dos laboratórios da cultura de células e do BIOFAMATOX especialmente à Karla Mirella, Cidália, Sandrine Arruda, Natali, Temístocles Ìtalo, Cínthia, Bruno, André, Jeyce Kelle, Larissa Cardoso, Eduardo, Rodrigo, Nelson e Thiago pela presença na luta diária, nos momentos difíceis onde pudemos nos apoiar e incentivar e também nas horas de alegria e descontração.

Muito obrigada!

RESUMO

A busca por novos compostos bioativos é motivada pela necessidade de se obter resultados terapêuticos mais eficazes que atuem de forma seletiva minimizando os efeitos colaterais. Há um grande interesse na descoberta de novos antibióticos em virtude do aumento dos casos de infecções hospitalares e aparecimento de cepas de micro-organismos resistentes aos antibióticos atualmente existentes. Neste cenário os derivados das tiazolidinas-2,4-dionas têm sido bastante explorados devido a sua vasta gama de atividades biológicas descrita na literatura tais como, antimicrobiana, hipoglicemiante, anti-inflamatória e antitumoral. Estes derivados constituem uma classe de compostos derivados das tiazolidinas que possuem um heterociclo de cinco membros contendo um átomo de enxofre e um átomo de nitrogênio nas posições 1 e 3 respectivamente e duas carbonilas nas posições 2 e 4. Neste trabalho seis compostos substituídos na posição 5 do anel foram testados para a atividade anticancerígena usando seis linhagens de células tumorais humanas NCI-H292 (Carcinoma mucoepidermoide de pulmão), HL60 (Leucemia promielocítica aguda), HT-29 (Adenocarcinoma de cólon), K562 (Leucemia mielocítica crônica), HEp-2 (Carcinoma epidermoide de laringe) e MCF-7(Adenocarcinoma de mama). Para melhor verificar o mecanismo de ação envolvido na citotoxicidade do composto 5 foram realizados estudos de mecanismos de morte celular por citometria de fluxo e os possíveis efeitos genotóxicos através dos ensaios cometa e micronúcleo. A atividade antimicrobiana foi realizada pela técnica de difusão em disco em cepas selecionadas do grupo de bactérias Gram-positivas, Gram-negativas, álcool-ácido resistentes e leveduras. Os testes antimicrobianos foram ainda complementados com os valores da CMI e CMB. O composto 5 mostrou os melhores resultados com valores de IC_{50} entre 1,26 e 3,5 µg/mL para a citotoxicidade nas células tumorais. Nenhum derivado testado apresentou efeito citotóxico com (IC_{50} > 25 µg/mL) nas células mononucleadas de sangue periférico humano (PBMC) nem atividade hemolítica contra eritrócitos humanos (EC_{50}> 125 µg/mL). Também não foi verificado alterações sigificativas na análise das fases do ciclo celular embora tenha sido observado um aumento significativo do percentual de células com fragmentação do DNA após o tratamento na concentração de 16 µg/mL do composto 5 por 48 horas. A análise morfológica após coloração de May - Grunwald - Giemsa revelou integridade da membrana, redução do volume celular, e alguns restos celulares na concentração de 16 µg/mL. O composto também aumentou de forma significativa o número de células com despolarização da membrana mitocondrial e em apoptose de forma significativa nas concentrações de 8 e 16 µg/mL. No estudo da genotoxicidade foi visto que o composto 5 apresentou danos significativos ao DNA das células tumorais sem causar danos significativos às células de PBMC. Estes dados sugerem que as células NCI - H292 sofreram apoptose após tratamento com o composto 5, e que a classe dos derivados das 5- (benzileno-tiazolidina-2,4-diona) analisadas apresentam atividade anticancerígena para os cânceres de cólon, pulmão, mama e leucemia e potente atividade antimicrobiana para as cepas de bactérias Gram-positivas e álcool-ácido resistente, sem causar citotoxicidade ou genotoxicidade significativas para as células normais, demonstrando assim, uma das características requeridas ɪ uma molécula candidata a um novo fármaco.

Palavras-chave: Tiazolidina-2,4- dionas, Citotoxicidade, Atividade antimicrobiana, Genotoxicidade

ABSTRACT

The search for new bioactive compounds is motivated by the need to obtainore effective therapeutic results that act selectively minimizing the side effects. There is great interest in the discovery of new antibiotics due to the increase in cases of hospital infections and the appearance of strains of microorganisms resistant to antibiotics currently available. In this scenario the thiazolidine-2, 4-dione derivatives have been extensively explored because of their wide range of biological activities described in the literature such as, antimicrobial, hypoglycemic, anti-inflammatory and antitumor. These derivatives constitute a class of thiazolidine derivative compounds having a five membered heterocycle containing one sulfur atom and one nitrogen atom at the 1 and 3 positions respectively and two carbonyls at the 2 and 4 positions. In this work six compounds substituted at the 5-position of the ring were tested for anticancer activity using six human tumor cell lines NCI-H292 (mucoepidermoid carcinoma of the lung), HL60 (acute promyelocytic leukemia), HT-29 (colon adenocarcinoma), K562 (chronic myelocytic leukemia), HEp-2 (laryngeal epidermoid carcinoma) and MCF-7 (Breast adenocarcinoma). In order to better verify the mechanism of action involved in the cytotoxicity of compound 5, studies of cell death mechanisms were performed by flow cytometry and possible genotoxic effects through the comet and micronuclei assays. The antimicrobial activity was performed by the disc diffusion technique in strains selected from the Gram-positive, Gram-negative, alcohol-acid resistant and yeast groups. The antimicrobial tests were further supplemented with MIC and CMB values. Compound 5 showed the best results with IC_{50} values between 1.26 and 3.5 μg / ml for cytotoxicity in tumor cells. No derivative tested showed cytotoxic effect with (IC_{50}> 25 μg / mL) in human peripheral blood mononuclear cells (PBMC) or hemolytic activity against human erythrocytes (EC50> 125 μg / mL). No significant changes in the analysis of cell cycle phases were also observed although a significant increase in the percentage of cells with DNA fragmentation after treatment at the concentration of 16 μg / ml of compound 5 was observed for 48 hours. Morphological analysis after May - Grunwald - Giemsa staining revealed membrane integrity, cell volume reduction, and some cell debris at 16 μg / mL. The compound also significantly increased the number of cells with mitochondrial membrane depolarization and apoptosis significantly at concentrations of 8 and 16 μg / mL. In the genotoxicity study it was seen that compound 5 showed significant damage to the DNA of tumor cells without causing significant damage to PBMC cells. These data suggest that NCI-H292 cells underwent apoptosis after treatment with compound 5, and that the class of 5- (benzylene-thiazolidine-2,4-dione) derivatives analyzed have anticancer activity for colon, lung, breast and leukemia and potent antimicrobial activity for strains of Gram-positive and alcohol-acid resistant bacteria, without causing significant cytotoxicity or genotoxicity to normal cells, thus demonstrating one of the characteristics required for a molecule candidate for a new drug.

Key words: Thiazolidine-2,4-diones, Cytotoxicity, Antimicrobial activity, Genotoxicity

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LISTA DE ABREVIATURAS

CI_{50}	Concentração que inibe 50% do crescimento celular
CMB	Concentração mínima bactericida
CMI	Concentração mínima inibitória
CMSP	Células Mononucleares do Sangue Periférico
DMEM	Dulbeco's modified eagle medium
DMSO	Dimetilsulfóxido
DNA	Ácido desoxirribonucleico (deoxyribonucleic acid)
Dox	Doxorrubicina
EMAR	Espectrometria de Massas
IC	Intervalo de confiança
ID	Indice de dano
IV	Infravermelho
MN	Micronúcleo
MTT	Brometo de [3- (4,5-dimetiltiazol-2yl) -2,5-difenil tetrazolium
PBMC	Peripheral Blood Mononuclear Cells
MRSA	*Staphylococcus aureus* resistente a Meticilina
PPARɣ	Receptores ativados por proliferador de peroxissoma
$RMN^{13}C$	Ressonância Magnética Nuclear de Carbono treze
$RMN^{1}H$	Ressonância Magnética Nuclear de Hidrogênio
RNA	Ácido Ribonucléico (Ribonucleic ácid)
RPMI	Roswell Parrk Memorial Institute Medium

SUMÁRIO

1 INTRODUÇÃO

A descoberta e desenvolvimento de novos fármacos ativos em sistemas biológicos é um processo dinâmico, complexo, multidisciplinar, longo e de elevado custo envolvendo a identificação e caracterização de alvos biológicos, síntese e otimização estrutural de moléculas-protótipos, caracterização físico-química e análise frente a atividades biológicas in vitro (PANCHAGNULA; THOMAS, 2000).

Os derivados das tiazolidinadionas têm sido relatados em muitas pesquisas por apresentar diversas atividades como hipoglicemiante (GOMES, 2006), antiinflamatória (COUTO et al. 2006), antitumoral (PATIL et al. 2010) e como potentes antibióticos com ação contra um grande número de patógenos dentre eles: bactérias Gram-positivas, Gram-negativas, álcool-ácido resistentes e fungos (GOUVEIA et al., 2009; SILVA, 2014).

O câncer é representado por um grupo de mais de 100 doenças caracterizado pelo crescimento caótico de células com a capacidade de invadir tecidos e órgãos. No câncer as células se dividem rapidamente dando origem a tumores malignos capazes de disseminar para outras partes do corpo. Essas características do câncer ocorrem em virtude de alterações estruturais, comportamentais e moleculares das células afetadas pela doença tais como; autossuficiência na regulação de sinais de crescimento celular, insensibilidade aos sinais que inibem o crescimento, evasão da morte celular programada (apoptose), ilimitado potencial replicativo, indução da angiogênese, invasão tecidual e metástase. (INCA, 2015; HANAHAM; WEINBERG, 2011).

As previsões sobre novos casos de câncer são alarmantes, com a expectativa de um aumento em cerca de 70% do número de casos em todo o mundo para as próximas duas décadas (STEWART; WILD, 2014).

As drogas antineoplásicas promovem um aumento da sobrevida das pessoas portadoras de diversos tipos de câncer, porém o seu uso prolongado aumenta o risco de efeitos colaterais (CHAVES et al., 2007). Isso desperta o interesse em se descobrir novos agentes terapêuticos mais eficientes e seletivos.

A busca de novos fármacos também é motivada pelo aumento dos casos de infecções bacterianas. A descoberta de novos fármacos com amplo espectro bacteriano ocorrida nas últimas décadas tem contribuído para uma grande diminuição do número

de mortes por infecções em todo mundo. Porém o uso indiscriminado de antibióticos leva ao aparecimento de cepas bacterianas resistentes o que constitui um problema de saúde pública mundial, pois a seleção de bactérias resistentes limita o número de antibióticos disponíveis isso implica na necessidade considerável de novas classes de agentes antibacterianos (SANTOS, 2004; TOMASIC et al., 2010; SPELLBERG et al., 2008; GUZMÁNRODRIGUÉZ et al., 2015; VENUGOPAL; YARLA; UMADEV, 2014).

Diante dessas evidências, este trabalho tem por objetivos avaliar seis compostos arilidenos derivados da tiazolidina-2,4-diona substituída na posição 5 de seu núcleo quanto as atividades antimicrobiana, citotóxica e genotóxica contra linhagens de células tumorais e células normais com o intuito de descobrir novos agentes potencialmente ativos.

2 OBJETIVOS

2.1 Objetivo Geral

Avaliar os efeitos citotóxicos, antimicrobianos e genotóxicos de novos derivados das tiazolidinas -2,4-dionas.

2.2 Objetivos Específicos

- Determinar *in vitro* o efeito citotóxico dos compostos tiazolidínicos em linhagens de células tumorais e em linfócitos humanos;
- Investigar a ocorrência de atividade hemolítica das substâncias testadas em eritrócitos humanos;
- Analisar a morfologia das células tratadas pelos compostos derivados das tiazolidinadionas;
- Investigar os mecanismos subjacentes à citotoxicidade por determinação do conteúdo de DNA nuclear da célula, externalização da fosfatidilserina e alterações no potencial transmembrânico mitocondrial;
- Avaliar a atividade microbiológica dos diferentes compostos tiazolidínicos substituídos frente a micro-organismos dos grupos Gram-positivos, Gram negativos, álcool-ácido resistentes e leveduras;
- Analisar os possíveis efeitos genotóxicos de derivados tiazolidinas-2,4–dionas em linhagens de células tumorais e normais.

3 REVISÃO DE LITERATURA

3.1 Aspectos Gerais do Câncer

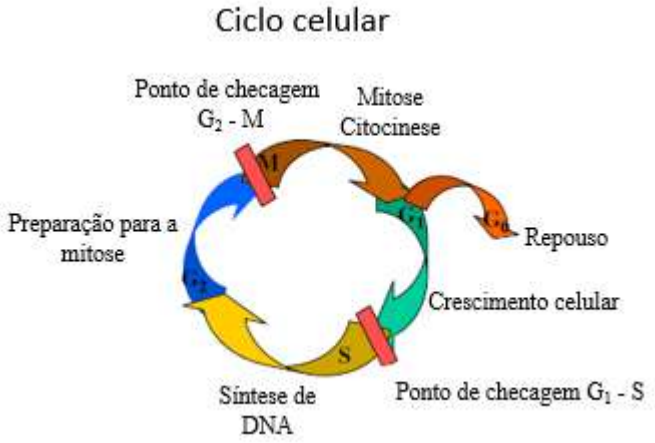
O termo câncer ou neoplasia se refere aos tumores malignos, caracterizado pelo crescimento descontrolado de células anormais capazes de invadir tecidos e órgãos. Estas células promovem a formação de tumores malignos dividindo-se rapidamente, podendo migrar para outros tecidos do corpo. O câncer constitui um grupo de aproximadamente 200 doênças que pode atingir os sistemas de células do corpo causando dor e muito temor entre as pessoas pelo sofrimento e alta mortalidade (ALMEIDA et al., 2005). É a segunda causa de morte no mundo e constitui um problema de saúde pública mundial devido à crescente taxa de incidência e mortalidade. As células do câncer apresentam múltiplas características estruturais, comportamentais e moleculares que definem a fisiopatologia do câncer e são responsáveis pelo crescimento maligno, tais como: Autossuficiência na regulação de sinais de crescimento celular, a insensibilidade aos sinais que inibem esse crescimento, evasão da morte celular programada (apoptose), ilimitado potencial replicativo, indução da angiogênese, invasão tecidual e metástase (INCA, 2015; HANAHAM; WEINBERG, 2000; 2011).

As causas de câncer são variadas, podendo ser externas ou internas ao organismo, estando inter-relacionadas. As causas externas referem-se ao meio ambiente e aos hábitos ou costumes próprios de uma sociedade. As causas internas são, na maioria das vezes, geneticamente pré-determinadas, e estão ligadas à capacidade do organismo de se defender das agressões externas. A maioria dos casos (cerca de 80%) está relacionada ao meio ambiente, onde encontramos um grande número de fatores de risco. Os tipos de ambiente são o meio em geral (água, terra e ar), o ambiente ocupacional (quando insalubre), o ambiente social e cultural (estilo e hábitos de vida) e o ambiente de consumo (alimentos, medicamentos) (INCA, 2009; 2015).

Existem vários mecanismos que estão envolvidos na evolução de uma célula normal para uma célula potencialmente maligna, mas a maior parte deles interfere na divisão celular e, assim, o conhecimento do ciclo celular ou dos seus mecanismos é importante para que haja a compreensão da etiologia do câncer. No estágio G0 o DNA apresenta-se super-enovelado e as células não estão se replicando, apresentando baixa atividade nuclear. A célula pode passar do estágio G0 para o estágio G1, neste caso ela será preparada para se multiplicar produzindo novos constituintes celulares para gerar

uma nova célula e se preparando para a síntese de DNA que acontecerá na fase S. Existem vários mecanismos que regulam a progressão do ciclo celular nas fases G1 e S. A multiplicação celular será ativada pelos fatores de crescimento, que são os produtos de oncogenes. E o controle do crescimento será realizado pelos inibidores da multiplicação celular, como os genes supressores tumorais que tem a função de parar a replicação celular para reparar algum dano ao DNA. A fase G2 prepara a célula para a mitose que é a divisão celular que mantém o número de cromossomos relativos a espécie) é onde gerado o fuso mitótico. A citocinese ocorre quando o material genético for duplicado, finalizando assim o ciclo de replicação celular com a formação de duas células filhas que retornaram ao estágio G0. Para as células tumorais o processo é contínuo e a célula sai da fase M e já entra numa nova fase G1. Desse modo a célula tumoral não retorna a fase G0 (ALMEIDA, 2005).

Figura 1. Ciclo de replicação celular



Legenda: Ciclo celular: A progressão no ciclo consiste numa série de eventos sequenciados e regidos por fatores de crescimento e supressores tumorais. O crescimento celular, a parada do ciclo ou a apoptose são eventos decididos nos pontos de verificação ou pontos de checagem entre as fases G1/S e G2/M. Fonte: Adaptado de Foster, 2008.

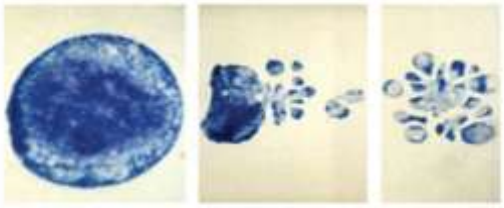
Outro mecanismo regulador é a apoptose (morte celular programada), que provoca a morte da célula em detrimento da possibilidade de a célula tornar-se alterada, podendo levar ao câncer (ALMEIDA, 2005). O nome ‘apoptose’ foi adotado para descrever um aspecto morfológico específico de morte celular. Neste processo a célula sofre redução do volume celular (picnose), condensação da cromatina, fragmentação nuclear, pouca ou nenhuma modificação ultraestrutural de organelas citoplasmáticas e a desintegração da membrana celular sem extravasamento do conteúdo celular para o

meio externo. Esse processo não gera inflamação pois, os restos celulares, após a fragmentação de uma célula afetada, são rapidamente eliminados por células próximas (KERR et al., 1972). Assim, o termo "apoptose" deve ser aplicado exclusivamente aos eventos de morte celular que ocorrem com a manifestação destas características morfológicas (KROEMER et al., 2009).

O corpo humano adulto gera em média 60 bilhões de células por dia e para manter a homeostase um número igual de células devem morrer por apoptose isso leva a crer que a desregulação da apoptose pode gerar a um acumúlo de células e contribuir para o desenvolvimento do tumor. A idéia de que o desenvolvimento e progressão do tumor poderia ser influenciada por apoptose remonta em 1972 quando Kerr mostrou que a taxa de crescimento de tumores foi menor do que o previsto como resultado de um elevado nível de apoptose endógena de células tumorais. Em estudos posteriores foi relatado que a apoptose desempenhou uma parte integrante do crescimento do tumor, progressão e resistência à terapia (KERR et al.,1994; COTTER, 2009).

A apoptose é uma via de morte celular que ocorre em várias etapas altamente reguladas e que controla parcialmente os números durante o desenvolvimento e também em organismos multicelulares adulto. O encolhimento celular, condensação da cromatina, e a fragmentação nuclear e celular levam à formação de corpos apoptóticos que são então engolidos por células fagocíticas vizinhas podendo ser observado na figura 2 a imagem de uma célula em processo de apoptose clássica visualizando a cromatina condensada, o encolhimento celular e a fragmentação da célula em corpos apoptóticos (COTTER, 2009). Uma outra marca bioquímica da apoptose é a clivagem do DNA internucleossomal. Outra característica marcante da apoptose é o movimento da fosfatidilserina na membrana lipídica do interior para o lado exterior da membrana plasmática, funcionando como um sinal de reconhecimento para células fagocíticas englobar células apoptóticas (COTTER, 2009). Também ocorrem mudanças na permeabilidade da membrana mitocondrial com perda do potencial de membrana (KROEMER; REED, 2000; RICCI; ZONG, 2006) e liberação de proteínas presentes no espaço intermembranar da mitocôndria (VAN LOO et al., 2002), dentre outras.

Figura 2. Célula em apoptose



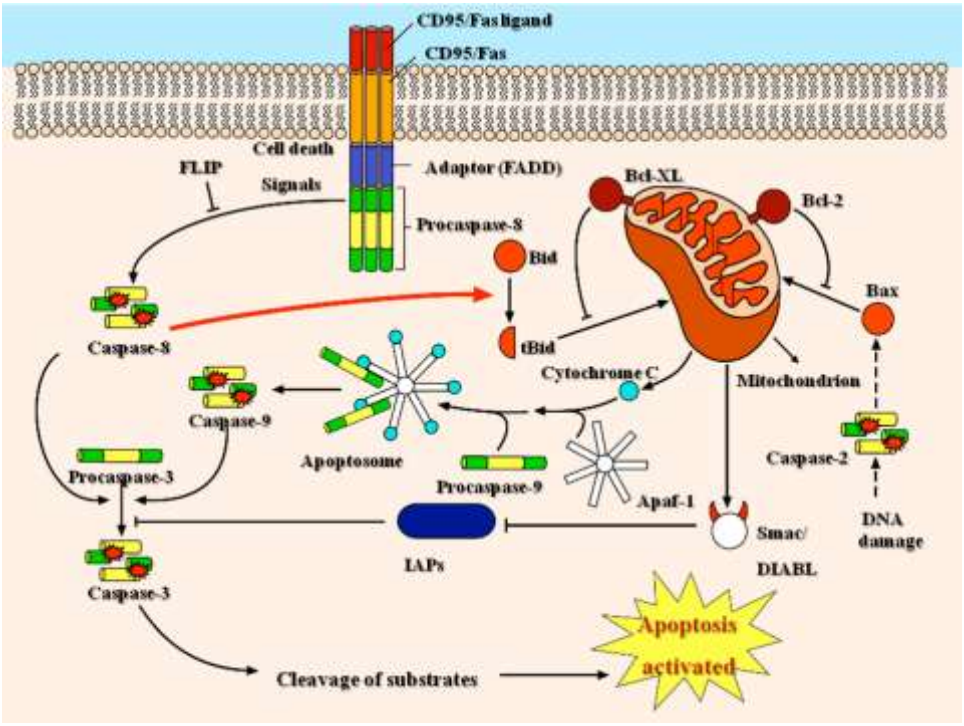
Legenda: Visualização da célula em processo da apoptose caracterizado pela condensação da cromatina, redução do volume celular e formação de corpos apoptóticos sem desintegração de organelas. Fonte: COTTER, 2009.

A morte celular por apoptose acontece através de um complexo mecanismo envolvendo uma cascata de reações moleculares dependentes de energia. O processo apoptótico é mediado pelas caspases que são proteases divididas em caspases iniciadoras (8, 9 e 10) e as caspases efetoras (3, 6 e 7). Existem duas vias principais: a via extrínseca ou de receptor de morte e a via intrínseca ou via mitocondrial (figura 3). Para dar início a apoptose, as caspases que se encontram na forma inativa (pró-caspases) precisam ser ativadas (ELMORE, 2007). A via intrínseca é ativada por estresse intracelular ou extracelular e a sinalização em resposta a esses insultos são direcionados para a mitocondria (GRIVICICH et al., 2007). Esta via é mediada pelos membros da família Bcl-2, uma importante classe de proteínas reguladoras que está dividida em membros anti apoptóticos como a Bcl-2 e Bcl-xL e pró-apoptóticos como a Bax e a Bak.

O Bcl-2 é um dos mais conhecidos proto oncogenes que está relacionado a supressão da apoptose. Um outro exemplo da família Bcl-2 é a proteína Bax que pode se apresentar sob a forma de homodímeros que determinam a apoptose ou como heterodímeros com a proteína Bcl-2, inibindo a apoptose e determinando a sobrevivência da célula (BONINE et al., 2000). A heterodimerização entre os membros proapoptótico e antiapoptóticos da família Bcl-2 tem função antiapoptótica e é mediada pela proteína BH3 (DEGTEREV et al., 2001). Estímulos, como dano ao DNA, levam ao aumento na expressão das proteínas pró-apoptóticas o que induz a célula a apoptose (GRIVICICH et al., 2007). Após ativação pelo estímulo apoptótico, a mitocondria libera o citocromo c, AIF (Fator indutor de apoptose) e outros fatores apoptogênicos do espaço intermembranar para o citosol (KRAMMER, 2002) esse processo envolve a formação de um megaporo que contém diversas proteínas e abrange as membranas interna e externa da mitocondria por onde ocorre a liberação do citocromo *c* para o

citoplasma, onde participará da ativação da apoptose através da formação do apoptossomo, que consiste em um complexo formado pela associação do citocromo *c* com a APAF-1 (Fator de ativação da protease associada a apoptose 1) e a caspase -9, esse complexo promove a clivagem e consequente ativação da caspase 9 que será então liberada para clivar a caspase efetora 3 ocasionando a apoptose (GRIVICICH et al., 2007). Na via extrínseca a apoptose é mediada por uma superfamília de receptores TNF (Fator de Necrose Tumoral). Esses receptores são ativados por seus ligantes naturais como CD95, TRAIL–R1 (ligante R1 indutor de apoptose relacionado ao TNF) ou TRAIL-R2. A ligação do receptor ao seu ligante irá atrair o FADD (Proteína do domínio de morte associado ao Fas) dando origem ao DISC (Complexo sinalizante de morte induzida). Através desse complexo as caspases iniciadoras 8 e 10 serão recrutadas e depois ativadas pela clivagem, isso permitirá a ativação das principais caspases efetoras, as caspases 3, 6 e 7 , que concluem o processo apoptótico (IGNEY; KRAMMER, 2002).

Figura 3. Vias de Transdução de sinais da apoptose.



Legenda: Vias de Transdução de sinais da apoptose. O sinal apoptótico é iniciado pela trimerização mediada pela ligação direta dos membros da família de receptores de morte como o Fas. Isso conduzirá a formação do complexo de DISC e a ativação da procaspase-8 induzida por proximidade. A caspase-8 ativa pode diretamente clivar a procaspase-3 começando assim a fase de execução da apoptose. Alternativamente, a clivagem do membro próapoptótico da família Bcl-2, Bid conduz a amplificação do

sinal pró-apoptótico por via mitocondrial. Esta via também é usada na apoptose induzida pelo stress citotóxico. Isso leva a liberação de fatores pró-apoptóticos como o citocromo c e a Smac/Diablo do compartimento intramembranar da mitocôndria para o citosol. A libertação do citocromo c conduz a formação do apoptossomo, que ativa a caspase-3. Fonte: Alenzi et al, 2010.

Em contraste com apoptose, em que a morte de células é quase invisível já que é rapidamente consumida pelas células vizinhas, as células que se tornam necróticas, incham e explodem, libertando o seu conteúdo para o microambiente local dos tecidos (HANAHAN; WEINBERG, 2011). A necrose é um tipo de morte celular que durante muito tempo foi atribuída à morte acidental e descontrolada, sendo caracterizada por um aumento do volume celular, inchaço das organelas, ruptura da membrana plasmática e consequente perda do conteúdo intracelular. No entanto, trabalhos recentes mostram que a necrose apresenta um controle genético e é regulada por um conjunto de vias de transdução de sinal e mecanismos catabólicos (KROEMER, et al., 2009). A necrose regulada conhecida como necroptose é regulada por um conjunto de vias de transdução de sinal e mecanismos catabólicos, por exemplo, o domínio de receptores de morte (TNFr1, Fas/CD95 e TRAIL-R) e receptores *Toll-like* (TLR3 e TLR4), também mostraram provocar necrose, principalmente se as capases estiverem inibidas. Este tipo de necrose estaria presente nas doenças neurodegenerativas, doenças inflamatórias, infecções e câncer, assim como em outros processos (KROEMER, 2009).

3.2 Estágios da Carcinogênese

O desenvolvimento do câncer ocorre através de um processo dinâmico que acontece por etapas determinadas de múltiplos estágios da carcinogênese, podendo ser considerado em três estágios: iniciação, promoção e progressão (figura 1) (HARRIS, 1991). O processo de carcinogênese, ou seja, de formação de câncer, em geral dá-se lentamente, podendo levar vários anos para que uma célula cancerosa origine um tumor detectável. Esse processo passa por vários estágios antes de chegar ao tumor (INCA, 2015).

A primeira etapa é a iniciação onde há a ocorrência de danos ao DNA pela exposição de uma população de células ao carcinógeno, causando mutações genéticas que constituem o primeiro passo para o desenvolvimento neoplásico Nesta fase as células se encontram, geneticamente alteradas, apresentando danos ao DNA, com

indução de mutação em genes alvo críticos, ativação de protooncogenes, inativação de genes supressores de tumor, replicação celular e fixação da mutação, porém ainda não é possível se detectar um tumor clinicamente. As células encontram-se "preparadas", ou seja, "iniciadas" para a ação de um segundo grupo de agentes que atuará no próximo estágio (JAKÓBISIAK et al., 2003; INCA, 2015). Em tecidos de células normais, eficientes mecanismos de controle acontecem quando são detectados danos ao DNA promovendo a destruição das células danificadas para evitar a progressão maligna. O gene p53, supressor de tumor, age impedindo a proliferação de células mutadas, desse modo, as mutações ocorridas nos genes supressores de tumor permitem que as células mutadas prossigam no ciclo celular (FOSTER, 2008).

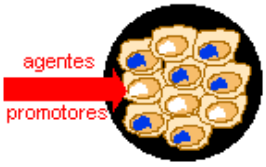
A promoção é o segundo estágio da carcinogênese. Nele, as células geneticamente alteradas, ou seja, "iniciadas", sofrem o efeito dos agentes cancerígenos classificados como oncopromotores. A célula iniciada é transformada em célula maligna, de forma lenta e gradual através da expansão clonal e desenvolvimento do tumor benigno. Para que ocorra essa transformação, é necessário um longo e continuado contato com o agente cancerígeno promotor. A suspensão do contato com agentes promotores muitas vezes interrompe o processo nesse estágio. Alguns componentes da alimentação e a exposição excessiva e prolongada a hormônios são exemplos de fatores que promovem a transformação de células iniciadas em malignas (JAKÓBISIAK et al., 2003; INCA, 2015).

O terceiro e último estágio é a progressão que é caracterizada pela multiplicação descontrolada, sendo um processo irreversível apresentando expressão alterada de enzimas, proteólises, invasão, migração e metástase. Nesta etapa o câncer já está instalado, evoluindo até o surgimento das primeiras manifestações clínicas da doença (JAKÓBISIAK et al., 2003; INCA, 2015).

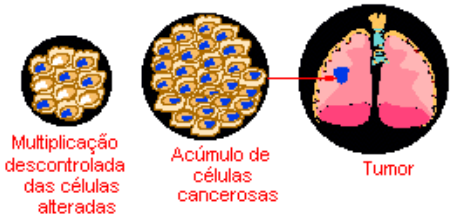
Figura 4: Etapas da carcinogênese.

1-Estágio da iniciação:

2-Estágio da promoção:



3-Estágio da progressão:



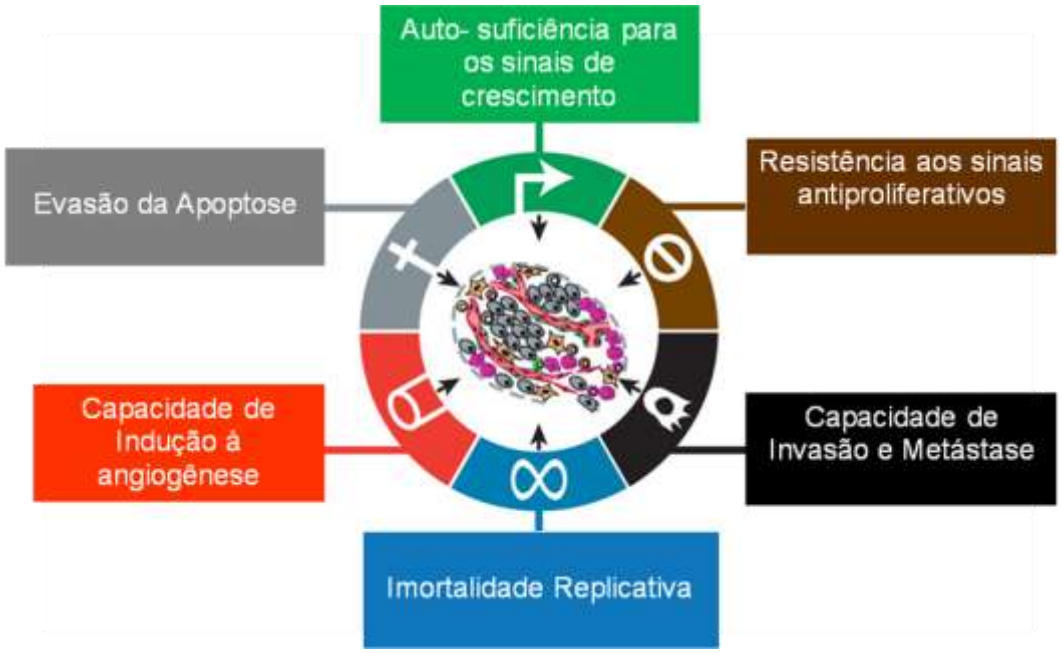
Legenda: Etapas da carcinogênese: **1- iniciação**: ocorrência de danos ao DNA pela exposição de uma população de células ao carcinógeno causando mutações genéticas; **2- promoção:** transformação em célula maligna de forma lenta e gradual através da expansão clonal e desenvolvimento do tumor benigno através de um longo e continuado contato com o agente cancerígeno promotor; **3- Progressão** que é caracterizada pela multiplicação descontrolada, sendo irreversível com expressão alterada de enzimas, proteólises, invasão, migração e metástase. **Fonte:** FOSTER, 2008; JAKÓBISIAK et al., 2003; INCA, 2015 http://www.inca.org.br/cancer, acessada em outubro de 2015.

3.3 Características Marcantes do Câncer

O câncer é uma doença genética que pode ocorrer nos diversos tecidos do organismo em razão de alterações patológicas ocorridas na informação contida no DNA. Tem origem a partir de mutações que modificam a expressão dos genes que codificam as proteínas envolvidas no controle da proliferação e da diferenciação celular ou comprometidas nos mecanismos de reparo do DNA. Como consequência, as células adquirem as principais características de células cancerígenas que são: a auto-

suficiência em sinais de crescimento; insensibilidade aos inibidores de crescimento; evasão da morte celular programada (apoptose); potencial de replicação ilimitado; capacidade para desenvolver novos vasos sanguíneos (angiogênese); invasão tecidual capacidade de migração (metástase) como descrito na figura 2 (HANAHAN; WEINBERG, 2000; MANTOVANI, 2009).

Figura 5. Marcos do câncer



Fonte: Adaptado de Hanahan e Weinberg, 2011

Auto-suficiência para sinais de crescimento: Para que células normais possam passar de um estado quiescente para um estado proliferativo ativo é necessário, a presença de sinais de crescimento mitogênicos que são transmitidos para a célula por receptores transmembrânicos que ligam diferentes classes de moléculas sinalizadoras como fatores de crescimento, componentes da matriz extracelular e moléculas de adesão/interação célula a célula. Nas células cancerígenas o processo ocorre de maneira diferente, pois estas são auto-suficientes na sinalização dos fatores de crescimento e para isso produzem e liberam fatores de crescimento que estimulam seus próprios receptores por sinalização autócrina e para receptores de células vizinhas por sinalização parácrina. Além disso, ocorre alteração do número, estrutura e função dos receptores de fator de crescimento, aumentando a propensão destes a enviar sinais de

crescimento para o núcleo. Nessas células também há a desregulação da via de sinalização dos receptores de crescimento para que esses estejam constantemente ativados (HARRINGTON 2007; HANAHAN; WEINBERG 2000).

Resistência aos sinais antiproliferativos: Outra característica marcante do câncer é a resistência aos inibidores de crescimento. No tecido de células normais a quiescência celular e homeostase do tecido são mantidos por vários sinais antiproliferativos, que podem bloquear a proliferação forçando as células a sair do ciclo ativo proliferativo para o estado quiescente (G_0) de onde a célula pode voltar ao ciclo ativo por sinalização extracelular. Ou ainda, os sinais antiproliferativos podem induzir as células a entrar num estágio pós-mitótico geralmente relacionado com a aquisição de características específicas associados à diferenciação (HANAHAN; WEINBERG 2000). A via de sinalização antiproliferativa acontece principalmente pelo controle do ciclo celular sendo mediada por proteínas que incluem a retinoblastoma (Rb), ciclinas, cinases dependentes de ciclinas (CDK) e as proteínas inibidoras das cinases dependentes de ciclinas (CDKi). As células do câncer frequentemente apresentam anormalidades na sinalização antiproliferativa através de alterações nessas proteínas, sendo comum em vários tumores a perda de Rb e de membros da família CDKi ou ainda pela superexpressão de determinadas ciclinas e CDK (HARRINGTON, 2007).

Evasão da apoptose: Numa célula normal, quando ocorre um dano ao DNA, ocorre a interrupção do ciclo celular para se avaliar a possibilidade de reparo. Quando não é possível reparar o dano, a célula é induzida à apoptose, impedindo que as mutações ocorridas sejam transmitidas às células descendentes pela divisão celular. Os principais genes envolvidos na apoptose são o pró-apoptótico p53 (supressor tumoral) e o anti – apoptótico Bcl-2 (oncogene). Diferentemente do que ocorre nas células normais, as células cancerosas conseguem evadir-se da apoptose, ignorando os sinais enviados pela via extrínseca ou ainda por reconfigurar o equilíbrio das moléculas pró e anti – apoptóticas em detrimento da apoptose (HARRINGTON, 2007).

Capacidade de indução à angiogênese: O crescimento do tumor maligno está relacionado com a capacidade de um fornecimento de sangue necessitando da formação de novos vasos que é realizada pela perda do equilíbrio entre os fatores positivos pró-angiogênico e negativos anti-angiogênicos que ocorre em células do tecido normal. Nas células cancerosas há um aumento da produção das proteínas pró- angiogênicas como o

fator de crescimento endotelial vascular (VEGF) e/ou por diminuir a produção das proteínas anti-angiogênicas como a trombospondina-1 (HARRINGTON, 2007).

Imortalidade replicativa: As células somáticas normais, após passar por um número finito de divisões celulares entram em senescência replicativa, isso acontece por que essas células são incapazes de replicar as extremidades dos seus cromossomos (telômeros) a cada divisão, como resultado seus telômeros sofrem encurtamento a cada ciclo celular atuando como relógios moleculares com a contagem regressiva do tempo das células. Diferentemente, as células do câncer conseguem manter a replicação dos telômeros a cada divisão celular, adquirindo desse modo a imortalidade replicativa (HARRINGTON, 2007).

Invasão tecidual e metástase: As células malignas conseguem atravessar a membrana basal MB e penetrar a matriz extracelular com o objetivo de invadir estruturas adjacentes. Esse poder de invasão leva a séria consequência da produção da metástase, pois desse modo essas células também conseguem invadir vasos sanguíneos e linfáticos e serem levadas pela circulação para um tecido íntegro onde irão emigrar do vaso e proliferar. Para haver a degradação da MB é necessário a presença de enzimas proteolíticas. Nos processos de invasão e metástases, as metaloproteinases da matriz extracelular (MMP) são expressas em altos níveis e apresentam importante ação proteolítica atuando diretamente sobre os componentes da MB. Desse modo, essas enzimas são cruciais na progressão tumoral (PEREIRA et al., 2006).

3.4 Estatisticas do Câncer

Atualmente as neoplasias malignas constituem um problema de saúde publica dada sua crescente importância como causa de morbidade e mortalidade. Com a expectativa de um aumento em cerca de 70% do número de casos em todo o mundo para as próximas duas décadas, e cerca de 60% desses ocorrerão nos paises emergentes (SEIXAS et al., 2010; STEWART; WILD, 2014).

O número de casos de câncer continuará aumentando nos países em desenvolvimento e crescerá ainda mais em países desenvolvidos se medidas preventivas não forem amplamente aplicadas. Nesses países, os tipos de canceres mais frequentes na população masculina foram próstata, pulmão e cólon e reto; e mama, cólon e reto e pulmão entre as mulheres. Nos países em desenvolvimento, os três cânceres mais

frequentes em homens foram pulmão, estômago e fígado; e mama, colo do útero e pulmão nas mulheres. Em 2030, a carga global será de 21,4 milhões de casos novos de câncer e 13,2 milhões de mortes por câncer, em consequência do crescimento e do envelhecimento da população, bem como da redução na mortalidade infantil e nas mortes por doenças infecciosas em países em desenvolvimento. No Brasil, a estimativa para o ano de 2014, válida também para o ano de 2015, previu a ocorrência de aproximadamente 576 mil casos de câncer, incluindo os casos de pele não melanoma, reforçando a magnitude do problema do câncer no país.

O câncer de pulmão matou aproximadamente 1.590.000 pessoas em 2012 e atualmente é a principal causa de morte por câncer em todo o mundo (ISLAMI et al., 2015).

O câncer de pele do tipo não melanoma (182 mil casos novos) será o mais incidente na população brasileira, seguido pelos tumores de próstata (69 mil), mama feminina (57 mil), cólon e reto (33 mil), pulmão (27 mil), estômago (20mil) e colo do útero (15mil). Sem considerar os casos de câncer de pele não melanoma, estimam-se 395 mil casos novos de câncer, 204 mil para o sexo masculino e 190 mil para sexo feminino. Em homens, os tipos mais incidentes serão os cânceres de próstata, pulmão, cólon e reto, estômago e cavidade oral; e, nas mulheres, os de mama, cólon e reto, colo do útero, pulmão e glândula tireoide. É incontestável que o câncer é hoje, no Brasil, um problema de saúde pública, cujos controles e prevenção deverão ser priorizados em todas as regiões, desde as mais desenvolvidas – cultural, social e economicamente – até às mais desiguais. As abordagens orientadas para enfrentar esse problema de saúde são, necessariamente, múltiplas, incluindo: ações de educação para saúde em todos os níveis da sociedade; prevenção orientada para indivíduos e grupos; geração de opinião pública; apoio e estímulo à formulação de legislação específica para o enfrentamento de fatores de risco relacionados à doença; e fortalecimento de ações em escolas e ambientes de trabalho. O câncer de pulmão é o mais comum de todos os tumores malignos, apresentando aumento de 2% por ano na sua incidência mundial. A última estimativa mundial apontou incidência de 1,82 milhão de casos novos de câncer de pulmão para o ano de 2012, sendo 1,24 milhão em homens e 583 mil em mulheres. (INCA, 2015).

3.5 Tratamentos contra o câncer

As pricipais formas de tratamento contra o câncer são a cirurgia, a radioterapia e aquimioterapia e o objetivo de cada um destes tratamentos é erradicar o câncer utilizando-os em forma da terapia combinada através da associação de mais que um tipo de tratamento (ALMEIDA, 2005).

O tratamento cirúrgico pode ser subdividido em três tipos: cirurgia curativa, indicada para os tumores em fases iniciais e sólidos; cirurgia preventiva, utilizada como primeiro passo na redução da população cancerígena quando está já está disseminada tem por finalidade impedir que a célula maligna se propague (INCA, 2012). A técnica cirúrgica pode levar à remoção de tumores com eficácia, se não houver metástase; no caso da leucemia, por exemplo, costuma ser necessário o uso de outros tipos conjuntos de terapia, incluindo o transplante de medula. A radioterapia (geralmente raios gama, radioisótopos como cobalto-60, raios-X e até prótons e mésons pi negativos) é usada comumente em conjunto com a cirurgia, com incremento da eficiência do tratamento. Mesmo isoladamente, a radioterapia pode diminuir tumores grandes, diminuir a recorrência e a chance de metástase, sendo uma metodologia antineoplásica muito usada; entretanto, mesmo que sejam usados os sensitizadores (que diminuem os efeitos colaterais) o tratamento por radiação é sujeito a severas limitações (ALMEIDA, 2005; TONETO et al., 2012). As diversas pesquisas promoveram um maior entendimento da biologia da célula do câncer e permitiu a exploração de novos mecanismos de ação para o desenvolvimento de novos fármacos com diversas abordagens moleculares como estudo de drogas que visam à alteração da expressão gênica, à inibição da invasão do tumor e metástase, à inibição da angiogênese, ou ainda o estudo de novas drogas citotóxicas que induzem a apoptose (BERNARDI et al., 2003).

No tratamento feito por quimioterapia o objetivo primário é destruir as células neoplásicas, sem destruir as normais. Entretanto, a maioria dos agentes quimioterápicos atua de forma não específica, lesando tanto células malignas quanto normais particularmente as células de rápido crescimento, como as gastrointestinais, capilares e as do sistema imunológico. (ALMEIDA, 2005).

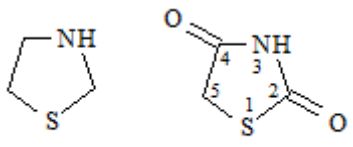
Existem vários quimioterápicos utilizados no tratamento do câncer como os agentes alquilantes polifuncionais, que são compostos capazes de substituir em outra molécula um átomo de hidrogênio por um radical alquil; os antimetabólitos: que afetam

as células inibindo a biossíntese dos componentes essenciais do DNA e do RNA; os antibióticos antitumorais: que são um grupo de substâncias com estrutura química variada que, embora interajam com o DNA e inibam a síntese desta macromolécula ou de proteínas, não atuam especificamente sobre uma determinada fase do ciclo celular; e os inibidores mitóticos: que podem paralisar a mitose na metáfase, devido à sua ação sobre a proteína tubulina, formadora dos microtúbulos que constituem o fuso espiralar, pelo qual migram os cromossomos, dentre outros (INCA, 2012). Existem também os quimioterápicos antineoplásicos que atuam por diferentes mecanismos de ação em virtude da presença de diferentes grupos funcionais na estrutura molecular destas substâncias (COSTA; CHAVES, 2013). Os heterocíclicos sintéticos derivados das tiazolidinadionas pertencem as novas classes de compostos em estudo para o tratamento do câncer.

3.6 Derivados das tiazolidinas

A química orgânica e medicinal tem como um dos principais objetivos desenhar, sintetizar e produzir moléculas que possuam propriedades terapêuticas em humanos. Compostos contendo sistemas de anéis heterocíclicos pertencem a uma classe de compostos com utilidade comprovada em química medicinal e por isso recebem atenção especial. Como exemplo, o anel heterocíclico de cinco membros contendo três átomos de carbono, um átomo de Nitrogênio e um átomo de Enxofre, conhecido como tiazóis (Figura 1) são de considerável interesse em diferentes áreas da química medicinal. Tiazolidina-2,4-diona (B) é o derivado da tiazolidina (A) com os dois grupos carbonila nas posições 2 e 4. Os substituintes nas posições 3 e 5 podem ser variados. (JAIN, 2013).

Figura 6. Núcleo Tiazolidina (A) e derivados Tiazolidina-2,4-diona (B)



(A) (B)

As Tiazolidinadionas (TZDs) Rosiglitazona, Ciglitazona, Pioglitazona e Troglitazona são uma classe de medicamentos orais usados para baixar a glicose no

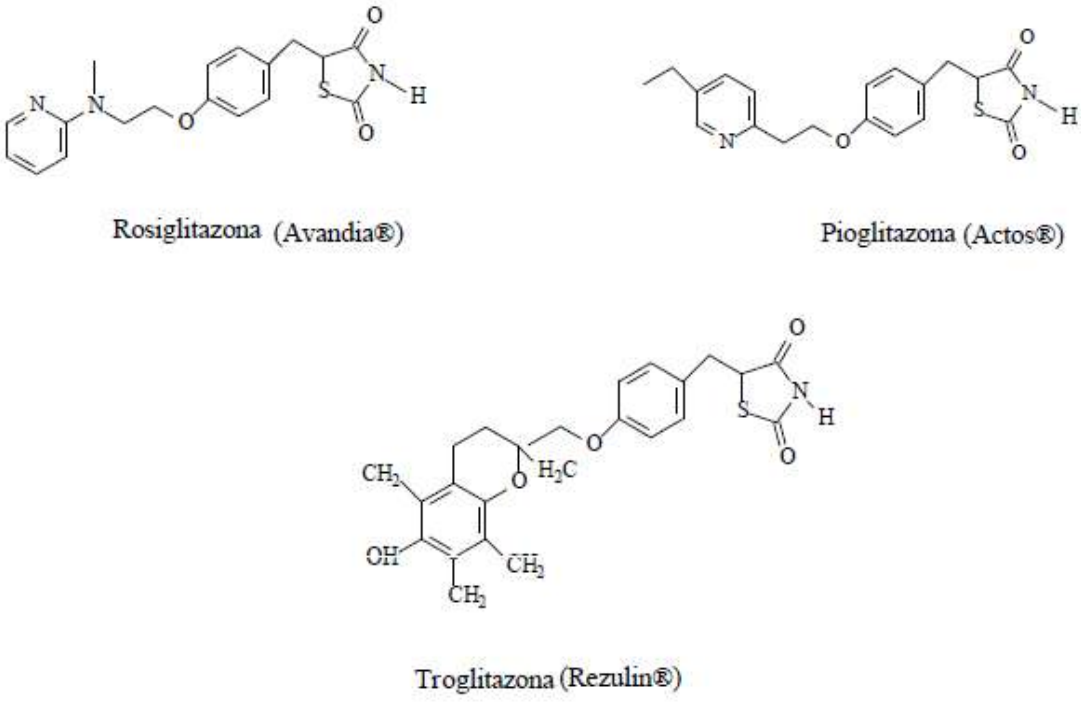
sangue. A Troglitazona foi retirada posteriormente por causar grave lesão hepática. A ação hipoglicemiante das TZDs é mediada principalmente através da redução da resistência à insulina no músculo e no tecido adiposo e inibindo a gliconeogênese hepática (MAGGS, 1998) esses fármacos atuam principalmente através dos receptores ativados por proliferadores de peroxissoma (PPARɣ), um receptor nuclear que tem um papel regulador na diferenciação celular, particularmente em adipócitos. Assim as TZDs, agindo através desses receptores provocam inúmeros efeitos além de diminuir os níveis de glicose no sangue (AFZAAL, 2005). O papel de PPAR-γ em células tumorais tem sido amplamente investigado. O tratamento com agonistas de PPAR- γ exerce efeitos biológicos tais como controle do crescimento celular, motilidade, diferenciação e apoptose (DIVYA, 2013).

3.7 Atividades biológicas das tiazolidinadionas

3.7.1 Hipoglicemiante

As TDZs glitazonas são drogas sensibilizadoras da ação da insulina. A sua estrutura química apresenta um anel diona, que confere a atividade anti-hiperglicêmica que, entretanto, depende da presença da insulina. O restante da molécula difere entre as drogas do grupo e é responsável pela especificidade farmacodinâmica e farmacocinética (GOMES, 2006).

Figura 7. Estrutura química das principais glitazonas



3.7.2 Atividade Antimicrobiana

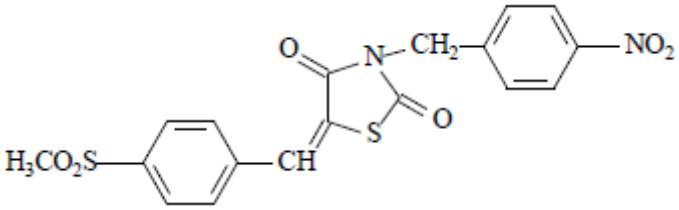
Derivados das tiazolidinadionas mostraram atividade antimicrobiana frente a bactérias gram-positivas, gram-negativas e álcool-acido resistentes, também foram ativos contra leveduras. O estudo foi realizado com nove compostos onde todos apresentaram atividade contra bactérias gram-positivas. Um dos compostos foi mais ativo inibindo as bactérias Gram-positivas, Gram-negativas, álcool-ácido resistentes e também contra leveduras. Os compostos que continha cloro na molécula apresentaram melhor atividade antibacteriana, demonstrando assim o poder deste átomo contra bactérias (SILVA, 2014).

A atividade antimicrobiana foi relatado por Mohanty et al. (2014), onde derivados das tiazolidinadionas foram avaliados quanto à sua atividade antibacteriana *in vitro* contra a *Escherichia coli* e *Pseudomonas aeruginosa* representando bactérias Gram-negativas e *Staphylococcus aureus* e *Bacillus subtilis* representando bactérias Gram-positivas. Os resultados revelaram que em geral, os compostos contendo os substituintes piridina e piperazina mostraram excelentes atividade anti-bacteriana.

3.7.3 Atividade Anti-inflamatória

Couto et al. (2006) analisaram derivados da série5-arilideno-3-(4-nitro-benzil)-tiazolidina-2,4-diona quanto ao seu potencial anti-inflamatório, verificando que este derivado apresentou ação anti-inflamatória investigada no modelo de bolsão de ar induzido por carragenina, reduzindo em 60% a migração celular para o local da inflamação.

Figura 8: 5- (4-metil-sulfonil-arilideno) - 3- (4-nitro-benzil) -tiazolidina-2,4-diona)



3.7.4 Atividade Antitumoral

Patil et al. (2010) sintetizaram uma nova série de derivados 5-benzilideno-2,4-tiazolidinadiona e avaliaram sua atividade antiploriferativa. Entre os compostos testados, o derivado 2-[4-[(2,4-dioxotiazolidin-5-ilideno) metil] fenoxi]-N-[3-(triflurometil)- fenil] acetamida, mostrou-se o mais promissor no teste preliminar de atividade antiproliferativa. Tal derivado apresentou potente citotoxicidade frente a cinco linhagens testadas: MCF7 (câncer de mama), PC3(câncer de próstata), KB (câncer nasofaríngeo), GURAV (câncer oral) e K562 (leucemia).

Compostos derivados das tiazolidinadionas também foram analisados quanto a sua estrutura função e sua capacidade para inibir a proliferação de células cancerosas que contêm a sinalização ERK ativada. A cascata de proteínas quinases ativadas por mitógenos (MAPK) é a principal via de sinalização envolvida na regulação da proliferação celular normal, sobrevivência e diferenciação. A regulação aberrante da cascata de MAPK contribui para o câncer e outras doenças humanas. Em particular a via de sinalização extracelular reguladas por quinases ERK (MAPK) tem sido objeto de intensa investigação em trabalhos de pesquisas conduzindo ao desenvolvimento de inibidores farmacológicos de tratamento do câncer (ROBERTS; DER, 2007). Tiazolidinas foram identificadas com várias características químicas como a inibição seletiva e potente de linhagens de células cancerosas que contém a sinalização ERK ativa. Também foi observada em vários dos compostos a capacidade de inibirem o

crescimento na linhagem de células de melanoma que apresenta resistência a medicamentos. Estudos recentes mostram que o uso de substâncias com sinalização alvo-ERK são uma alternativa contra a resistência a drogas (JUNG et al., 2013).

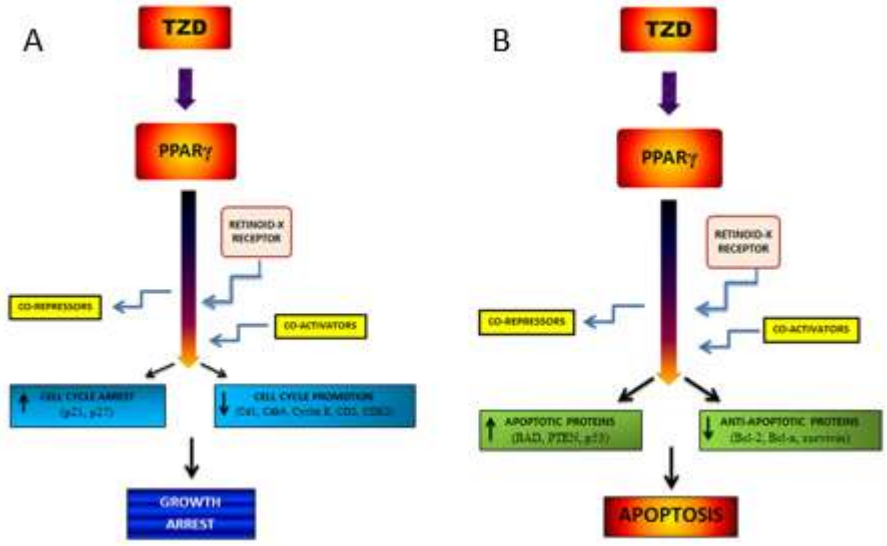
3.8 Mecanismos de ação envolvendo as tiazolidinadionas

As glitazonas têm como mecanismo de ação uma ligação aos receptores nucleares PPARs (receptores ativados por proliferador peroxissoma) do tipo γ. Após a ligação, há uma mudança conformacional no receptor, que permite a ligação com receptor do ácido retinóico (RXR) e recrutamento de um ou mais co-ativadores. A interação deste complexo heterodímero com regiões nucleares responsivas irá determinar a transcrição de aproximadamente 500 genes, porém, apenas uma pequena minoria é conhecida, e estão associados principalmente ao metabolismo lipídico, glicídico e diferenciação celular (GOMES, 2006).

- Ação anticâncer de tiazolidinadionas dependentes de PPAR ɣ (receptores ativados por proliferador de peroxissoma).

As tiazolidinadionas atuam principalmente pela ativação dos receptores nucleares PPARɣ (receptores ativados por proliferador de peroxissoma) levando a heterodimerização com o receptor do retnóide X, o recrutamento de co-ativadores e a dissociação de co-repressores levando a determinados eventos celulares. O PPARɣ é um receptor nuclear que tem um papel regulador na diferenciação celular e na morte celular programada (AFZAAL, 2005; BLANQUICETT et al., 2008; DIVYA et al., 2013; JAIN et al., 2013).

Figura 9. Mecanismo de ação das tiazolidinas (TDZ) induz a parada do ciclo celular e apoptose.



Legenda: ligação das TZD aos receptores nucleares PPARs (receptores ativados por proliferador peroxissoma) do tipo γ: Após a ligação, há uma mudança conformacional no receptor que permite a ligação com receptor do ácido retinóico (RXR) e recrutamento de um ou mais co-ativadores. A interação deste complexo heterodímero com regiões nucleares responsivas irá determinar a transcrição de aproximadamente 500 genes. Em (A) promovendo a parada do ciclo celular através do aumento de proteínas envolvidas no controle do ciclo celular (p21 e p27) e diminuição das proteínas promotoras do ciclo (CD1, CDk4, Ciclina E e CDkE). Em (B) há um aumento das proteínas pró-apoptóticas (BAD, PTEN e p53) associada com a diminuição das proteínas antiapoptóticas (Bcl-2, Bclx e survivina) (GOMES, M., B.; 2006) Fonte**:** Jain et al., 2013.

Parada do ciclo celular:

A parada do ciclo celular ocorre em virtude da diminuição do nível de proteínas ciclinas que regulam o ciclo celular, tais como; a ciclina D1 (CD1), ciclina E e Cd2. Os inibidores de CDK (ciclinas dependentes de kinases) bloqueiam a progressão do ciclo celular, por inativação, à formação de complexos de ciclina-CDK, que são cruciais para a fosforilação. As TZD aumentam a expressão dos inibidores de ciclinas dependentes de kinases CDKi como as p18, p21 e p27 causando por fim a parada do ciclo celular (DIVYA, 2013; JAIN et al., 2013).

Apoptose:

A ação apoptótica ocorre pela diminuição de proteínas anti-apoptóticas tais como Bcl-2 e Bcl-xL através da ativação da caspase 3, enquanto que aumenta os níveis

das proteínas pró-apoptóticas p53, Bad (promotor de morte associado ao Bcl-2) e fosfatase homólogo de tensina (PTEN). A troglitazona e ciglitazona podem inibir a função antiapoptótica de Bcl-xL/Bcl2 por bloqueio do domínio de morte BH3 que é o domínio mediador da heterodimerização com membros pró-apoptóticos de Bcl-2 interagindo como agonista (DIVYA et al., 2013; JAIN et al., 2013). Em Degterev et al. (2001), já havia sido verificado que derivados tiazolidinonas inibiram a heterodimerização Bcl-2/Bcl-xL mediada pelo domínio BH3 na função antiapoptótica, liberando membros pró-apoptóticos da família Bcl-2, os quais dão início à apoptose (DEGTEREV et al., 2001).

Diferenciação celular:

Foi relatado na literatura que a combinação do agonista de RXR (receptor do retnóide X) bexaroteno, com o agonista de PPARɣ, rosiglitazona causou o aumento da expressão de marcadores de diferenciação em células de câncer de cólon CEA (Antígeno Carcinoembrionário) ao mesmo tempo diminuindo a expressão da ciclooxigenase-2 (COX-2) e de prostaglandinas E2 (PGE-2). O tratamento das células HT-29 de câncer cólon retal com TZDs inibiu o crescimento celular e a metástase através da promoção de efeitos de diferenciação (DIVYA, 2013).

Redução da transcrição do gene do Recptor de insulina (IR)

Vários estudos têm demonstrado que os IR (Receptores de Insulina) é aumentado na maioria dos cânceres de mama humanos, o aumento da expressão do IR funcional também foi envolvido na carcinogênese de tireóide. O IR pode exercer o seu potencial oncogênico em células malignas através da estimulação anormal de múltiplas cascatas de sinalização celular aumentando o crescimento dependente de fator de proliferação. A transcrição do gene do IR e o teor de proteína receptora foram reduzidas em células com a super expressão forçada de PPARɣ ou com a ativação do PPARɣ induzida por TZDs. Desse modo, o IR pode ser considerado um novo gene alvo que representa a resposta antimitogênica para PPARɣ e seus agonistas (DIVYA, 2013).

- Ação anticâncer de tiazolidinadionas independentes de PPAR ɣ (receptores ativados por proliferador de peroxissoma).

Inibição de CDK e Ativação das proteínas p27 e p21:

Estudos mostram que a supressão da ciclina D1 em células de câncer de mama por TZDs se dá por efeito independente de PPARɣ. Outro estudo relata que o efeito de ciglitazona na transcrição do gene de P27 foi independente de PPARɣ e provavelmente mediada pelo elemento de ligação 1-binding a proteína de especificidade do promotor p27. E em células de câncer do pâncreas as troglitazonas induziram a ativação do promotor de p21 (DIVYA et al., 2013).

Supressão da atividade da telomerase:

A atividade da telomerase encontra-se muito baixa ou ausente em tecidos não neoplásicos, mas, na maioria das células cancerosas, sua atividade está presente ocasionando “imortalidade" celular (DUARTE e PASCHOAL, 2006). Foi relatado em trabalhos anteriores que a troglitazona reduziu a expressão de RNAm de hTERT (transcriptase reversa da telomerase humana) e a atividade da telomerase na linhagem celular de câncer de mama MDA-MB-231, mesmo na ausência de PPARɣ. Verificou-se também que não há nenhuma correlação entre PPARɣ e os níveis de transcrição RNAm de hTERT em pacientes com câncer de mama (DIVYA et al., 2013).

Inibição de metástase celular:

As tiazolidinadionas têm efeito inibidor na adesão a matriz extracelular e invasividade das células cancerosas. TZDs afetam a atividade gelatinolitica e fibrinolítica com um mecanismo independente de ativação de PPAR ɣ, agindo através da inibição da invasão de células no câncer de pâncreas. Elas causam uma significativa inibição da expressão do gene MMP-2 (matriz metalopeptidase 2), regula negativamente a expressão de MMP-9 (matriz metalopeptidase 9), e inibe a produção de MMP-7(matriz metalopeptidase 7), em ambas as culturas celulares que expressam e que não expressam PPARɣ (DIVYA et al., 2013).

Diminuição de prostaglandina E2:

As prostaglandina E2 (PGE2) são conhecidas por desempenharem um importante papel na tumorigénese por causa do sua atividade imunossupressora e de mediar a ação anti-apoptótica. As células de câncer de pulmão produzem prostaglandina E2 (PGE2) e as TZDs inibiram a produção da PGE2 em NSCLC (câncer de pulmão de não- pequenas células), através da inibição de ciclooxigenase-2 (COX-2) por uma via independente de PPARɣ (DIVYA et al., 2013).

3.9 Estudos de Genotoxicidade

Uma substância é considerada como agente genotóxico por atuar produzindo modificações estruturais e funcionais nas moléculas do DNA que se forem fixadas e transmitidas levam a mutações (KIRSCH-VOLDERS et al., 2003; HOUTGRAAFF et al., 2006). Os seres vivos sofrem exposição frequênte a agentes ambientais químicos, físicos ou biológicos tóxicos que podem causar alterações moleculares e celulares ao DNA célular (HEDDLE et al., 1983). As mutações sofridas pela molécula de DNA promovem a variabilidade genética das populações, porém, elas também podem induzir a um aumento da virulência de microorganismos patológicos, a doenças que são geneticamente herdadas e ao câncer (GONZALES et al., 2011). Por isso faz-se imprescindível o estudo de genotoxicidade das substâncias (FERREIRA-MACHADO et al., 2004).

Para a avaliação de genotoxicidade em células de mamíferos diversos ensaios podem ser utilizados tais como o ensaio cometa e o teste do micronúcleo, de acordo com os objetivos do estudo (BRUSICK, 1987; RIBEIRO et al., 2003).

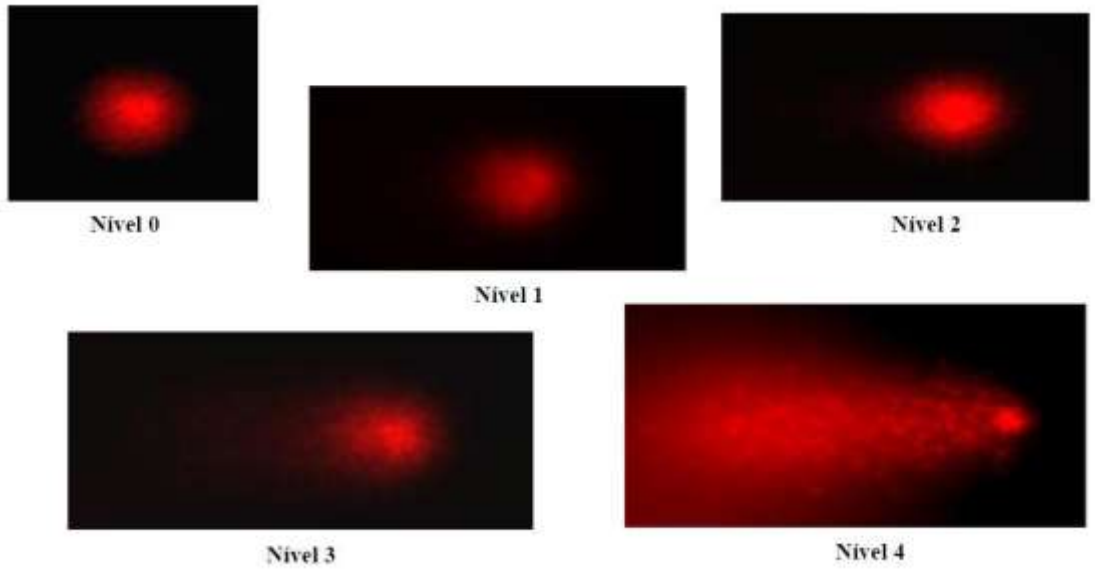
Ensaio Cometa: O Ensaio Cometa alcalino ou Single Cell Gel Electrophoresis é um teste muito utilizado para identificar danos causados ao DNA por agentes genotóxicos. É uma técnica rápida e eficiente a um custo relativamente baixo. Apresentando ainda certas vantagens em relação a outros ensaios, tais como: alta sensibilidade para detectar danos ao DNA com a utilização de pequenas quantidades de células podendo ser realizado em diversos tipos celulares e sem a necessidade destas células estarem em divisão. O teste tem por base a capacidade do DNA sofrer migração em uma matriz de agarose durante uma eletroforese (SINGH et al., 1988; FAIRBAN et al., 1995; TICE et al., 2000; HARTMANN et al., 2003).

O ensaio cometa pode ser realizado nas versões neutra e alcalina. A versão alcalina (pH>13) do cometa identifica quebras simples e duplas no DNA, sítios álcali-lábeis, sítios incompletos de reparo e ligações cruzadas enquanto a versão neutra identifica quebras duplas (TICE el al., 2000; WOJEWÓDZKA et al., 2002).

A análise dos danos ao DNA é feita pela visualização das lâminas com microscópio de fluorescência em aumento de 400X com um total de 100 células por grupo. O grau do dano causado ao DNA foi avaliado de acordo com a intensidade e tamanho da cauda do cometa, que é proporcional ao nível da fragmentação do DNA

(Figura 10). A avaliação foi realizada pelo padrão de escores de acordo com Mota et al. (2011) onde avaliou-se em cinco níveis de lesão gerada de acordo com o escore atribuído a cada cometa de acordo com o dano ao DNA: 0 = sem danos ao DNA, portanto, sem cauda (< 5 %); 1 = baixo nível de danos, com a cauda menor que o diâmetro da cabeça (5-20 %); 2 = médio nível de danos, com a cauda representando 1-2 vezes o diâmetro da cabeça (20-40 %); 3 = alto nível de danos, com a cauda representando mais de 2 vezes o diâmetro da cabeça (40-95 %); 4 = dano total (> 95 %).

Figura 10. Demonstração do padrão de escores para análise do ensaio cometa.

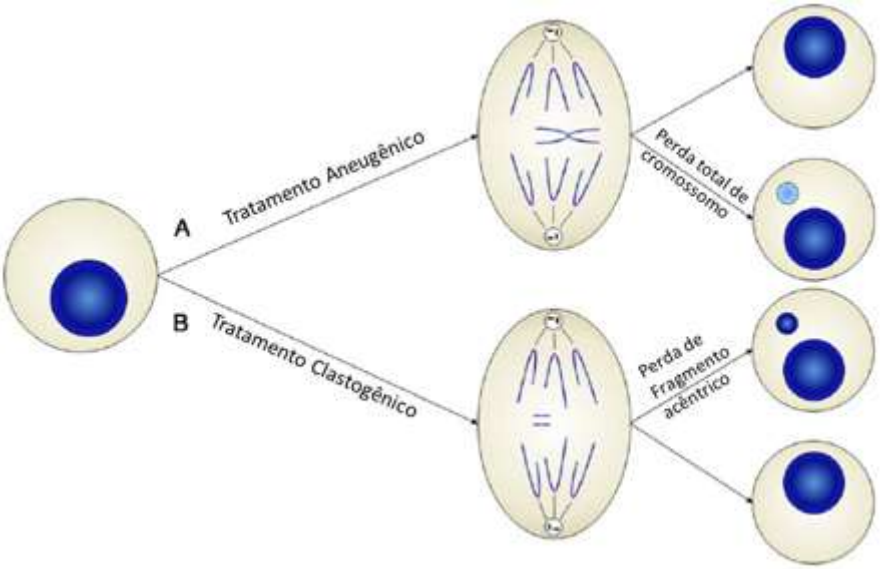


Legenda: Considera-se nível 0 = sem danos (<5%), nível 1 = baixo nível de danos (5-20%), nível 2 = médio nível de danos (20-40%), nível 3 = alto nível de danos (40-95%) e 4 = dano total (95%). Fonte: MOTA et al. 2011

Ensaio do Micronúcleo

O ensaio do micronúcleo (MN) é utilizado para detectar danos genômicos que não permitem reparos (FENECH, 2000). Este teste é capaz de detectar aberrações numéricas e cromossomais com resultados rápidos, com baixo custo e sem uso de produtos químicos prejudiciais permitindo a identificação de quebras nos cromossomos originadas pela ação de substâncias clastogênicas e detecção de segregação cromossômica anormal causadas por agentes aneugênicos com alta confiabilidade o que torna esse ensaio muito utilizado como biomarcador do dano genômico (MARZIN, 1997; FENECH, 2000; RIBEIRO et al., 2003; BONASSI et al., 2003).

Figura 11. Mecanismos de formação de micronúcleos (MNs).



Legenda: Mecanismos de formação de MNs por ação de agentes aneugênicos em (A) e de formação de MNs por exposição a agente clastogênicos (B). Fonte: TERRADAS et al, 2010.

Os micronúcleos são identificados como estruturas arredondadas com bordas evidentes e com diâmetro inferior a 1/5 do volume do núcleo principal, com cromatina semelhante a do núcleo e a mesma intensidade de cor ou mais fraca e localizado dentro dos limites citoplasmático (figura 12). (SCHMID, 1975; RAMIREZ & SALDANHA, 2002; TOLBERT et al., 1992).

Figura 12. Fotomicrografia de ensaio micronúcleo.

Legenda: Célula binucleada com um micronúcleo (seta). Fonte: Fenech (2006).

4 CAPÍTULO I - ARTIGO CIENTÍFICO 1

Accepted Manuscript

Title: Selective cytotoxic and genotoxic activities of 5-(2-Bromo-5-methoxybenzylidene)-thiazolidine-2,4-dione against NCI-H292 human lung carcinoma cells

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PII: S1734-1140(17)30061-0
DOI: https://doi.org/10.1016/j.pharep.2017.11.008
Reference: PHAREP 818

To appear in:

Received date: 26-1-2017
Revised date: 29-9-2017
Accepted date: 20-11-2017

Please cite this article as: Maria do D.Rodrigues, Priscila B.G.S.Santiago, Karla M.R.Marques, Valéria R.A.Pereira, Maria C.A.B.de Castro, Jeanne C.L.L.Cantalice, Teresinha G.da Silva, Mônica L.Adam, Silene C.do Nascimento, Julianna F.C.de Albuquerque, Gardenia C.G.Militao, Selective cytotoxic and genotoxic activities of 5-(2-Bromo-5-methoxybenzylidene)-thiazolidine-2,4-dione against NCI-H292 human lung carcinoma cells (2010), https://doi.org/10.1016/j.pharep.2017.11.008

Selective cytotoxic and genotoxic activities of 5-(2-Bromo-5-methoxybenzylidene)-thiazolidine-2,4-dione against NCI-H292 human lung carcinoma cells

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ABSTRACT

Background: Thiazolidine-2,4-dione ring system is used as a pharmacophore to build various heterocyclic compounds aimed to interact with biological targets. In the present study, benzylidene-2,4-thiazolidinedione derivatives (compounds **2** to **5**) were synthesized and screened against cancer cell lines and the genotoxicity and cytotoxicity of the most active compound **(5)** was investigated on normal and lung cancer cell line. *Methods:* For in vitro cytotoxic screening, the MTT assay was used for HL60 and K562 (leukemia), MCF-7 (breast adenocarcinoma), HT29 (colon adenocarcinoma), HEp-2 (cervix carcinoma) and NCI-H292 (lung carcinoma) tumor cell lines and Alamar-blue assay was used for non-tumor cells (PBMC, human peripheral blood mononuclear cells) were used. Cell morphology was visualized after Giemsa-May-Grunwald staining. DNA content, phosphatidylserine externalization and mitochondrial depolarization were measured by flow cytometry. Genotoxicity was assessed by Comet assay. *Results:* 5-(2-Bromo-5-methoxybenzylidene)-thiazolidine-2,4-dione **(5)** presented the most potent cytotoxicity, especially against NCI-H292 lung cancer cell line, with IC_{50} value of 1.26 µg/mL after 72 h incubation. None of the compounds were cytotoxic to

PBMC. After 48 h incubation, externalization of phosphatidylserine, mitochondrial depolarization, internucleosomal DNA fragmentation and morphological alterations consistent with apoptosis were observed in NCI-H292 cells treated with compound **(5)**. In addition, compound **(5)** also induced genotoxicity in NCI-H292 cells (2.8-fold increase in damage index compared to the negative control), but not in PBMC. *Conclusion:* Compound 5 presented selective cytotoxic and genotoxic activity against pulmonary carcinoma (NCI-H292 cells)

Keywords: Cytotoxicity, genotoxicity, 5-benzylidene-2,4-thiazolidinedione, lung cancer

Introduction

Cancer is one of the leading causes of mortality worldwide characterized by sustained chronic proliferation of immortal cells, evasion of growth suppressors' programs, resistance to cell death, development of angiogenesis, tissue invasion and metastasis [1]. The most common sites of cancers diagnosed among men are lung, prostate, colorectal, stomach and liver. Meanwhile, most common sites of cancer in women are breast, colorectal, lung, cervix and stomach. The total number of cancer related deaths worldwide raised from 8.2 million in 2012 to 8.8 million in 2015 and lung cancers caused approximately 1.69 million deaths in 2015 [2–4].

Thiazolidine-2,4-dione ring (Figure 1) system exhibits a broad spectrum of biological activities, such as hypoglycemic [5,6], anti-mycobacterial [7], anti-inflammatory [8], antileishmanial [9], antibiotics [7,10] and antitumor [11]. Much attention has been given to the construction of new derivatives of thiazolidinediones with anticancer activity [12–14].

Heterocyclic molecules belonging to the thiazolidinedione class represents a new alternative in the search for drugs with antineoplastic potential. Thiazolidinediones exhibit antitumor effects through both PPARγ-dependent and -independent mechanisms [15,16]. The role of PPARγ (peroxisome proliferator-activated receptors) in tumor cells has been extensively investigated and treatment with PPARγ agonists exert biological effects such as cell growth control, motility, differentiation and apoptosis [16,17] PPARγ-independent mechanism of tumor cell death induced by thiazolidinediones treatments includes proteasome-dependent degradation of cyclins (D1 and D3) with consequent cell cycle blockage at G_1-S transition, induction of cellular acidosis through inhibition of the Na^+/H^+ exchanger and release of apoptotic factors from the mitochondria due to the production of reactive oxygen species (ROS) [15].

Among many thiazolidinediones derivatives with distinct biological activities, 5-benzylidene-2,4-thiazolidinedione and its analogues presented cytotoxic activity against a variety of different cell lines: compound 2-[4-[(2,4-Dioxothiazolidin-5-ylidene) methyl] phenoxy]-N- [3-(trifluromethyl) phenyl] acetamide was active against K562 leukemia, MCF-7 (breast cancer), PC3 (Prostate cancer), KB (Nasopharyngeal cancer) and GURAV (Oral cancer) [18]; 5-(4-(2-(piperidin-1-yl)ethoxy)benzylidene)thiazolidine-2,4-dione was active against a panel of 60 human tumor cell [19]. In fact, a wide variety of substituents in the thiazolidine-2,4-dione

(TZD) nucleus lead to a huge diversity of compounds targeting different signaling pathways related to cell proliferation or cell death [11].

One of the most important characteristic for a new molecule with action against cancer is its selectivity toward tumor cells, since many anticancer drugs cause diverse cytotoxic and genotoxic damages also in normal cells. Although there are many reports indicating a wide range of biological activities for compounds with a thiazolidine-2,4-dione nucleus, genotoxicity studies in normal cells are still insufficient. In this context, this study aimed to evaluate *in vitro* antitumor effects of 5-benzylidene-2,4-thiazolidinedione derivatives, as well as to investigate the possible mechanisms of action, and genotoxic outcomes of these compounds on human normal and tumor cells.

Material and Methods

Synthesis of thiazolidine-2,4-dione derivatives compounds

The synthesis methodology of compounds 1 to 5 was already described [9]. Briefly, the thiazolidine-2,4-dione **(1)** was obtained by the method already described [20,21]. This reaction occurs by condensation of monochloroacetic acid and thiourea in an aqueous medium under reflux for 24 h. The series of thiazolidine-2,4-dione derivatives were originated by changes in the 5 position of the heterocyclic ring **(Fig. 1).** The compounds5-(2-bromo-6-fluorobenzylidene)-thiazolidine-2,4-dione **(2)**, 5-(2-Hydroxy-3-bromo-5-chlorobenzylidene)-thiazolidine-2,4-dione **(3)**, 5-(2-Hydroxy-5-clorobenzylidene)-thiazolidine-2,4-dione **(4)** e 5-(2-bromo-5-methoxybenzylidene)-thiazolidine-2,4-dione **(5)** were synthesized according to protocol described previously [9] **(Fig. 2)**. A solution of thiazolidine-2,4-dione (0.2 g, 1.70 x 10^{-3} mol) in ethanol, (7.0 mL) containing piperidine (2 drops) and aromatic aldehyde (0.184 g, 2.25 x 10^{-3} mol) was heated (70ºC), under stirring, for 5-9 h. Afterward, the product was cooled in an ice bath, filtered and recrystallized with an appropriate solvent. The resulting precipitate was filtered off and recrystallized from acetic acid to give the compounds (2-5). All compounds were identified by IR spectroscopy method, NMR and HRMS (Supplementary material**).** The yields obtained ranged from 73 to 79%.

Cell lines

The cell lines NCI-H292 (Mucoepidermoid pulmonary carcinoma), HL60 (Promyelocytic leukemia), HT-29 (Colon adenocarcinoma), K562 (Chronic myelogenous leukemia), HEp-2 (cervix carcinoma) and MCF-7 (Breast adenocarcinoma) were obtained from The Rio de Janeiro cell bank, Brazil. The cells were maintained in DMEM or RPMI 1640 medium. All cell lines were supplemented with 10% fetal bovine serum (GIBCO), 1% antibiotic solution (penicillin 5000 Units/mL + streptomycin 5000 µg/mL) and 1% L-glutamine 200 mM.

Cytotoxic activity in tumor cells

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma Aldrich Co., St. Louis, MO/USA) reduction assay was used for cytotoxicity. Tumor cells were plated in 96-well plates (10^5 cells/mL for adherent cells or 3×10^5 cells/mL for leukemia). Tested compounds (0.39–25 µg/mL) dissolved in DMEM with DMSO 0.007% for the lowest concentration (0.39 µg/mL) to 0.5 % for the highest concentration (25 µg/mL) of compounds were added to each well. Subsequently, cells were incubated for 72 h. The compound **5** was also evaluated after 24 and 48 h of incubation in NCI-H292 cells. Control groups received the same amount of DMSO. After 69 h of treatment 25 µL of MTT (5 mg/mL) was added, three hours later the supernatant was removed and the MTT-formazan product was dissolved in 100 µL of DMSO, and absorbance was measured at 570 nm in plate spectrophotometer. Doxorubicin (0.01–5 µg/mL) was used as positive control. Data are presented as IC_{50} (concentration that cause 50% of cell growth inhibition) values with their 95% confidence intervals (CI 95%) obtained by non linear regression after normalize the absorbance results against untreated control samples [22].

Peripheral blood mononuclear cells isolation and cytotoxic activity (PBMC)

Mononuclear cells (lymphocytes and monocytes) were obtained from the peripheral blood of healthy volunteers collected in sterile tubes with heparin solution (BD Vacutainer ™) as an anticoagulant. The protocol was approved by The Human Research Ethics Committee (Nº CAAE 48809515700005208). The total blood (6 ml) was diluted in 3 ml PBS and layered onto 2 ml Ficoll®-Hystopaque (Sigma). For phase separation of the solution, the tube was centrifugated at 1500 rpm for 30 minutes. After centrifugation the mononuclear cells were concentrated in the middle layer located between the plasma (light phase) and erythrocytes (dark phase). Afterwards the PBMC were transferred to another tube with PBS to a final volume of 11 ml. The cells were pelleted by centrifugation and resuspended in RPMI 1640 medium supplemented with 20% fetal bovine serum and 1% antibiotics 1% antibiotic solution (penicillin 5000 Units/mL + streptomycin 5000 µg/mL). Phytohemagglutinin 2% (Sigma) was added to the medium to stimulate the proliferation of lymphocytes. Cells were seeded at 10^6 cells/mL in 96 well plates. After 24 hours of incubation, compounds were dissolved in DMEM with DMSO and added to each well. Doxorubicin (0.078 to 10 µM) was used as a positive control. The negative control received the same amount of DMSO. Twenty-four hours before the end of the incubation period (total time of 72h), 10 µL of Alamar Blue stock solution (0.312 mg/mL) was added to each well. The absorbances were measured at 570 nm (oxidized state) and 595 nm (reduced state) in a plate spectrophotometer. Cell proliferation that was calculated by the following formula: % proliferating = ALW – (AHW x R0) x 100, where ALW is the absorbance at the lowest wavelength (570 nm) e AHW is the absorbance at highest wavelength (595 nm), respectively. The R0 was calculated according to the formula: R0 = (absorbance of medium with Alamar Blue minus absorbance of medium without Alamar blue at 570 nm)/(absorbance of medium with Alamar Blue minus absorbance of medium without Alamar blue at 595 nm) [23].

Evaluation of hemolytic potential in human erythrocytes

Blood was collected from healthy volunteers. Erythrocytes were pelleted by centrifugation (3000rpm/5min) and resuspended in saline (0.85% NaCl + 10 mM $CaCl_2$) to obtain 2% erythrocytes solution (SE) 2%. The 96-well plates were prepared according to the following: 100 µl of saline (negative control); 50 µl of saline solution and 50 µl of vehicle with 1% DMSO (Blank); 80 µL saline + 20 µL Triton X - 100 1% (positive control); 50 µl of saline solution and 50 µl of compounds (1.95 to 250 µg/ mL) diluted in 1% DMSO. Then 100µl of the solution of erythrocytes was plated in each well. After incubation for 1 h under constant agitation at room temperature, the supernatant was analyzed in automatic plate reader (450nm). The 50% effective concentration (EC_{50}) and their 95% confidence intervals were determined from nonlinear regression.

*Morphological changes induced compound **5** (*5-(2-bromo-5-methoxybenzylidene)-thiazolidine-2,4-dione) *on lung adenocarcinoma (NCI-H292)*

Compound **5,** the most active compound, was selected for further investigation of cytotoxicity on lung carcinoma NCI-H292. Cells cells (10^5 cells/mL) were treated for 48 h with Compound 5 at 8.0 and 16.0 µg/mL, the IC_{50} and 2x IC_{50} for 48h, respectively. Afterward, slides were prepared by cytospin centrifugation. To evaluate the morphology, the cells were fixed with methanol for 1 minute and stained with May-Grunwald-Giemsa. Doxorubicin (0.5 µg/mL) was used as a positive control.

Analysis by Flow Cytometry

Externalization of phosphatidylserine in NCI-H292 after treatment with compound 5

The externalization of phosphatidylserine was evaluated by annexin/propidium iodite staining. NCI-H292 cells were treated with compound 5 at 8 and 16 µg/mL for 48 hours, the IC_{50} and 2x IC_{50} for 48 h, respectively. Doxorubicin (0.5 µg/mL) was used as positive control. Cells were harvested and incubated with AnnexinFitV-FITC kit according to the manufacturer (Sigma). Cell fluorescence was then determined by flow cytometry on a BD cytometry - FACS Calibur - CBA. Twenty thousand events per experiment were acquired and cell debris were omitted from the analysis. The percentages of viable cells, early apoptotic, late apoptotic and necrotic cells were graphed using Prism 5.0 (GraphPad Software Inc.)

*Measurement of mitochondria transmembrane potential in NCI-H292 after treatment with compound **5***

NCI-H292 cells treated with compound 5 at 8 and 16 µg/mL for 48 hours, the IC_{50} and 2 x IC_{50} for 48 h, respectively and doxorubicin (0.5 µg/mL) were centrifuged at 2000 rpm for 5 minutes, washed with PBS and stained with 200 µL of rhodamine 123 (1 µg/ml) for 15 min. Subsequently, cells were pelleted again and incubated with PBS for 30 minutes. Cell fluorescence was then determined by flow cytometry. Twenty

thousand events per experiment were acquired and cell debris were omitted from the analysis [24].

*DNA content of NCI-H292 cells (DNA fragmentation and cell cycle) after treatment with compound **5***

Cells were collected, pelleted and fixed with cold ethanol (70%) for 1h. After washing with phosphate buffer saline to remove ethanol, treated and untreated cells were incubated with 100 µL of lysis solution (0.1% sodium citrate, 0.1% triton X - 100 and 2 µg/ml of propidium iodide). After 30 minutes cell fluorescence of the samples were analyzed on a BD cytometry - FACS Calibur – CBA. FlowJo was used to calculate the cell cycle parameters, based on the cells PI emission (analyzed in the FACSCalibur FL2 channel that captures 564-606 nm emissions). Twenty thousand events per experiment were acquired and cell debris were omitted from the analysis [25].

*Genotoxic effect of compound **5** in tumor and normal cell lines - Comet assay*

We evaluated the genotoxic effect of compound **5** using the comet assay [26]. Approximately 2 x 10^5 cells/mL of the NCIH-292 were treated with compound 5 for 48h. To investigate the initial effects on DNA damage induced by compound 5 we used the 72 h IC_{50} and 2 x IC_{50}, 1.26 and 2.5 µg/mL, respectively. For normal cells, we choose 2 x IC_{50} and 4 x IC_{50} found in cancer cells. PBMC were treated with compound **5** with 2.5 and 5.0 µg/mL for 48 h. Doxorubicin (0.5 µg / mL) was used as a positive control. The negative control received the same amount of DMSO as treated samples (0.5%). After treatment, cells were harvested and 15 µL of the cell suspension was embedded in low melting point agarose at 37°C. The homogenate was then casted on agarose-coated glass slide. Slides were stored in the dark at 4ºC for 20 min before adding electrophoresis buffer. Gel electrophoresis was performed at 40 V for 20 minute and 300 mA). The slides were neutralized in 0.2 M Tris buffer (pH 7.5) for 15 minutes and fixed with 98% ethanol for 5 minutes. After drying, the slides were stored in a refrigerator until staining. Staining was performed through 1:1000 Gel Red (BiotargetGelRed®) addition onto each slide. We analyzed the slides using a fluorescence microscope (Olympus – BX series). Approximately 100 cells per treatment were analyzed and scored from 0 to 4 points depending on the degree of damage in the nucleoid as the following criteria: (a) class 0: undamaged, with no tail; (b) class 1: with tail shorter than the diameter of the head (nucleus); (c) class 2: with tail length between one and two times the diameter of the head; (d) class 3: with tail longer than two times the diameter of the head; and (e) class 4: comets with no heads. The damage index (DI) can vary from 0 (0x100) to 400 (4x100) [27]. Examples of comets from our experiment are provided for better understanding of the classification used (**Fig. 3**).

Statistical analysis

For cytotoxicity assays, IC_{50} values and their 95% confidence intervals were obtained by non-linear regression. In order to determine the differences, the data were

compared by analysis of variance (ANOVA) followed by Dunnett`s test (p <0.05). The GraphPad Prism version 5.00 was used for statistical analysis.

Results

Cytotoxic activity of thiazolidine-2,4-dione derivatives in tumor cells

Four compounds were tested in six human tumor cell lines HEp-2, HT-29, HL-60, MCF-7, K562, and NCI-H 292 and cytotoxicity was determined by MTT assay after 72 hours of incubation. Compounds **2**, **3** and **4** showed low cytotoxic activity with IC_{50} values ranging from 9.0 µg/ml to 25.0 µg/ml (**Table 1**). Compound **5** was the most active compound with lowest IC_{50} value (1.2 µg/mL) for lung cancer (NCI-H292). All cell lines were inhibited by the positive control doxorubicin, being HEp-2 and HT-29 the most resistant cell lines. In order to investigate the time dependency of compound 5 cytotoxic activity, NCI-H292 cells were treated for 24 h and 48 h. Compound **5** presented IC_{50}> 25 µg/mL after 24 hours of treatment and IC_{50} of 7.9 µg/mL after 48 hours of treatment **(Fig 4)**

Cytotoxic activity of thiazolidine-2,4-dione derivatives on peripheral blood mononuclear cells (PBMC) and hemolytic activity

Compounds that cause non-specific cell death targeting the cell plasmatic membrane are very toxic. A positive hemolytic assay excludes the compound from being used as therapeutic drug. None of the compounds tested were toxic to human erythrocytes (EC_{50}> 125 µg/mL). In addition, the compounds were tested in a model of human normal cells: peripheral blood mononuclear cells. After isolation from human blood, PBMC cultivated under mitogenic stimulus works as a model of normal cells. Compounds **2** to **5** were not cytotoxic to PBMC after 72 h of treatment (IC50> 25 µg/mL). The results indicated that the compound **5** showed selectivity for tumor cell lines since it causes tumor cell growth inhibition but it does not cause hemolysis of human erythrocytes neither cytotoxicity toward PBMC.

Analysis of cell morphology -May - Grünwald – Giemsa staining

The cell morphology was analyzed by optical microscopy after 48 h of incubation. NCI-H292 untreated cells (negative control - CN) showed typical cellular morphology of adherent cells with intact nuclear and plasmatic membrane and the presence of mitotic cells (**Fig. 5**). Cells treated with compound **5** at 8.0 µg/mL and 16.0 µg/mL exhibit morphological changes consistent with apoptosis including the cell volume reduction and nuclear fragmentation (**Fig. 5**). Doxorubicin (0.5 µg/mL) reduced the number of cells, induced reduction of cell volume, chromatin condensation and nuclear fragmentation in NCI- H292 cells.

Phosphatidylserine externalization analysis and mitochondrial transmembrane potential by flow citometry

The percentage of NCI-H292 apoptotic cells was significantly higher ($p<0.05$) after 48h treatment with compound **5** at 16 µg/mL (64%) compared to the negative control (15%). Doxorubicin 0.5 µg/mL also induced apoptosis (51%) compared to the negative control ($p<0.05$). None of the treatments induced increase on necrotic cells (**Fig. 6 and Fig. 7**). The mitochondrial membrane potential evaluation was performed by flow cytometry. Rhodamine 123, a green-fluorescent dye, is sequestered by active mitochondria. When mitochondria depolarizes due to membrane damage, the dye is lost and the average fluorescence decrease. After exposure of NCI-H292 to compound **5** (16.0 µg/mL) for 48 hours a significant increase ($p<0.05$) in mitochondrial depolarization was observed (**Fig. 6 and Fig. 7**). Doxorubicin (0.5 µg/ml) was also able to promote significant mitochondrial depolarization at the concentration tested ($p<0.05$).

Analysis of cell cycle and DNA fragmentation

NCI-H292 cells were treated with compound **5** for 48 h (**Fig. 6**) and 72 h (**Fig. 9**) at IC_{50} and 2 x IC_{50} and DNA content was quantified by flow cytometry. After 48 h of treatment, an increase on subdiploid DNA was observed when cells were treated with compound **5** at 16 µg/mL (16.12% after 48h) compared to negative control (2.4% after 48h) ($p<0.05$). After 48 h there were no significant differences at cells cycle phases (G0/G1, S and G2/M) between treated and control cells (**Fig. 7**). After 72 h of treatment compound **5** at 1.26 µg/mL caused an increase of cells on G0 / G1 (59.8 % after 72 h) vs. negative control (41.3 % after 72 h) ($p<0.05$). When cells were treated with 2.52 µg/mL of compound **5** during 72 h, an increase on subdiploid DNA (27.4 % after 72 h) was observed (negative control 3.79 % after 72 h) ($p<0.05$).

Genotoxicity assessment in PBMC and NCI-H292

The in vitro genotoxic activity on both PBMC and lung cancer cell line NCI-H292 was assessed after 48 h by the Comet assay. To analyze the extent of damage to DNA molecules, data were expressed as damage index (ID) ranging from 0 (no damage) to 400 (maximum damage) (**Fig. 10**). No DNA damage was detected on PBMC after 48 h of treatment at any tested concentration of Compound **5** (2.52 µg/mL e 5.0 µg/mL) compared to the negative control; Conversely, Doxorubicin (0.5 µg/mL) caused significant DNA damage. For lung cancer cell line NCI-H292, compound **5** (1.26 µg/mL e 2.52 µg/mL) caused an increase on damage index of DNA at both tested concentrations (1.26 µg/mL e 2.52 µg/mL). Doxorubicin also increased DNA damage on NCI-H292.

Discussion

Recently, compounds 2, 3, 4 and 5 were synthesized in satisfactory yields and tested against pteridine reductase 1 (PTR1), a promising enzyme as target for the development of antileishmanial drugs. Compound 3, the first non-competitive inhibitor of PTR1, presented a two-digit micromolar potency (less than 50 uM) in comparison with compounds 2, 4 and 5 [9]. Nonetheless, cytotoxic and genotoxic potentialities on neoplastic or normal cells have not been described yet.

Compounds 2 to 5 were screened against six tumor cell lines (HEP-2, HL-60, HT-29, K562, MCF-7 and NCI-H292) and 5 presented lowest IC_{50} values. The most sensitive cell line treated with compound 5 was NCI-H292 (IC_{50} value of 1.26 µg/mL). The presence of an electron releasing substituent methoxy group increased the activity of compound 5, while in compound 2 the presence of two strong electron withdrawing atoms (bromine and fluorine) reduced the cytotoxic activity. Previously, some studies have reported *in vitro* cytotoxic activities of thiazolidine-2,4-dione derivatives upon tumor lines of different histological types [18,28,29]. Amongst ten compounds synthesized, (2-[4-[(2,4-Dioxothiazolidin-5-ylidene) methyl] phenoxy]-N-[3-(trifluromethyl) phenyl] acetamide) showed potent cytotoxicity against five of the seven cell lines tested (breast cancer MCF-7, prostate cancer PC3, nasopharyngeal KB, cancer oral cancer GURAV and leukemia K562) [18]. Another group of derivatives where the substitution reaction occurs at the third position of the 2,4- thiazolidinedione ring presented two active compounds against MCF-7 cell line [28]. Recently, two series of thiazolidinediones were synthesized and their cytotoxicity evaluated in prostate adenocarcinoma PC-3, breast adenocarcinoma MDA-MB-231, and fibrosarcoma HT-1080 cancer cell lines. Most of the compounds synthesized were active against at least one cell line, being 5-{4-[(3-(4-Chlorophenyl)-3,4-dihydro-4-oxoquinazolin-2-yl)methoxy]benzylidene}thiazolidine-2,4-dione the most active compound. Moreover, mechanistic investigation revealed pro-apoptotic activity of the most cytotoxic compound [29] .

In order to verify whether the cytotoxicity is related to direct cell membrane damage, the compounds were incubated with human erythrocytes. None of the compounds induced lysis of human erythrocytes (EC_{50}> 125 µg/mL). Probably, the cytotoxicity of compound 5 toward tumor cells occurs by a more specific mechanism other than a immediate and direct disruption of cell membrane. In fact, treatment of NCI-H292 cells for a brief period such as 24 h with compound 5 did not reduce cell growth. Only after 48 hours of treatment, a significant cytotoxicity on NCI-H292 was observed with maximum effect achieved after 72 h. Lung cancer is currently the leading cause of cancer death worldwide and for most patients with this disease current treatments do not promote cure [3,30]. Additionally, cytotoxic action on normal cells (peripheral blood mononuclear cells) was not observed with compounds 2 to 5 (IC_{50}> 25 µg/ml). Compound 5 demonstrated antiproliferative selectivity to lung cancer cells, since the tumor cells were affected by the treatment but not normal cells.

Morphological and biochemical patterns in NCI-H292 human lung cancer cells incubated with compound 5 were investigated. NCI-H292 treated cells exhibited cell volume reduction and DNA fragmentation (8 and 16 µg/mL), suggesting activation of apoptotic events. The process of cell death by apoptosis has some characteristics such as cell volume reduction, chromatin condensation, and nuclear fragmentation without extravasation of the cellular content [31]. So, unlike what happens in necrosis, no inflammatory process occurs [32]. Mitochondrial membrane depolarization and loss of plasma membrane phospholipid asymmetry also occur during the apoptosis process [33].

To confirm if morphological alterations in NCI-H292 treated cells were consistent with apoptosis, phosphatidylserine (PS) externalization was evaluated. An increase on NCI-

H292 apoptosis was observed after 48 h of treatment with compound 5 and such findings were accompanied by mitochondrial depolarization and DNA fragmentation (16 µg/mL). After 72 h of incubation, compound 5 caused DNA fragmentation in NCI-H292 cells at 6-fold lower concentration (2.5 µg/mL) than that used for 48 h analyses and cell cycle arrest at G0/G1 at 1.26 µg/mL. A report has shown that hybrid molecules containing 5-benzilidene thiazolidine-2, 4-dione induced apoptosis on leukemia cells by activation of the extrinsic and the intrinsic pathways of cell death [14]. Pioglitazone (PGZ), a thiazolidinedione compound, induced growth inhibition and apoptosis of human B lymphocytic leukemia (SD1 cells). Treatment of these cells with the PPARγ ligand pioglitazone resulted in growth inhibition in a dose-dependent manner which was associated with a G1 to S cell cycle arrest after 3 days of treatment. After 4 days of treatment PGZ caused significant apoptosis in lymphocytic leukemic cell lines [34]. TZD18, Another PPARα and PPARγ agonist structurally related to the thiazolidinedione, induced G1 cell cycle arrest in Ph+ lymphocytic leukemia cell line at 10 uM and at higher concentration (20 µM) induced apoptosis in a time-dependent fashion [35]. Thus, G1 arrest may represent one of the underlying mechanisms for subsequent cell apoptosis.

Thiazolidine-2,4-dione (TZDs) were evaluated regarding the expression of proteins that control the transition from the G1 to S cell cycle´s phase in human prostate cancer cell lines [36]. Inhibitory concentrations of TZDs rosiglitazone and ciglitazone induced expression of p21 (Inhibitor of cyclin-dependent kinase) and decrease of cyclin D1 levels in PC-3 cells. Cell cycle arrest occurred due to the decrease in the level of cyclin proteins that regulate cell cycle progression [36].

The genotoxicity of compound 5 was accessed by alkaline comet assay. This technique allows quantitative evaluation of recent damage to DNA by single and double breaks [37] and has advantages over others DNA damage methods such as the micronucleus test because of its high sensitivity and early detection of DNA injury. Compound 5 did not cause genotoxicity in PBMC even for a prolonged period of treatment with 5 µg/mL (48 h), but NCI-H292 cells presented significant DNA damage at 4-fold lower concentrations than that used for normal cells (1.25 µg/mL). Doxorubicin (0.5 µg mL) also produced DNA damage in normal cells. DNA damage induced by compound 5 in NCI-H292 initiated with low concentration as detected by the comet assay and became more extensive with higher concentrations of compound 5, as detailed by flow cytometry assay. Although 5-benzylidene-thiazolidine-2,4-dione derivatives have been reported in a large number of biological activities, there are still a few studies that investigate possible genotoxic effects in normal lines. Recently, some thiazacridine derivatives (ATZD), a new class of cytotoxic agents combining one acridine nucleus with thiazolidine group, were described as non-genotoxic upon human lymphocytes [38]. Progress in the treatment of locally stage III Non–Small Cell Lung Cancer has been achieved with two chemotherapy doublets in combination with thoracic radiotherapy: 1) full-dose cisplatin plus etoposide (PE) for two cycles; and 2) weekly low-dose paclitaxel plus carboplatin (PC). However, advanced lung cancer has been resistant to traditional chemotherapy [39]. This data reinforce the need for encouragement of smoking cessation as a preventive approach and the development of new chemotherapeutic agents to treat lung cancer. Hence, we have identified compound 5- (2-bromo-5-methoxybenzylidene) thiazolidine-2,4-dione as a selective cytotoxic and

pro-apoptotic agent against lung carcinoma cells. Furthermore, compound 5 did not show genotoxicity to normal cells.

Conflict of interest

None

Funding

The authors thank Brazilian National Research Council (CNPq) and Research Foundation of Pernambuco State (FACEPE) for financial support.

Acknowledgements

We thank Maria Cicalese for English editing.

References

[1] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–74. doi:10.1016/j.cell.2011.02.013.

[2] Islami F, Torre LA, Jemal A. Global trends of lung cancer mortality and smoking prevalence. Transl Lung Cancer Res 2015;4:327–38. doi:10.3978/j.issn.2218-6751.2015.08.04.

[3] IARC-WHO, Stewart BW, Wild CP. World Cancer Report 2014. International Agency for Research on Cancer; 2014. doi:9283204298.

[4] WHO | Cancer. WHO 2017. http://www.who.int/mediacentre/factsheets/fs297/en/ (accessed September 26, 2017).

[5] Nazreen S, Alam MS, Hamid H, Yar MS, Dhulap A, Alam P, et al. Thiazolidine-2,4-diones derivatives as PPAR-γ agonists: Synthesis, molecular docking, in vitro and in vivo antidiabetic activity with hepatotoxicity risk evaluation and effect on PPAR-γ gene expression. Bioorg Med Chem Lett 2014;24:3034–42. doi:10.1016/j.bmcl.2014.05.034.

[6] Maggs DG, Buchanan TA, Burant CF, Cline G, Gumbiner B, Hsueh WA, et al. Metabolic effects of troglitazone monotherapy in type 2 diabetes mellitus. A randomized, double-blind, placebo-controlled trial. Ann Intern Med 1998;128:176–85.

[7] da Silva IM, da Silva Filho J, Santiago PBG da S, do Egito MS, de Souza CA, Gouveia FL, et al. Synthesis and antimicrobial activities of 5-Arylidene-thiazolidine-2,4-dione derivatives. Biomed Res Int 2014;2014:316082. doi:10.1155/2014/316082.

[8] Barros CD, Amato AA, Oliveira TB de, Iannini KBR, Silva AL da, Silva TG da, et al. Synthesis and anti-inflammatory activity of new arylidene-thiazolidine-2,4-diones as PPARγ ligands. Bioorg Med Chem 2010;18:3805–11. doi:10.1016/j.bmc.2010.04.045.

[9] Leite FHA, Santiago PBG da S, Froes TQ, da Silva Filho J, da Silva SG, Ximenes RM, et al. Structure-guided discovery of thiazolidine-2,4-dione derivatives as a novel class of Leishmania major pteridine reductase 1 inhibitors. Eur J Med Chem 2016;123:639–48. doi:10.1016/j.ejmech.2016.07.060.

[10] Mohanty S, Reddy GS, Karmakar AC. Synthesis of New 5-Substituted – Aminomethylene-Thiazolidine-2,4-dione Derivatives As Potential Antibacterial Agents. JOAC 2014;3:82–90.

[11] Asati V, Mahapatra DK, Bharti S. Thiazolidine-2,4-diones as multi-targeted scaffold in medicinal chemistry: Potential anticancer agents. Eur J Med Chem 2014;87:814–33. doi:10.1016/J.EJMECH.2014.10.025.

[12] Patil V, Tilekar K, Mehendale-Munj S, Mohan R, Ramaa CS. Synthesis and primary cytotoxicity evaluation of new 5-benzylidene-2,4- thiazolidinedione derivatives. Eur J Med Chem 2010;45:4539–44. doi:10.1016/j.ejmech.2010.07.014.

[13] Jung K-Y, Samadani R, Chauhan J, Nevels K, Yap JL, Zhang J, et al. Structural modifications of (Z)-3-(2-aminoethyl)-5-(4-ethoxybenzylidene)thiazolidine-2,4-dione that improve selectivity for inhibiting the proliferation of melanoma cells containing active ERK signaling. Org Biomol Chem 2013;11:3706. doi:10.1039/c3ob40199e.

[14] Romagnoli R, Baraldi PG, Salvador MK, Camacho ME, Balzarini J, Bermejo J, et al. Anticancer activity of novel hybrid molecules containing 5-benzylidene thiazolidine-2,4-dione. Eur J Med Chem 2013;63:544–57. doi:10.1016/j.ejmech.2013.02.030.

[15] Blanquicett C, Roman J, Hart CM. Thiazolidinediones as anti-cancer agents. Cancer Ther 2008;6:25–34.

[16] Jain VS, Vora DK, Ramaa CS. Thiazolidine-2,4-diones: Progress towards multifarious applications. Bioorg Med Chem 2013;21:1599–620. doi:10.1016/j.bmc.2013.01.029.

[17] Divya GS, Mansoor KP, Rasheed SP, Kumar A. PPAR gamma agonists: an effective strategy for cancer treatment. JPSI 2013;2:1–3. doi:10.7897/2277-4572.02575.

[18] Patil V, Tilekar K, Mehendale-Munj S, Mohan R, Ramaa CS. Synthesis and primary cytotoxicity evaluation of new 5-benzylidene-2,4- thiazolidinedione derivatives. Eur J Med Chem 2010;45:4539–44. doi:10.1016/j.ejmech.2010.07.014.

[19] Laxmi SV, Anil P, Rajitha G, Rao AJ, Crooks PA, Rajitha B. Synthesis of thiazolidine-2,4-dione derivatives: anticancer, antimicrobial and DNA cleavage studies. J Chem Biol 2016;9:97–106. doi:10.1007/s12154-016-0154-8.

[20] Libermann D, Himbert J, Hengl L. Thiazolidinone, a starting point for a synthesis of thiopyruvic and thioglyoxilic substituted acids.[in French]. Bull Soc Chim Fr 1948:1120–4.

[21] Albuquerque JFC, Azevedo CC, Galdino SL, Chantegrel J, Pitta IR, Luu-DucC. Synthesis and structural study of 5-arylidene thiazolidine-2,4-diones and 4-thioimidazolidin-2-ones-3-substituted. [in French]. Ann Pharm Fr 1995;53:209–14.

[22] Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63. doi:10.1016/0022-1759(83)90303-4.

[23] O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity.

Eur J Biochem 2000;267:5421–6. doi:10.1046/j.1432-1327.2000.01606.x.
[24] Militão GCG, Dantas INF, Pessoa C, Falcão MJC, Silveira ER, Lima MAS, et al. Induction of apoptosis by pterocarpans from Platymiscium floribundum in HL-60 human leukemia cells. Life Sci 2006;78:2409–17. doi:10.1016/j.lfs.2005.09.044.
[25] Ferreira PMP, Bezerra DP, Do Nascimento Silva J, Da Costa MP, De Oliveira Ferreira JR, Alencar NMN, et al. Preclinical anticancer effectiveness of a fraction from Casearia sylvestris and its component Casearin X: In vivo and ex vivo methods and microscopy examinations. J Ethnopharmacol 2016;186:270–9. doi:10.1016/j.jep.2016.04.011.
[26] Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988;175:184–91. doi:10.1016/0014-4827(88)90265-0.
[27] Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol 2004;26:249–61. doi:10.1385/MB:26:3:249.
[28] Akshaya TT, Arunlal VB, Babu G, Biju CR. Synthesis, characterisation and. JDDT 2014;2:42–7.
[29] Metwally K, Pratsinis H, Kletsas D. Novel 2,4- thiazolidinediones: Synthesis, in vitro cytotoxic activity, and mechanistic investigation. Eur J Med Chem 2017;133:340–50. doi:10.1016/j.ejmech.2017.03.052.
[30] Islami F, Torre LA, Jemal A. Global trends of lung cancer mortality and smoking prevalence. Transl Lung Cancer Res 2015;4:327–38. doi:10.3978/j.issn.2218-6751.2015.08.04.
[31] Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26:239–57.
[32] Fadeel B, Orrenius S. Apoptosis: A basic biological phenomenon with wide-ranging implications in human disease. J Intern Med 2005;258:479–517. doi:10.1111/j.1365-2796.2005.01570.x.
[33] Leist M, Jäättelä M. Four deaths and a funeral: from caspases to alternative mechanisms. Nat Rev Mol Cell Biol 2001;2:589–98. doi:10.1038/35085008.
[34] Zang C, Liu H, Posch MG, Waechter M, Facklam M, Fenner MH, et al. Peroxisome proliferator-activated receptor γ ligands induce growth inhibition and apoptosis of human B lymphocytic leukemia. Leuk Res 2004;28:387–97. doi:10.1016/j.leukres.2003.07.005.
[35] Liu H, Zang C, Fenner MH, Liu D, Possinger K, Koeffler HP, et al. Growth inhibition and apoptosis in human Philadelphia chromosome – positive lymphoblastic leukemia cell lines by treatment with the dual PPAR alpha/gamma ligand TZD18. Blood 2006;107:3683–92. doi:10.1182/blood-2005-05-2103.Supported.
[36] Lyles BE, Akinyeke TO, Moss PE, Stewart L V. Thiazolidinediones regulate expression of cell cycle proteins in human prostate cancer cells via PPARγ-dependent and PPARγ-independent pathways. Cell Cycle 2009;8:268–77. doi:10.4161/cc.8.2.7584.
[37] Araldi R., Melo TC, Mendes TB, Sá Júnior P, Nozima BH, Ito ET, et al. Using the comet and micronucleus assays for genotoxicity studies : A review. Biomed Pharmacother 2015;72:74–82.
[38] Barros FWA, Bezerra DP, Ferreira PMP, Cavalcanti BC, Silva TG, Pitta MGR, et al. Inhibition of DNA topoisomerase I activity and induction of apoptosis by thiazacridine derivatives. Toxicol Appl Pharmacol 2013;268:37–46. doi:10.1016/j.taap.2013.01.010.

[39] Wakelee H, Kelly K, Edelman MJ. 50 Years of progress in the systemic therapy of non-small cell lung cancer. American Society of Clinical Oncology Educational Book / ASCO American Society of Clinical Oncology Meeting 2014:177–89. doi:10.14694/EdBook_AM.2014.34.177.

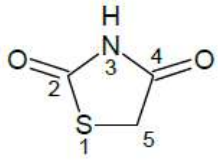

Fig. 1. Thiazolidine-2,4-dione ring

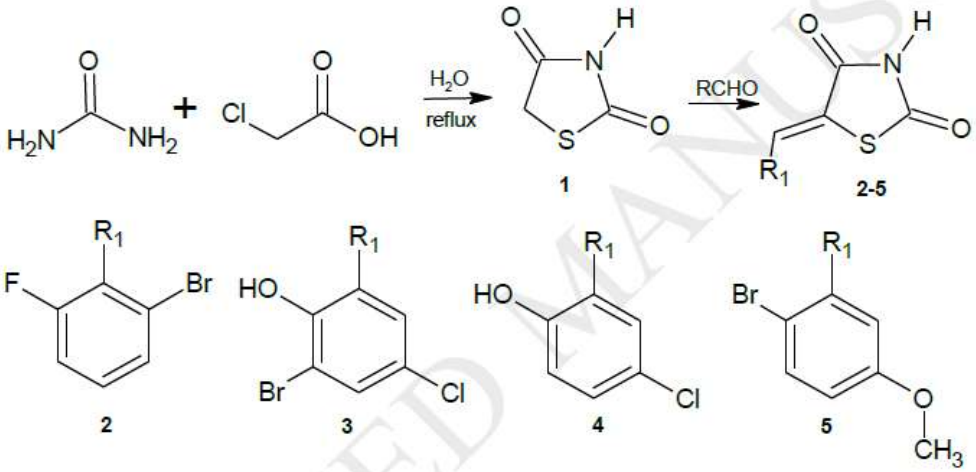

Fig. 2. Synthetic routes and structure of compounds 2 to 5

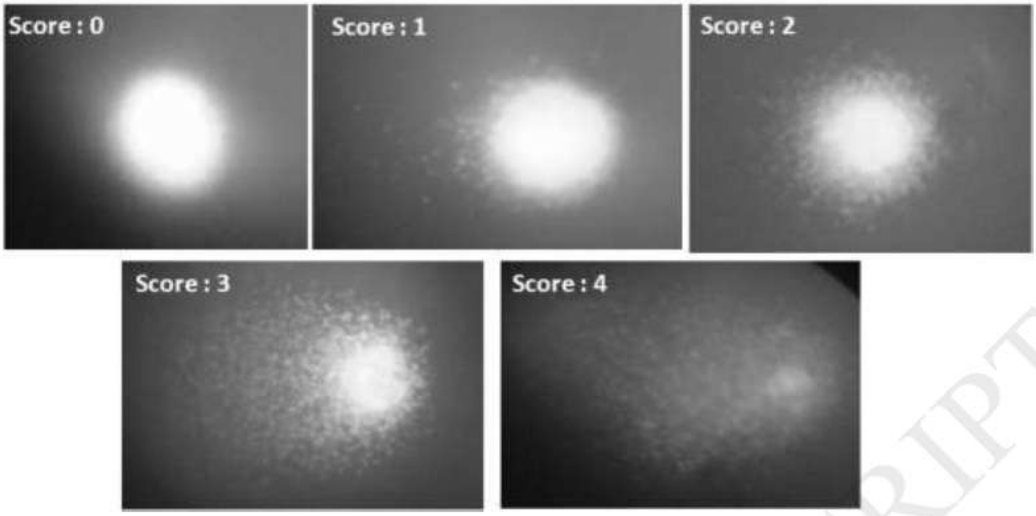

Fig. 3. Photomicrograph of comets classified by visual inspection into five categories: score zero representing undamaged cells and score 1 to 4 representing increasing damage. 1000 x magnification.

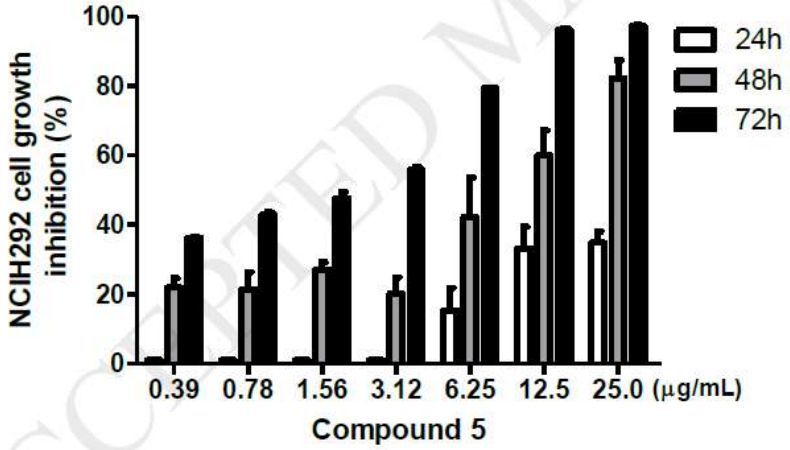

Fig. 4. Time-dependent growth inhibition effect of compound 5 in NCI-H292 cell line. Tumor cells were treated with 7 different concentrations at 24, 48 and 72 h.

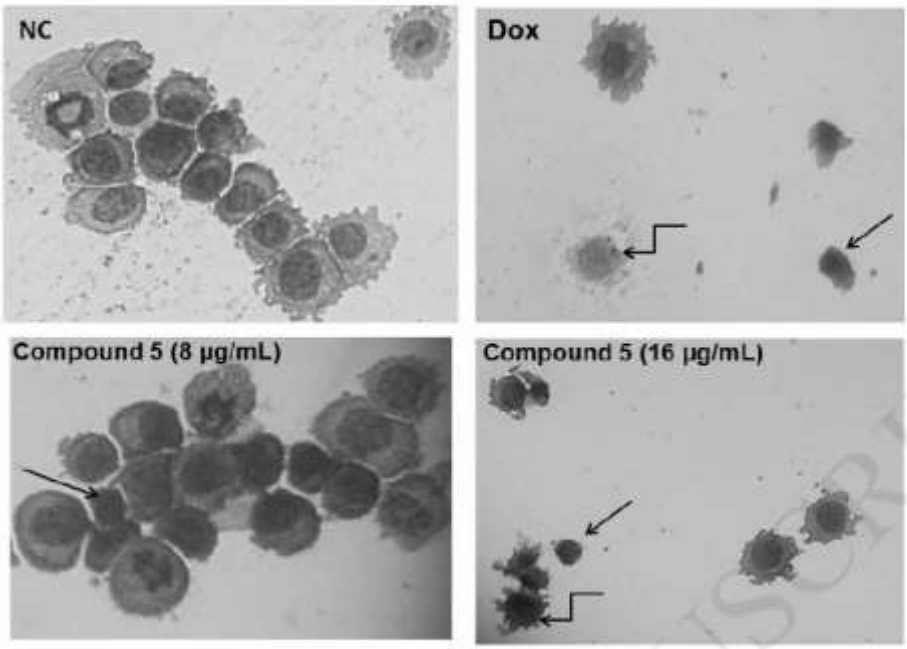

Fig. 5. Cell Morphology of NCI-H292 cells after 48 h of treatment. (NC) negative control treated with the vehicle alone DMEM 0.5% DMSO. Doxorubicin 0.5 µg/mL was used as positive control (Dox). Cells were treated with compound **5** at the concentrations 8.0 µg/mL and 16.0 µg/mL. Arrows indicate the aspects observed: volume reduction (short arrows) and cellular debris (curved arrows). Cells were stained with May - Grunwald - Giemsa and visualized by light microscopy with 400X magnification.

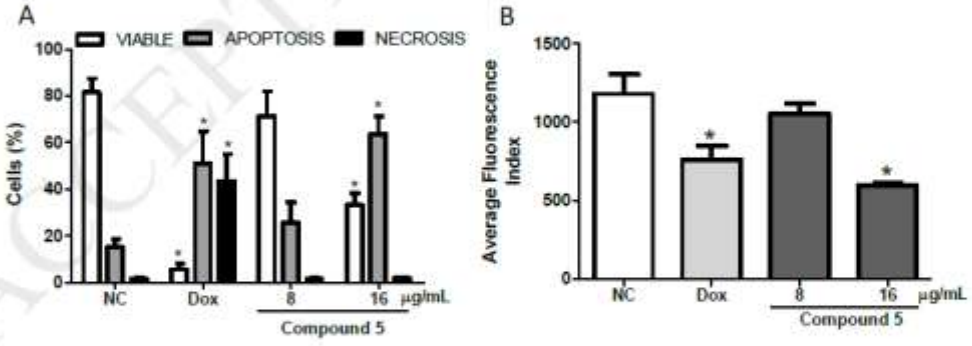

Fig. 6. **A**- Externalization of phosphatidylserine on lung cancer cell line NCI-H292 analyzed by flow cytometry after 48 hours incubation with compound **5**. **B**-Effect of compound **5** in NCI-H 292 transmembrane mitochondrial potential determined by flow cytometry after 48 hour of treatment. The negative control (NC) was treated only with DMEM 0.5% DMSO. Doxorubicin (0.5 µg/mL) was used as positive control (Dox).

Values are mean ± standard deviation of 3 independent experiments (n = 6). *p<0.05 compared to negative control by ANOVA followed by Dunnett's test.

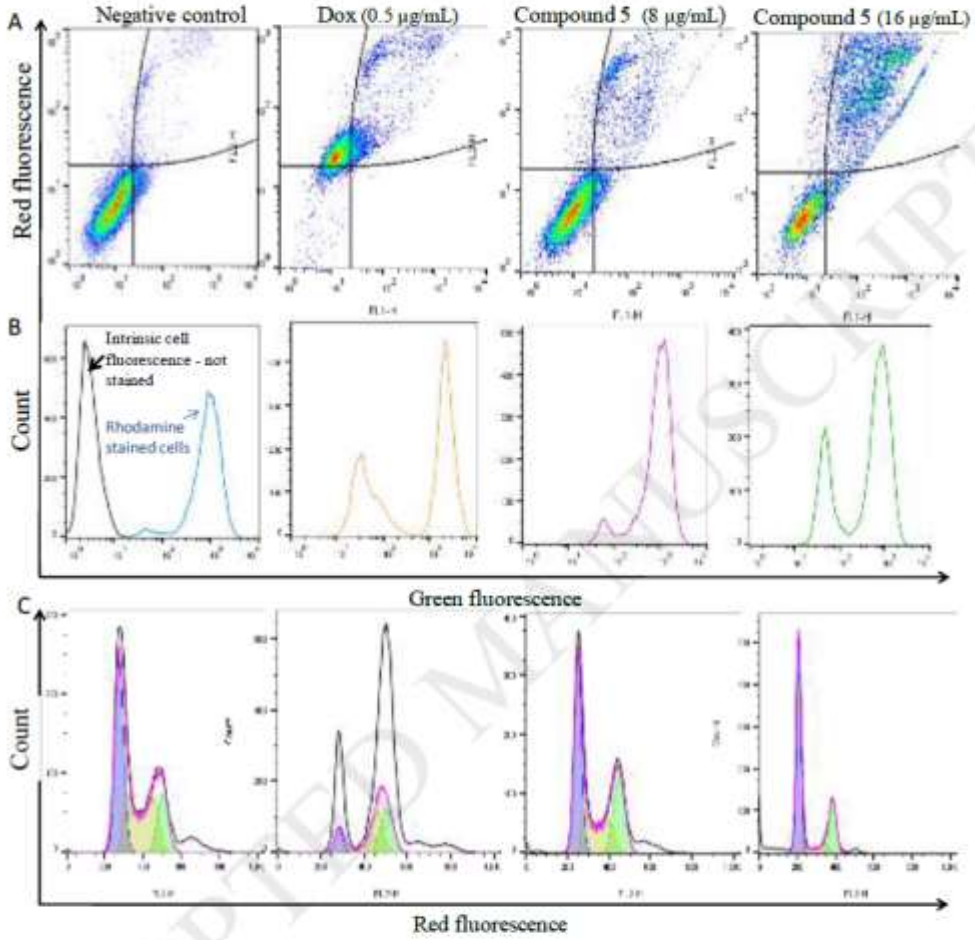

Fig. 7. Effect of Compound 5 in NCI-H292 cell line apoptosis parameters and DNA content after 48 h treatment. **A)** Representative flow cytometric dot plots showing the percentage of cells in viable, apoptotic and necrotic stages. **B)** Representative flow cytometric histograms of cells with mithochondrial despolarization. **C)** Cell cycle histograms. Dox: doxorubicin.

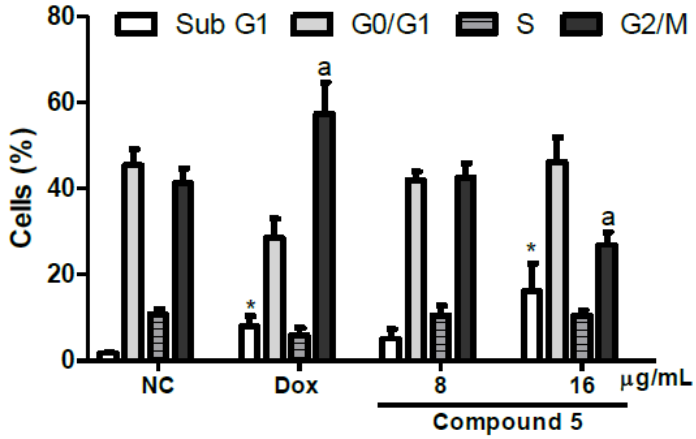

Fig. 8. The effect of compound 5 at IC_{50} and 2 x IC_{50} concentrations on the cell cycle of NCI -H292 was determined by flow cytometry after 48 h of incubation. The negative control (NC) was treated only with DMEM 0.5% DMSO. Doxorubicin (0.5 µg/mL) was used as positive control (Dox). Values are mean ± standard deviation of 3 independent experiments (n = 6). *p<0.05 when Sub G1 was compared to negative and ^{a}p<0.05 when G2/M was compared to negative control by ANOVA followed by Dunnett's test.

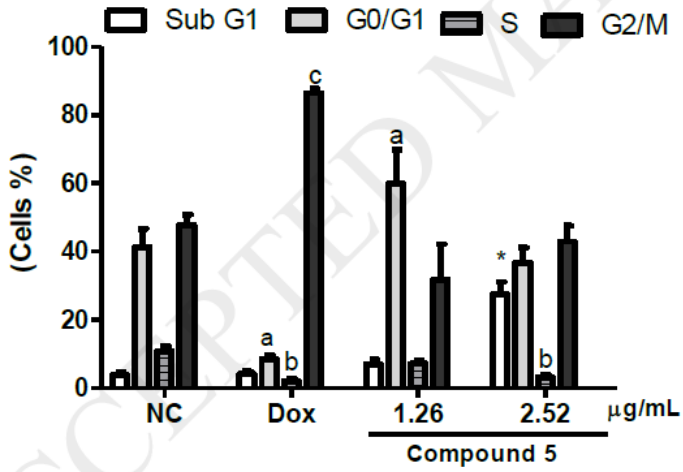

Fig. 9. The effect of compound 5 at IC_{50} and 2 x IC_{50} concentrations on the cell cycle of NCI-H292 determined by flow cytometry after 72 h of incubation. The negative control (NC) was treated only with DMEM 0.5% DMSO. Doxorubicin (0.5 µg/mL) was used as positive control (Dox). Values are mean ± standard deviation of 3 independent experiments (n = 6). ^{a}p<0.05 when G0/G1 was compared to negative control. ^{b}p<0.05 when S phase was compared to negative control. ^{c}p<0.05 when G2/M was compared to negative control. *p<0.05 when Sub G1 was compared to negative control. All of them were compared by ANOVA followed by Dunnett's test.

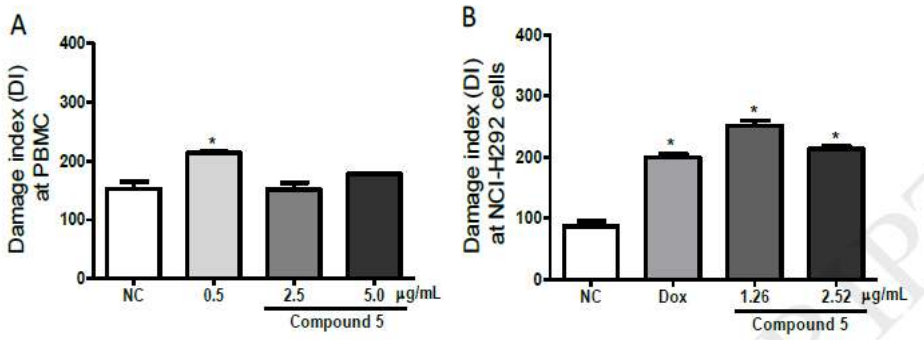

Fig. 10. Damage Index (ID) obtained by Comet assay. A) Peripheral blood mononuclear cells (PBMC) isolated from healthy human volunteers. B) NCI-H292 lung cancer cells. Both normal (PBMC) and cancer cells (NCI-H292) were treated with DMEM 0.5% DMSO (NC); Doxorubicin 0.5 µg/mL as positive control (Dox) and compound **5** for 48 hours. Values (damage index of 100 cells at 2 slides; n = 2) corresponds to the mean ± SD of two independent experiments.* $p<0.05$ compared to the negative control (NC) and analyzed by ANOVA followed by Dunnett´s post test.

Table 1 Cytotoxicity of thiazolidine-2,4-dione derivatives after 72 hours of incubation. The compound concentration that causes 50% inhibition of cell growth and their 95% confidence interval (CI95%) are expressed in µg/mL. Doxorubicin was used as positive control (DOX).

	Tumor cell line IC_{50} (CI95%) µg/mL					
Compound	**HEp-2**	**HL-60**	**HT-29**	**K-562**	**MCF-7**	**NCI-H292**
2	>25	9.0 (6.8-11.9)	>25	>25	20.2 (16.4-24.7)	15.4 (12.9-18.4)
3	>25	16.1 (13.9-18.9)	>25	11.6 (8.7-15.4)	17.9 (15.6-20.6)	10.9 (8.8-13.5)
4	>25	10.2 (7.7-13.3)	>25	17.0 (8.2-35.1)	>25	16.9 (12.8-22.4)
5	>25	2.0 (1.5-2.7)	>25	6.8 (0.2-14.5)	3.5 (1.8-6.3)	1.26 (0.9-1.7)
DOX	1.2 (0.5-2.4)	0.03 (0.02-0.03)	0.7 (0.3-1.0)	0.24 0.16-0.39	0.5 0.36-0.74	0.3 0.17-0.9

IC_{50}: Concentration that causes 50% inhibition of cell growth, and (CI95%) confidence interval. Dox: doxorubicin

5 CAPÍTULO II - ARTIGO CIENTÍFICO 2

ARTIGO CIENTÍFICO 2:

Artigo a ser submetido ao periódico Drug Development Research

Journal Impact: 1.06

Evaluation of Microbiological and Cytotoxic Activities of Derivatives of thiazolidines-2,4-diones

Evaluation of Microbiological and Cytotoxic Activities of Derivatives of thiazolidines-2,4-diones

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ABSTRACT The novel 5-thiazolidine-2,4-dione (a-f) derivatives were synthesized to evaluate antimicrobial activity in vitro against a panel of a pathogenic fungal strain, an alcohol-acid resistant bacterium, four Gram-positive bacteria and three strains of Gram-negative bacteria. The results revealed that the compounds (a-f) showed activity against at least two strains tested. However, no compound inhibited the growth of Gram-negative bacteria and yeast. Compounds (e) and (f) were also analyzed in tumor cells and normal to evaluate their cytotoxic potential, showing inhibition of cell growth higher than 75% and values of CI_{50} below 4μg / mL for lung tumors (NCIH-292), colon (HT-29), breast cancer (MCF-7) and laryngeal cancer (HEp-2). The compounds tested were not cytotoxic to normal cells, this reveals an important characteristic for a new compound because they are considered interesting to fill the chemotherapy gap with selectivity to tumor cells.

Key words: Microbiological activity, Cytotoxicity, Thiazolidines-2,4-diones.

INTRODUCTION

Bacterial resistance represents a global public health problem, the treatments of bacterial infections are a challenge to include emerging infectious diseases and infections by bacteria resistant to multiple drugs. Although there are many antibiotics and chemotherapeutics available today for medical use, there is a great need to discover new classes of antibacterial agents due to the emergence of strains resistant to existing antibiotics [Tomasic et al., 2010].

Cancer is another worldwide public health problem due to the rising incidence and mortality rate. There are more than 100 types of cancers, having as a common feature the disordered growth of cells, capable of invading tissues and organs. Cancer cells have multiple structural, behavioral, and molecular characteristics that define the pathophysiology of cancer and are responsible for malignant growth such as: Self-sufficiency in the regulation of cell growth signals, the insensitivity to the signals that inhibit this growth, evasion of programmed cell death (apoptosis), unlimited replicative potential, induction of angiogenesis, tissue invasion and metastasis [INCA, 2015; Hanaham e Weinberg, 2011].

The use of antineoplastic drugs provides an increase in the survival of patients with various types of câncer, but prolonged exposure to these drugs increases the risk of side effects in different organs and tissues [Chaves et al., 2007].

Despite the increase in global investments for research and development of new drugs the approval rate is declining and the main reason for this decline is due to toxicity and undesirable side effects [Morrow et al., 2010].

In this context, compounds derived from thiazolidines appear as an alternative in the research of new bioactive molecules. The biological activities exhibited by the

thiazolidines-2,4-diones are described as antihyperglycaemic, antiinflammatory, antiarthritic, antimicrobial and antitumor. The thiazolidine derivatives have a heterocyclic ring system with multiple applications and represent a class of drugs and patented substances in several phases of the research. These derivatives have become very important for organic synthesis and the development of new drugs [Jain, 2013].

Faced with this evidence, researchers around the world have been engaged in the search for new drugs with antibiotic action and antitumor drugs in order to find a more effective treatment that causes less adverse reactions. Thus, in this study, studies were carried out to verify antibiotic and antitumor activities *in vitro* of the compounds containing the core thiazolidines-2,4-diones, envisioning the development of new drugs.

MATERIAL AND METHODS

Compounds

The synthesis methodology of the compounds isolated in this article was described by Leite et al., 2016. The thiazolidine-2,4-dione was obtained by the method described by Liebermann (1948) and modified by Albuquerque et al. (1995) and Gouveia et al. (2009). This reaction occurs by condensation of monochloroacetic acid and thiourea in an aqueous medium under reflux for 24 h [Bozdag-Dündar et al., 2007; Bruno et al., 2002]. The thiazolidine-2,4-dione derivatives were obtained by the changes in the 5-position of the heterocyclic ring (Fig. 1). Compounds (a) (Z) -5- (2-bromo-6-fluorobenzylidene) -thiazolidine-2,4-dione; (b) (Z) -5- (2-Hydroxy-3-bromo-5-chlorobenzylidene) -thiazolidine-2,4-dione, (c) (Z) -5- (2-Hydroxy-5-chlorobenzylidene) -tiazolidine-2,4-dione , **(d)** (Z) -5- (2-bromo-5-methoxybenzilidene)-tiazolidine-2,4-dione; **(e)** (*Z*)-5-(2,4-dichlorobenzylidene-

thiazolidine-2,4-dione e **(f)** (Z)5-(3,4-dichlorobenzylidene-thiazolidine-2,4-dione, were synthesized according to the protocol described previously [Vicini et al., 2006; Silva et al., 2014; Pikkemaat et al., 2002] (Fig. 1). A solution of tiazolidine-2,4-dione (0,2 g, 1,70 x 10-3 mol) was heated in ethanol (7,0 mL) containing piperidine (2 gotas) and aromatic aldehyde (0,184 g, 2,25 x 10-3 mol) (70 ° C), under stirring, for 5-9 h. The product was then cooled in an ice bath, filtered and recrystallized with a suitable solvent. The resulting precipitate was removed by filtration and recrystallized from acetic acid to give the compounds (a - f). The chemical structures were proved by physical methods of Hydrogen and Carbon Nuclear Magnetic Resonance spectroscopy thirteen (RMN1H; RMN13C, Infra-red (IV) and Mass Spectrometry (EMAR). The yields ranged from 73 a 79%.

Fig. 1. Synthetic pathways of the compounds **(a-f)**.

Evaluation of Microbiological Activity

The tests to evaluate the antimicrobial activity of the synthesized compounds were carried out at the Laboratory of Microbiology of Drugs of the Department of Antibiotics - UFPE. The microorganisms of the Collection of Cultures of the Department of Antibiotics - UFPE, representatives of groups of bacteria Gram positive: *Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis, Enterococcus faecalis*; Gram-negativas: *Pseudomonas aeruginosa, Escherichia coli, Serratia marcescens*;

álcool-ácido resistentes: *Mycobacterium smegmatis* e levedura: *Candida albicans*. The inoculums were obtained from recent cultures of the test microorganisms, with 18-24 hours of incubation at 35 ° C in the following culture media: Müeller-Hinton ágar – bactérias Gram-positivas e Gram-negativas; Sabouraud ágar – *Candida albicans;* Glucose yeast extract agar (GL) – *Enterococcus faecalis* e *Mycobacterium smegmatis*.

As suspensões desses inóculos foram padronizadas à densidade óptica de 0,2 de absorbance at 600 nm, equivalent to 0.5 turbidity of the McFarland scale, in physiological solution, corresponding to approximately 10^7 CFU for bacteria and 10^6 CFU yeasts, according to the methods described by Barry et al. [2002] e Koneman et al. [2001].

Paper disc diffusion method

The in vitro evaluation of the antimicrobial activity of the synthesized compounds was initially performed by the paper disc diffusion technique, according to Bauer et al. [1966]. The tests were performed with a solution of 30,000 μg / mL of the test substance, using DMSO (Dimethylsulfoxide) as the solvent. Suspensions of test microorganisms were seeded in Petri dishes containing 10 mL of the culture medium appropriate for the microorganism. 6 mm diameter disks impregnated with 10 μl of the solution were used, resulting in a concentration of 300 μg per disc. The discs were placed on the surface of the medium seeded with the test microorganism, the plates being incubated according to the requirement of the microorganism under study (Tabela 1).

Table 1. Growth conditions of the microorganisms used in antagonism tests performed on the compounds obtained by synthesis.

Microorganisms	Culture mediums	Temp. (º C)	Time (h)
Staphylococcus aureus	Müeller-Hinton	35	24
Micrococcus luteus	Müeller-Hinton	35	24
Bacillus subtilis	Müeller-Hinton	35	24
Pseudomonas aeruginosa	Müeller-Hinton	35	24
Mycobacterium smegmatis	Glicose-extrato de levedura ágar	35	48
Enterococcus faecalis	Müeller-Hinton	35	24
Escherichia coli	Sabouraud-ágar	35	24
Serratia marcescens	Müeller-Hinton	27	24
Candida albicans	Sabouraud-ágar	30	48

After the incubation period the results were read by measuring the diameter of the inhibition halos formed around the discs. The tests were performed in triplicate and the results expressed by the arithmetic mean of the inhibition halos in millimeters and calculated the standard deviation. The positive controls used as standards were ampicillin (10 μg / disc), cefotaxime (30 μg / disc) and kanamycin (30 μg / disc).

Well Methodology: Determination of Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (CMB).

The CMI determination was assessed according to the criteria adopted by the Clinical and Laboratory Standards Institute CLSI [2013]. Multiwell plates (96 wells) were used, listed from 1 to 12 horizontally and in alphabetical order, from A to H, vertically. The substances were totally dissolved in DMSO to form a homogeneous mixture at 1280 μg / mL titled stock solution, and a standardized suspension of the test microorganisms was made according to the 0.5 MacFarland.

With the exception of the third column, in the other columns was placed 100 μL of the medium Müeller-Hinton broth, the second column being the negative control, that

is, containing only 100µl of the Müeller-Hinton broth; in the first one, 10 µl of the standardized microbial suspension was added to the 100 µL of the Müeller-Hinton broth (positive control). In the third column, 180 µL of the Müeller-Hinton broth was placed and then 20 µL of the stock solution was added. From this column serial dilutions of 1: 2 were made, ie, 100µL of the current column was removed and added in the next column, to the twelfth column. To the wells of the dilutions were placed 10 µL of the suspension of the test microorganisms. The plate was incubated for 18-22 hours, after the incubation period a developer dye (resazurin sodium) was applied, able to more accurately show whether there was turbidity in the well. The CMI value was determined at the concentration of the last well where there was no visible turbidity. From this well seeds were made by exhaustion on plates with Müller-Hinton agar medium to establish CMB, which will be the concentration where there is no growth of colonies. The MIC was determined where there was 50% of the bacterial growth in relation to the positive control. All analyzes were performed in triplicate or quadruplicate.

Cytotoxic Activity in Tumor Cell Lineages

Tumor cells were seeded in 96-well plates (10^5 cells / ml for adherent cells or 3 × 10^5 cells / ml for leukemia). The tested compounds (0.39-25 µg / ml), dissolved in 1% DMSO, were added to each well and incubated for 72 h. Control groups received the same amount of DMSO. After 69 h of treatment, 25 µl of MTT (5 mg / ml) was added, three hours later, the MTT-formazan product was dissolved in 100 µl of DMSO, and the absorbance was measured at 595 nm in plate spectrophotometer. Doxorubicin (0.01-5 µg / mL) was used as a positive control [Mosmann, 1983; Alley et al., 1988]. Data are

presented as IC 50 values with their 95% confidence intervals obtained by non-linear regression.

Evaluation of Cytotoxicity

The tumor cell lines used, NCI-H292 (mucoepidermoid lung carcinoma), HL60 (acute promyelocytic leukemia), HT-29 (colon adenocarcinoma), K562 (chronic myelocytic leukemia), HEp-2 (human laryngeal squamous cell carcinoma) and MCF -7 (Breast adenocarcinoma), were obtained from the Cell Bank of Rio de Janeiro, and were cultured in DMEM and RPMI medium for leukemic lines, supplemented with 10% fetal bovine serum and 1% antibiotic solution (penicillin 1000 IU / ml + 250 µg / ml streptomycin) and 1% of 200 mM L-glutamine. They were kept in an oven at 37 ºC and humid atmosphere containing 5% CO_2. Cells were plated at 105 cells / mL for the HEp-2, HT-29, MCF-7, and NCI H-292. For HL 60 and K562, the concentration was 3 x 10^5 cells / mL. Substances previously dissolved in DMSO were diluted in the culture medium to obtain serial final concentrations (0.39µg / mL- 25µg / mL) and distributed in a 96-well plate (100 µL / well). Plates were incubated for 69 hours in a 5% CO2 oven at 37 ° C. Then, 25 µL of the MTT solution (tetrazolium salt) was added, and the plates were incubated for 3 h. Then the culture medium with the MTT was aspirated and 100 µL of DMSO was added to each well to dissolve the formazan crystals. Doxorubicin was used as standard. The absorbance, after dissolution of the precipitate and compared with the negative control, was read in a spectrophotometer at 595nm. The IC50 value was defined as the concentration at which 50% inhibition of cell proliferation was observed.

Avaliação do potencial hemolítico em eritrócitos humanos

Blood was collected from healthy volunteers. The erythrocytes were washed with saline (0.85% NaCl + 10mM $CaCl_2$) by centrifugation (3000 rpm / 5min), the supernatant discarded and the pellet resuspended in saline to obtain a 2% suspension of red blood cells. These experiments were performed on 96-well multiplates according to the following: 100µl of saline (negative control); 50 µl of saline and 50 µl of the vehicle with 1% DMSO (White); 80µL saline + 20 µL Triton X - 100 (positive control); 50 µl of saline and 50 µl of the samples (1.95 to 125 µg / ml) diluted in 1% DMSO. Then 100µL of the erythrocyte solution was added to all wells. After incubation for 1 h under constant stirring at room temperature, the supernatant was analyzed and the optical reading was done on automatic plate reader (450 nm). The 50% effective concentration (EC50) and its 95% confidence interval were determined from non-linear regression using the GraphPad Prism® program (version 5.0).

Evaluation of cytotoxicity in Peripheral Blood Mononuclear Cells obtaining Peripheral Blood Mononuclear Cells (PBMC).

Mononuclear cells (lymphocytes and monocytes) were obtained from the peripheral blood of healthy volunteers collected in Vacutainer-type tubes containing Heparin solution (BD Vacutainer®) as anticoagulant. After collection, 6 mL of blood were diluted in 3 mL of PBS and added slowly into another tube containing 2 mL of Ficoll®-Hystopaque (Sigma). Then, centrifugation was performed at 1500 rpm for 30 minutes to separate the solution phases. Ficoll promotes the formation of a density gradient, mononuclear cells concentrated in the layer located in the middle, between plasma (light phase) and erythrocytes (dark phase). The PBMCs were transferred to another tube to which PBS was added to the final volume of 11 ml and then centrifuged

for 20 minutes at 1000 rpm. The supernatant was discarded and the PBMC pellet resuspended in RPMI 1640 medium supplemented with 20% fetal bovine serum and 1% antibiotics (to obtain a final concentration of 100 U / ml penicillin and 100 μg / ml streptomycin). 2% phytohemagglutinin (Sigma), which is a mitogenic agent, was added to the medium to stimulate lymphocyte proliferation. The cells were counted and diluted to a final concentration of 10^6 cells / ml. The procedures involving blood collection were submitted and approved by the human ethics committee of the Federal University of Pernambuco.

Cytotoxic activity in human peripheral blood mononuclear cells

Recently identified as resazurin, alamar Blue [O´Brien et al., 2000] is a fluorescent and colorimetric indicator with redox properties. Alamar blue is reduced to resofurin in the presence of viable cells. The reduced form is pink (fluorescent / viable cell) and the oxidized form is blue (non-fluorescent / non-viable cell). Initially, 10^6 cells / ml were plated in 96-well plates. After 24 h of incubation, the samples were dissolved in DMSO and added to each well and incubated for 72 h. Doxorubicin (0.078 to 10 μM) was used as a positive control. The negative control received the same amount of DMSO. Twenty-four hours before the end of the incubation period, 10 μl of stock solution of alamar blue (0.312 mg / mL) was added to each well. Absorbances were measured at wavelengths of 570 nm and 595 nm using a plate spectrophotometer.

Análise dos Dados

The reduction of alamar blue reflects cell proliferation. Cell proliferation was calculated using the following formula: % proliferation = ALW – (AHW x R0) x 100, at where, ALW e AHW are the absorbances at the lowest and highest wavelength, respectively. R0 was calculated using the following formula: R0 = AOLW/AOHW,

where AOLW and AOHW are the absorbances of the isolated medium subtracted from the absorbances of the medium added to the alamar blue at the smaller and larger wavelengths, respectively.

Results and discussion

Synthesis of the compounds

1 H-NMR spectra showed peaks confirming the molecular structure of the compounds (Fig. 2) with the properties described below:

(a) 5-(2-bromo-6-fluorobenzylidene)-thiazolidine-2,4-dione

MF C10H5BrFNO2S, MW 302.1196, Yield. 78 (%), Rf 0.40 (9.8:0.2 CHCl3/MeOH), MP 115e116 _C, IR, (KBr, cm_1): 3164 (NeH),1703(C]O4), 1445 (C]O2), 1322 (C]C). 1H RMN (400 MHz, DMSO-d6):d 7.09e7.15 (t, 1H(3), Ar, J ¼ 9 Hz); 7.23e7.30 (m, 1H(4), Ar); 7.46 (d,1H(5), Ar, J ¼ 7.8 Hz); 7.64 (s, 1H(5), C]CH); 11.71 (s broad, 1H, NH). 13C RMN and DEPT (75.5 MHz, DMSO-d6): d 115.58, 127.28, 127.32,128.94, 129.98, 131.88, (115.80 heterocycle); CH]C 142.29; 167.19(C]O2); 166.19 (C]O4.). HRMSþ: Calculated: 302.1678; found:302.1096.

(b) 5-(2-Hydroxy-3-bromo-5-chlorobenzylidene)-thiazolidine-2,4-dione (2d)

MF C10H5BrClNO3S, MW 334.5736, Yield 73%,Rf 0.47 (9.6:0.4 CHCl3/MeOH), MP 197 _C; IR, (KBr, cm_1): 3157(NeH), 1698 (C]O4), 1592 (C]O2), 1449 (C]C). 1H NMR (400 MHz,DMSO-d6): d, 7.07 (s, 1H(4), Ar), 7.35 (s, 1H(5), Ar); 5.18 (s, 1H, OH),8.05 (s, 1H, C]CH). 11.98 (s 1H, NH). 13C NMR and DEPT (DMSO-d6,75.4 MHz, dppm): (d 119.83, 134.57, 122.85, 130.40, 123.59, 136.57Ar), 123.40

heterocycle, 143.79 (C]CH) 168.32 (C]O2), 166.47 (C]O4). HRMSþ, calculated: 334.5736; found: 335.2612.

(c) 5-(2-Hydroxy-5-chlorobenzylidene)-thiazolidine-2,4-dione (2e)

MF C10H6ClNO3S, MW 255.6775, Yield 79%, Rf 0.45(0.9:0.1 CHCl3/MeOH) MP 207 _C. IR, (KBr, cm_1): 3128 (NeH), 1723(C]O4), 1645 (C]O2), 1588 (C]C). 1H RMN (400 MHz, DMSO-d6):d 7.07 (d, 1H(3), Ar, J ¼ 7.7 Hz), 7.38 (dd, 1H(4), Ar, J ¼ 7.5 Hz, J ¼ 1.5 Hz), 7.34 (s, 1H(6), Ar), 7.98 (s, 1H, C]CH), 5.15 (s 1H, OH),10.86 (s, 1H, NH). 13C NMR and DEPT (75.5 MHz, DMSO-d6):d (119.74, 121.67, 123.09, 123.86, 125.57, 127.37 Ar); 118.59 heterocycle,147.02 (C]CH), 167.43 (C]O2), 165.8 (C]O4). HRMSþ, calculated: 255.6725; found: 254.092.

(d) 5-(2-bromo-5-methoxybenzylidene)-thiazolidine-2,4-dione (2g)

MF C11H8BrNO3S, MW 314.1551, yield 79%, Rf 0.5(9.6:0.4, CHCl3/MeOH), MP 212e213 _C; IR, (KBr, cm_1): 3125(NeH), 1702 (C]O4), 1603 (C]O2), 1471 (HC]C). 1H MNR (400 MHz, DMSO-d6 d ppm): 3.81 (s, 3H, OCH3), 7.31 (d, 1H (3), Ar,J ¼ 7.3 Hz), 7.17 (dd,1H(4), Ar, J ¼ 7.5 Hz, J ¼ 1.3 Hz), (s 1H(5). Ar); 7.78(s, 1H, C]CH), 12.76 (s broad, 1H, NeH). 13C NMR and DEPT (75.5 MHz, DMSO-d6): d 55.63 (OCH3); 134.03,114.01,134.91,127.61,117.36, 159.39 (Ar); 142.28 (C]CH); 166.12, (C]O(2), 165.49 (C]O(4). HRMSþ, calculated: 314.1551; found: 314.1081.

(e) 5-(2,4-dichlorobenzylidene-thiazolidine-2,4-dione

MF C10H5Cl2NO2S, MW 274.1232, Yield. 85 (%), Rf 0.51 (9.8: 0.2 CHCl3/MeOH), MP 203 _C Recrystallization: ethanol; IR, (KBr, cm_1): 3054 (NeH), 1712 (C]O), 1433 (C]C). 1H RMN (400 MHz, DMSO-d6, d ppm): 7.81 (s, 1H, C]CH),12.64 (s broad, 1H, NeH). 7.23(s, 1H(3) Ar), 7.26 (d, 1H(5), Ar J ¼ 7.52 Hz), 7.36 (d, 1H(6), Ar,J ¼ 7.51 Hz). 13C RMN and DEPT (75.5 MHz, DMSO-d6; d

ppm):119.18, 135.58, 128.31, 137.12, 127.91, 128.97, 142.12 CH]C, 125.31(heterocycle), 167.20 (C]O(2), 166.19 C]O(4). HRMSþ, calculated:274.1233, found: 274.1073.

(f) 5-(3,4-dichlorobenzylidene-thiazolidine-2,4-dione

MF C10H5Cl2NO2S, MW 274.1232, Yield. 73 (%), Rf 0.50 (9.5: 0.5CHCl3/MeOH), MP 174 _C. Recrystallization: ethanol; IR, (KBr, cm_1): 3056 (NeH), 1712 (C]O), 1433 (C]C). 1H RMN (400 MHz, DMSO-d6, d ppm): 7.26 (s, 1H(2), Ar), 7.18 (d, 1H(5), Ar J ¼ 7.355 Hz),7.14 (d, 1H(6), Ar, J ¼ 7.14 Hz), 7.76 (s, 1H, CH]C), 11.98 (s broad, 1H,NH). 13C RMN and DEPT (75.5 MHz, DMSO-d6; d ppm): (134.21,133.75, 129.11, 131.15, 130.11, 127.12 Ar); (117.79, heterocycle) 146.15CH]C); 169.12 (C]O(2), 167.23 C]O(4). HRMSþ, calculated:274.1234, found: 274.1215.

Microbiological Activity

The 5-arylidene-thiazolidine-2,4-dione derivatives (a-f) showed activity against Gram-positive and Alcohol-acid resistant bacteria, but did not show activity against Gram-negative bacteria (Table 2). The compounds showed better response to *S. aureus* with inhibition halo of **(a)** (21.66 mm) and **(b)** (20.33 mm) and **(e)** (20mm). These results were close to the kanamycin standard with 22.33 mm. The best results against the strain of *M. luteus* was evidenced with the compounds **(a), (b)** e **(e)**, the compounds **(a)** and **(b)** presenting halo superior to the kanamycin standard for this microorganism. For the microorganism *B. subtilis*, all compounds except compound **(d),** showed greater inhibition halo than the Kanamycin standard. The strain of *E. faecalis* was more sensitive to the compounds **(c), (b), (e)** e **(f)** with higher results than the Cefotaxime (12

mm) and Kanamycin (13 mm) standards. The compounds that showed activity against the alcohol resistant acid bacteria (*M. smegmatis*) were the **(b)** (15 mm), **(a)** (14 mm), **(c)** (14.33 mm) e **(e)** (14mm), these results were similar to the Cefotaxime standard (15 mm). Results similar to those found in this analysis were obtained in recent studies conducted by our research group and published by Silva et al. [2014], where it was observed that the 5-arylidene-thiazolidine-2,4-diones derivative showed inhibition halo greater than 30 mm for the microorganisms *M. luteus* and *B. subtilis*, the compound 5-(2,4-Dichlorobenzylidene) -thiazolidine-2-one (32 mm and 30.7 mm) for *M. luteus* e *B. subtilis*, respectively and the compound 5- (3,4-Dichlorobenzylidene) thiazolidine-2-one showed (34 mm) for *M. luteus.*

Boja et al. [2012] showed thiazolidine-2,4-dione derivatives substituted at the 3 and 5 positions synthesized, showed activity against two Gram-negative bacteria (*P. aeruginosa* e *E. coli*) and two fungi (*C. albicans* e *A. niger*), besides Gram-positive (*S. aureus* e *B. subtilis*). This result suggests that the substitution at the 3-position of the thiazolidinic ring may confer such activity for these molecules, since only the substitution at position 5 did not present activity for these types of microorganisms.

Table 2. Antimicrobial activity of compounds tested against strains bacterial and yeast, using the disk diffusion method.

Codes of Compounds	Microorganisms: Bacteria Gram positive				Gram-negative bacteria			Bacterium Alcohol-resistant acid	Yeast
	Sa	***Ml***	***Bs***	***Ef***	***Pa***	***Ec***	***Sm***	***Ms***	***Ca***
(a)	21.66 ± 3.78	30	19.33 ± 0.57	9.66 ± 0.57	0	0	0	14	0
(b)	20.33 ± 0.57	35.33 ± 0.57	20	15.66 ± 0.57	0	0	0	15	0
(c)	19	18.66 ± 1.15	20.33 ± 0.57	16.66 ± 0.57	0	0	0	14.33 ± 0.57	0
(d)	16.33 ± 0.57	15.66 ± 0.57	11	0	0	0	0	0	0
(e)	20	22	17	14	0	0	0	15	0
(f)	12	7	27	18	0	0	0	0	0
Kanamycin(30 μg/disc)	22,33 ± 0,58	27 ± 1,0	13,33 ± 0,58	13 ± 1,0	14,6 ±0,58	14,66 ± 0,58	14,66 ± 0,58	40 ± 1,0	NT
Cefotaxime(30 μg/disc)	34	45	25	12	22	38	36	15	24±1
Ampicillin(1 0 μg/disc)	38	45	28	24	NT	20	NT	NT	NT

The results represent the mean ± standard deviation of the diameters of the inhibition halos in mm of the microorganisms: *Sa, S. aureus; Bs, B. subtilis; Ml, M. luteus; Ef, E. faecalis; Pa, P. aeruginosa; Ec, E. coli; Sm, S. marcescens; Ms, M. smegmatis; Ca, C. albicans.*

Compounds (a), (b), (c) and (d) were subjected to the tests to determine MIC and CMB (Table 3). The results showed significant inhibitory effects, and most compounds exhibited MIC values between 8-16 μg / mLThis group of thiazolidine derivatives showed a high activity against *M. luteus*. In addition, it was observed that for the *S. aureus*, Compound (b) showed a MIC of 16 μg / mL and compound (d) was as active as the cephalexin standard (8 μg / mL).Compounds (b) (MIC: 16 μg / mL and CMB 32 μg / mL), and (c) (MIC: 32 μg / mL and CMB 64 μg / mL), achieved potency higher than the cephalexin standard (MIC> 128 μg / mL and CMB> 128 μg / mL) for *E. faecalis*.

The (Z) -5- (2,3,4-Trifluorobenzylidene) - rhodanine compound synthesized by Tomasic et al. [2010] inhibited the growth of *S. aureus* a 0,5 μg/mL and *S. aureus* methicillin-resistant (MRSA) of 32 μg / mL. A strong antimicrobial activity against *S. aureus* was observed for compounds bearing the rhodanine ring, higher than those containing other heterocyclic groups. None of the 5-Benzylidene-thiazolidine-4-one and 5-benzylidene-pyrimidine-4,6-dione compounds inhibited the growth of Gram-negative bacteria *E. coli* ou *P. aeruginosa*.

Table 3: Antimicrobial activity of compounds tested against strains of Gram-positive bacteria.

	Microorganisms							
	S. aureus		*M. luteus*		*B. subtilis*		*E. faecalis*	
Compounds	CMI	CMB	CMI	CMB	CMI	CMB	CMI	CMB
(a)	>128	>128	32	64	128	>128	-	-
(b)	16	32	16	32	32	32	16	32
(c)	128	>128	8	16	128	128	32	64
(d)	8	>128	16	>128	-	-	-	-
Cefalexina	8	16	<2	2	<2	<2	>128	>128

Values represent MICs - Minimal Inhibitory Concentration and CMB-Minimum Bactericidal Concentration (μg / mL) against strains of Gram-positive bacteria.

Cytotoxic Activity

In the present study cytotoxic activity for substances (e) and (f) was performed against six tumor cell lines as well as for human peripheral blood mononuclear cells PBMC. The in vitro cytotoxic activity of compounds (e) and (f) was performed by the

MTT assay against NCI-H 292 cells (mucoepidermoid carcinoma of the lung), HL60 (promyelocytic leukemia), HT-29 (colon adenocarcinoma), K562 (chronic myelocytic leukemia), HEp-2 (laryngeal squamous cell carcinoma) and MCF-7 (breast adenocarcinoma). The two thiazolidine derivatives substituted at the 5-position of the heterocyclic ring were synthesized and tested. The two compounds showed inhibition of cell growth greater than 75%, compared to the six lines of tumor cells with a single concentration of 25μg / mL. The compounds were serially diluted and retested to find the concentration that inhibited 50% of cell proliferation. The results obtained in this test are described in Table 4 and show that the chronic myelocytic leukemia (K-562) strain was the most resistant to compounds with IC 50 above 10 μg / mL for the two substances tested. Compound (e) showed better cytotoxic activity for HT-29 strains (1.7 μg / mL); NCI-H292 (3.5 μg / ml) and HEP-2 (4.0 μg / ml). Compound (f) was more active in MCF-7 (3.6 μg / mL) and NCI-H (4.9 μg / mL) cells.

Table 4. Evaluation of cytotoxic activities of 2,4-dione thiazolidine derivatives after 72 hours of incubation.

Compounds - IC_{50} μg/ mL (Confidence Interval - 95% CI)			
Cellular lineages	**dox**	**(e)**	**(f)**
HEp-2	**0,7** (0,3-1,4)	**4,0** (3,1-5,2)	**5,3** (5,7-7,6)
HT-29	**0,4** (0,3-0,5)	**1,7** (0,8-3,5)	**5,9** (4,8-7,3)
MCF-7	**0,2** (0,1-0,2)	**5,7** (4,5-7,3)	**3,6** (2,8-4,7)
NCI-H292	**0,2** (0,1-0,5)	**3,5** (2,9-4,3)	**4,9** (4,2-5,4)
HL-60	**0,02** (0,01-0,02)	**6,9** (4,7-10,1)	**9,5** (5,2-17,2)
K562	**0,8** (0,7-0,9)	1**7,9** (8,6-37,2)	**17,5** (16,1-18,9)

IC_{50} values (Concentration causing 50% inhibition of cell growth) and CI (confidence of compounds (e) and (f) against the Hep-2, HT-29, MCF-7, NCI-H292, HL-60 and K562. Doxorubicin (DOX) was used as a positive control.

In recent work, Akshaya et al. [2014] synthesized and tested thiazolidine-2,4-dione derivatives and verified cytotoxicity of the compounds against the MCF-7 breast cancer line, corroborating our results. In the protocol established for this experiment The compound is active when the IC 50 has a value of 4μg / mL or less [Geran et al., 1972; Ferrari et al., 1992]. The cytotoxic action of thiazolidinediones can occur by several mechanisms of action that are described in the literature, one of them occurs due to the activation of the PPARɣ (peroxisome proliferator-activated receptors gamma), leading to certain cellular events such as cell cycle arrest and apoptosis [Divya et al., 2013; Jain et al., 2013]. Another relevant aspect for the biological activity of these molecules is the presence of halogen atoms in their chemical structures. There is a prevalence of halogens in the current drugs because of their ability to promote a more stable binding with the receptor site because they have the ability to form intermolecular bonds similar to the hydrogen bonds so as to facilitate the interaction and stability of the bonds [Hernandes et al. al., 2010]. In order to verify whether the activity of the compounds are dependent time or even dose dependent the MTT test was performed for the 24 and 48 hour periods for the colon and lung cancer strains. It was observed that compound (e) did not cause cytotoxicity within 24 hours with IC 50 above 25μg / mL for the two tested strains and that compound (f) also showed concentrations above 15μg / mL for both strains, indicating low cytotoxicity in this period. After 48 hours of incubation the IC 50 values for compound (e) were 7.7 μg / mL and 9.6 μg / mL and for compound (f) the values were 7.9 and 8.3 for the colon and lung strains respectively (table 5). Showing that the action of these compounds are time dependent.

Table 5. Evaluation of cytotoxic activities of thiazolidine-2,4-diones derivatives after 24 and 48 hours of incubation.

COMPOUNDS CI_{50} µg/mL (Confidence Interval - 95% CI)				
	24 HOURS		48 HOURS	
Cell lineages	**(e)**	**(f)**	**(e)**	**(f)**
HT-29	**>25**	**23.2**	**7.78**	**7.9**
		(19.7-27.2)	(6.0-10.0)	(6.2-10-1)
NCI-H292	**>25**	**15.71**	**9.6**	**8.3**
		(11.3-21.7)	(7.4-12.4)	(6.2-11.2)

IC 50: concentration that causes 50% inhibition of cell proliferation; CI: confidence interval;

The evaluation of hemolytic activity was performed to find out whether the compounds cause damage to the cell membrane. The results (Table 6) showed that the compounds did not cause membrane lysis at concentrations up to 125 µg / mL. This is in agreement with the results seen in the 24 hour period which demonstrated that cytotoxicity does not occur in short periods suggesting that cytotoxic activity occurs by more specific mechanism.

Table 6: Cytotoxic activity of thiazolidine-2,4-dione derivatives in human peripheral blood mononuclear cells (PBMC) and hemolytic activity in human erythrocytes.

	Normal Cells	
	PBMC	ERYTHROCYTE
Compounds (μg/mL)	Cytotoxicity IC_{50}	Hemolytic Activity EC_{50}
(e)	>25	>125
(f)	>25	>125

IC50: concentration that causes 50% inhibition of cell growth; EC50: Effective concentration that causes 50% damage in human erythrocytes; PBMC peripheral blood mononuclear cells;

One of the major challenges for developing new cancer drugs is to develop a molecule that presents cytotoxic potential for tumor cells and is inert to normal cells [Anazetti et al., 2003]. Thus, to analyze whether the action of these derivatives exhibit cytotoxicity to normal cells the compounds were analyzed against CMSP-peripheral blood mononuclear cells, where it was found that the compounds did not exhibit cytotoxicity with IC 50 values greater than 25 μg / mL when incubated for a period of 72 hours (Table 6), suggesting that the compounds show selectivity to the tumor cells since for these IC50 values were lower.

Conclusions

The 5- (benzylene-thiazolidine-2,4-dione (a-f) derivatives synthesized employing a simple procedure possess potent antimicrobial activities against strains of Gram-positive bacteria. The compounds tested are cytotoxic to colon, lung and breast cancer cell lines. The thiazolidinediones derivatives tested do not have cytotoxicity for

normal cells, demonstrating characteristics of promising molecules for the development of new drugs.

Financing source

The authors thank the CNPq and FACEPE Foundation for Research of the State of Pernambuco for financial support.

Conflict of interest

The authors declare that they have no conflict of interest to disclose.

References

Akshaya TT, Arunlal VB, Babu G, Biju CR. 2014. Synthesis, characterisation and *invitro* anticancer activity of thiazolidine-2, 4-dione derivatives, Drug Discov Ther. 2 (24): 42-7.

Albuquerque JFC, Azevedo CC, Galdino SL, Chantegrel J, Pitta IR, Luu-Duc C, 1995. Synthèse et étude structurale des 5-arilidène thiazolidine-2,4-diones et 4-thio-imidazolidine -2-ones-3-substituèes, Ann. Pharm. Fr, 53:209-14.

Alley MC, Scudiero DA, Monks A, Hursey ML, CzerwinskI MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH, Boyd MR. 1988. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res. (48):589-601.

Anazetti MC, Melo PS, Duran N, Haun M. 2003. Comparative cytotoxicity of dimethylamide-crotonin in the promyelocytic leucemia cell line (HL-60) and human peripheral blood mononuclear cells. Toxicology. (188): 261-74.

Barry AL, Pfaller MA, Rennie RP, Fuchs PC, Brown SD. 2002. Precision and Accuracy of Fluconazole Susceptibility Testing by Broth Microdilution, Etest and Disk Diffusion Methods. Antimicrob. Agents Chemo. 46(6): 1780-4.

Bauer, AW, Kirby WM, Sherris JC, Turck M. 1966. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 45 (4): 493– 96.

Boja P, Prajwal LL, Manjunatha K, Prathibha A, Kumari NS. 2012. Novel thiazolidine-2, 4-dione mannich bases: Synthesis, characterization and antimicrobial activity. Der Pharma Chemica. 4 (3): 867-71.

Böyum A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest (21): 77-89.

Bozdag-Dündar O, Ozgen O, Mentes A, Altanlar N, Ath O, Kendi E, Ertana RR. 2007. Synthesis and antimicrobial activity of some new thiazolyl thiazolidine-2, 4-dione derivatives, Bioorg. Med. Chem. (15) 6012-17.

Bruno G, Costantino L, Curinga C, Maccari R, Monforte F, Nicolo F, Ottana R, Vigorita MG. 2002. Synthesis and aldose reductase inhibitory activity of 5-Arylidene-2, 4-thiazolidinedionesy, Bioorg. Med. Chem. (10): 1077-84.

Chaves AP, Gomes JAP, Höfling-Lima AN. 2007. Alterações oculares induzidas pela quimioterapia. Arq. Brás. Oftalmol (70):718-725.

Clinical and Laboratory Standards Institute (CLSI). 2013. Performance Standards for Antimicrobial Disk Susceptibility Tests. Wayne. PA. 23rd informational supplement M100-S23, USA.

Divya GS, Mansoor KP, Shebina PR, Kuma A. 2013. PPAR Gamma agonist: An effective strategy for cancer treatment. JPSI. (2):1-3.

Ferrari M, Fornasiero MC, Isetta AM. 1992. MTT colorimetric assay for testing macrophase cytotoxic activity in vitro. J. Immunology Methods. 153 (1-2): 265-7.

Gouveia FL, Oliveira RMB, Oliveira TB, Silva IM, Nascimento SC, Sena KXFR et al. 2009. Synthesis, antimicrobial and cytotoxic activities of some 5-arylidene-4-thioxo-thiazolidine-2-ons, Eur. J. Med. Chem. (44) 2038-43.

Hanahan D & Weinberg RA. 2011. Hallmarks of cancer: The Next Genration, Cell. (144): 646- 74.

Hernandes MZ, Cavalcanti SM, Moreira DR, de Azevedo Junior WF, Leite AC. 2010. Halogen atoms in the modern medicinal chemistry: hints for the drug design. Curr Drug Targets. 11(3): 303-14.

INCA - Instituto Nacional do Câncer (Brasil). Estimativas 2012: incidência de câncer no Brasil. Disponível em <http://www.inca.gov.br> acessado em 24/09/2012.

INCA - Instituto Nacional do Câncer (Brasil). Estimativas 2014/2015: Incidência de câncer no Brasil. Disponível em <http://www.inca.gov.br> acessado em 14/10/2015.

Jain, VS, Vora, DK, Ramaa CS. 2013. Thiazolidine-2, 4-diones: Progress towards multifarious applications. Bioorg. Med. Chem. (21): 1599-1620.

Koneman EW, Allen SD, Janda WM. Schreckenberger PC, Winn WC. 2001.2. Diagnóstico microbiológico: Texto e Atlas colorido. 5. ed. Rio de Janeiro: MEDSI.

Leite FHA, Santiago PBGS, Froes TQ, Da Silva Filho J, Da Silva SG, Ximenes RM, et al. 2016. Structure-guided discovery of thiazolidine-2, 4-dione derivatives as a novel class of Leishmania major pteridine reductase 1 inhibitors. Eur J Med Chem. (123): 639-48.

Liebermann D, Himbert J, Hengl L, 1948. La thiazolidione, point de depart d'une synthèse des acides thiopyruviques et thioglyoxiliques substituèes, Bull. Soc. Chim. Fr: 1120-24.

Morrow JK, Tian L, Zhang S. 2010. Molecular networks in drug discovery. Crit Rev Biomed Eng. 38 (2): 143-56.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicy assays. Journal lmmunol. Methods. (65):55-63.

Nazreen S, Alam MS, Hamid H, Yar MS, Dhulap A, Alam P, et al. 2014. Thiazolidine-2,4-diones derivatives as PPAR-ɣ agonists: Synthesis, molecular docking, in vitro and in vivo antidiabetic activity with hepatotoxicity risk evaluation and effect on PPAR-c gene expression. Bioorg. Med. Chem. Lett. (24): 3034–42.

O'brien J, Wilson I, Orton T, Pognan F. 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxibcity. Eur. J. Biochem. (267): 5421-26.

Pikkemaat MG, Linssen ABM, Berendsen HJC, Janssen DB. 2002. Molecular dynamics simulations as a tool for improving protein stability, Protein Eng. (15):185-192.

Silva IM, Silva Filho J, Santiago PBGDS, Egito MS, Souza CA, Gouveia FL et al. 2014. Synthesis and antimicrobial activities of 5-arylidene-thiazolidine-2, 4-dione derivatives, Biomed. Res. Int. (2014): 1-8.

Tomasic T, Zidar N, Mueller-Premru M, Kikelj D, Masic LP. 2010. Synthesis and antibacterial activity of 5-ylidenethiazolidin-4-ones and 5-benzylidene-4, 6-pyrimidinediones. 2010. Eur. J. Med. Chem. 45: 1667–72.

Vicini P, Geronikaki A, Anastasia K. 2006 Synthesis and antimicrobial activity of novel 2-thiazolylimino-5-arylidene-4-thiazolidinones. Bioorg. Med. Chem. (14): 3859-64.

Geran RI, Greenberg NH, Mcdonald MM, Schumacher AN, Abbot BJ. 1972. Protocols for screening clinical agents and natural products against animal tumors and other biological systems.

6 CAPÍTULO III - ARTIGO CIENTÍFICO 3

ARTIGO 3

Study of the genotoxicity of the 5 - (2-bromo-5-methoxybenzylidene) thiazolidine-2,4-dione

A ser submetido ao periódico:

ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY

Study of the genotoxicity of the 5 - (2-bromo-5-methoxybenzylidene) thiazolidine-2,4-dione

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Highlights

- Studies with the 5- (2-Bromo-5-methoxybenzylidene) thiazolidine-2,4-dione compound demonstrated cytotoxic and antimicrobial activities.
- There was no significant difference in the concentrations tested for normal cells with the 5- (2-Bromo-5-methoxybenzylidene) thiazolidine-2,4-dione compound.
- In tumor cells the damage index was significant suggesting compound selectivity.

Abstract

Thiazolidine-2,4-dione derivatives comprise an important group of compounds having a range of biological activities such as hypoglycemic, anti-inflammatory, antimicrobial and antitumor. Previous studies have demonstrated cytotoxic and antimicrobial activities of the compound (5- (2-Bromo-5-methoxybenzylidene) thiazolidine-2,4-dione) with promising results, but its genotoxic effects have not yet been studied. In this work the mutagenic activity in tumor cells and normal by the micronucleus and comet assay tests were evaluated. The compound was used at concentrations based on the IC50 and 2x at the IC50. The damage index and micronucleus frequencies in the cells at each concentration were compared to the negative control by ANOVA with Dunnet's post test and the Chi-square test. It was observed that there was no significant difference in the concentrations tested for the normal cells, whereas in the tumor cells the damage index was significant suggesting the selectivity of the compound.

Key words: Thiazolidine-2,4-diones, Comet assay, Micronucleus test, Tumor cells.

1.0 INTRODUCTION

The thiazolidine derivatives have a heterocyclic ring that is being extensively investigated and appears in several studies. The compounds containing the thiazolidine-2,4-dione nucleus are described as having multiple biological actions such as antimicrobial (Alegaon and Alagawadi, 2012), anti-inflammatory, analgesic (Reanmongkol and Songkram, 2013), anticonvulsant (Amin et al.,2008), and its anticancer action has recently been extensively studied (Romagnoli et al., 2013).

The action of these compounds has been mainly related to the activation of PPARγ (receptors activated by peroxisome proliferators). PPAR-γ agonist substances cause several biological actions besides the hypoglycemic action also interfering in the control of cell growth, motility, differentiation and apoptosis (Divya, 2013). The 5- (2-Bromo-5-methoxybenzylidene) thiazolidine-2,4-dione) or compound (5) derivative was synthesized and tested in previous work showing excellent cytotoxic activity against tumor cell lines, however. its genotoxic effect is not yet described in the literature. The study of the genotoxic action of several compounds allows the knowledge of the possible threats to which the human population is exposed and for substances with possible uses as drugs, this analysis is indispensable (Aquino, 2010; Marzin, 1997). In this study, compound (5) was investigated in its ability to cause genotoxic effects through the in vitro micronucleus test (MNvit) which is a genotoxicity test for the detection of micronuclei (MN) in the cytoplasm. Micronuclei can originate from chromosomal fragments (without a centromere), or entire chromosomes that are unable to migrate to the poles during the anaphase stage of cell division. The compound was also evaluated by the Single Cell Gel Electrophoresis assay which is very sensitive and can be performed with any type of eukaryotic cell. The Cometa Assay is a rapid and efficient technique to detect genomic damage in mammalian cells exposed to genotoxic agents (Fairban et al., 1995; Singh et al., 1988). Thus, this work aimed to evaluate the mutagenic action of the derivative (5- (2-Bromo-5-methoxybenzylidene) thiazolidine-2,4-dione) in HL60 (acute promyelocytic leukemia), MCF-7 (Adenocarcinoma breast) and PBMC (peripheral blood mononuclear cells), through micronuclei tests and comet assay, since this compound presents important biological activities with potential pharmacological application.

2.0 Methods

2.1 Synthesis of the compounds

The synthesis methodology of compound 5 has already been described (Leite et al., 2016). Briefly, the compound was synthesized in a series of six molecules (Fig. 1). The thiazolidine-2,4-dione (1) was obtained by the method described by Lieberman et al. (1948) and modified by Albuquerque et al. (1995) and Gouveia et al. (2009). This reaction occurs by condensation of monochloroacetic acid and thiourea in an aqueous medium under reflux for 24 h (Bzdag-Dündar et al., 2007; Bruno et al., 2002). They were synthesized according to the protocol described previously (Pikkemaat et al., 2002; Silva et al., 2014; Vicini et al., 2006) (Figure 1). All compounds were identified by the method of IR spectroscopy, NMR and HRMS.

2.2 Maintenance of tumor lines

The tumor lines used in this study were: MCF-7 (breast adenocarcinoma), and HL-60 (human promyelocytic leukemia). Tumor cell lines were assigned by the Laboratory of Cell Cultures and Pharmacological Tests of the Department of Antibiotics of the Federal University of Pernambuco - UFPE. The strains were cultured (2 x 10^5 cells / ml) in flasks (25 cm 2/60 ml) in Sigma DMEM (Dulbecco's Modified Minimum Eagle Medium) supplemented with 10% fetal bovine serum (Gibco), 1% antibiotic solution (penicillin 1000 IU / mL + streptomycin 250 μg / mL) and 1% of 200 mM L-glutamine and incubated at 37 ° C in a humidified atmosphere containing 5% CO_2.

2.3 Obtaining Peripheral Blood Mononuclear Cells (PBMC)

Mononuclear cells (lymphocytes and monocytes) were obtained from the peripheral blood of healthy volunteers collected in Vacutainer-type tubes containing Heparin solution (BD Vacutainer®) as anticoagulant. The protocol was approved by the Human Research Ethics Committee Nº CAAE 48809515700005208 After collection, 6 mL of blood were diluted in 3 mL of PBS and added slowly into another tube containing 2 mL of Ficoll®-Hystopaque (Sigma). Then, centrifugation was performed at 1500 rpm for 30 minutes to separate the solution phases. Ficoll was used to promote the formation of a density gradient where the mononuclear cells were concentrated in the layer between

the plasma (light phase) and the erythrocytes (dark phase). The PBMCs were transferred to another tube to which PBS was added to the final volume of 11 ml and then centrifuged for 20 minutes at 1000 rpm. The supernatant was discarded and the PBMC pellet resuspended in RPMI 1640 medium supplemented with 20% fetal bovine serum and 1% antibiotics (to obtain a final concentration of 100 U / ml penicillin and 100 μg / ml streptomycin). Phytohemagglutinin (Sigma) 2%, a mitogenic agent, was added to the medium to stimulate lymphocyte proliferation. Cells were counted and diluted to a final concentration of 1 x 10^6 cells / ml.

2.4 Genotoxic effect of compound 5 on normal and tumor cell lines

The genotoxic effects of compound (5) were evaluated by the Comet and Micronucleus Assays according to the method described by Fenech et al. (1985); Singh et al. (1988); Tice et al. (2000) respectively. The MCF-7 and HL-60 strains were cultured at 2x105 cells / mL and PBMC at the concentration of 106 cells / mL in 24 well plates in medium (1 mL / well) Dulbecco's Modified Minimum Eagle Medium (DMEM, Sigma), supplemented with 10% fetal bovine serum (Gibco), 1% antibiotic solution (penicillin 1000 IU / ml + streptomycin 250 mg / ml) and 1% of 200 mM L-glutamine and incubated at 37 ° C in a humidified atmosphere containing 5% CO2, and incubated for 24 hours. After the incubation period, the solutions of compound 5 at concentrations relative to IC50 and double the IC50 for each lineage were added. Doxorubicin (0.2 μg / mL) was used as a positive control. After this time the medium was aspirated and the cells were washed with 300 μL of buffer (PBS, pH 7.2) and then treated with 300 μL of trypsin / EDTA. Subsequently, for the inactivation of the trypsin was added 600 μL of DMEM medium following the aspiration of the samples, which were transferred to Eppendorf tubes for further processing. Processing was started with the samples being washed with 60 μL buffer (PBS, pH = 7.2) and centrifuged at 2000 rpm for 20 minutes. As a negative control the culture medium (DMEM) was used with the same amount of DMSO used to dissolve the test substance. After centrifugation, the culture medium was discarded and the cells resuspended with 60 μl of PBS.

2.5 Comet test

About 15 μL of the cell suspension was homogenized in 100 μL of low melting point (LM) agarose preheated in a 37 ° C water bath. The homogenate was then transferred to

standard agarose coated glass sheets, covered with coverslips and incubated for 10 minutes under refrigeration at 4 ° C. After this period, the coverslips were removed and the slides were immersed in a lysis solution for 2 hours under refrigeration at 4 ° C. After this period, the slides were electrophoresed (40V for 20 minutes and at 300 mA). The slides were then immersed in neutralization solution for 15 minutes and then fixed in absolute ethanol solution for 5 minutes. After drying, they were stored in a refrigerator until the time of staining. For staining, Gel Red dye (Biotarget GelRed®) was used, where 1 μl of the dye was homogenized in 1000 μL of sterile deionized water (1: 1000). The slides were analyzed by fluorescence microscopy (Olympus-BX series) and then the cell counts were performed where about 100 cells per slide were analyzed, with scores of 0-4 being assigned according to the level of nucleoid damage.

2.6 Micronucleus Test

In this assay only two drops of the cell suspension were used after treatment with compound (5). The two drops of the cell suspension were transferred to previously washed slides, still wet and in the horizontal position. The slides were allowed to dry at room temperature. After drying, the slides were fixed with absolute ethanol for 5 minutes and then washed in running water and stained using Giemsa (Merck) dye, which was added evenly over the slides by a average time of 5 minutes. After staining, the slides were again washed with running water and put to dryness at room temperature. The counting was performed under an optical microscope with magnification of 400x. For each treatment, 1000 cells were counted per slide to determine the number of micronuclei. Considering as micronuclei the structures observed in the same focal plane, with the intensity of coloration similar to that of the associated nucleus and volume less than one third of the volume of the associated nucleus located within the cytoplasm.

2.7 Statistical tests

Statistical analyzes were performed using the ANOVA with Dunnett's post-test for comparisons between the damage indexes by the comet test and the chi-square (X2) for the analysis of the frequencies of micronucleated cells. The analysis was done by

treatment / lineage in relation to the negative control with a level of significance of 0.05.

3.0 Results and discussion

3.1 Comet test

The analysis of damage indices obtained in the Cometa Assay demonstrated that in the normal cell lines (PBMC) no significant damage index was observed (Figure 3). For tumor cell lines significant differences were observed in relation to the negative control. Faced with MCF-7 breast cancer cells, compound 5 was used at concentrations of 3.5 and 7.0 μg / mL showing significant damage to both concentrations (Figure 4). In the HL-60 line only at the concentration of 4.0 μg / mL presented a significant damage index (Figure 5). The results were analyzed by ANOVA followed by Dunnett's post-test. In previous study, a good cytotoxic activity of this derivative was observed for the lung cancer (NCI-H292), breast (MCF-7) and leukemia (HL-60) lines without cell cytotoxicity normal PBMC (peripheral blood mononuclear cells) (Rodrigues et al., 2017). This shows important features of a drug candidate molecule that selectively acts on cancer cells (Geran et al., 1972). The assays were performed with compound 5 at concentrations that do not provide cytotoxicity to the cells, since the cytotoxic action causes the formation of breaks of the DNA strands (Ribeiro et al., 2003). To evaluate the genotoxic action of the compound in normal cells, peripheral blood mononuclear cells (PBMCs) were used as the experimental model for this study because of the sensitivity to genotoxic agents, because they have a relatively long life and are easily obtained (Albertine et al. al., 2000). The thiazolidine derivatives are known to exhibit a diverse range of biological activities such as antihyperglycemic, anti-inflammatory, antimicrobial and lately much research has been done on its antitumor action (Gouveia et al., 2009; Verma and Saraf, 2008). However, information on its genotoxic activity is scarce, so there are no genotoxic studies in this literature with this class of compounds that serve as parameters for comparison with the results presented here.

3.2 Micronucleus test

In this study, micronuclear formations were observed (Figure 6) after exposure to the different concentrations of compound 5 (Table 1). The concentrations used were based on the IC 50 values and twice the IC 50 of compound 5 presented for each lineage in

cytotoxicity tests performed in previous work by our research group (Rodrigues et al., 2017). For the MCF-7 line (3.5 μg / mL and 7.0 μg / mL) and HL60 (2.0 μg / mL and 4.0 μg / mL). The compound did not present statistically significant genotoxic effects at tested concentrations (Table 2). Although an increase in the number of micronuclei related to the increase in concentration has been observed. In the analysis by the X^2 test it was observed that there was no significant difference between treatment with compound 5 and the negative control in any of the cell lines tested. Indicating that it did not present genotoxic effects in the concentrations tested in relation to the negative control (table 2).

4.0 Conclusions

Compound 5 - (2-bromo-5-methoxybenzylidene) thiazolidine-2,4-dione had no significant genotoxic effect on human normal lymphocytes (PBMCs) exposed for 48 hours at different concentrations of compound 5 as compared to the negative control. In tumor cells MCF-7 and HL-60, a significant increase in the damage index was observed in the cells analyzed after incubation with the compound. In the micronucleus test, no significant difference was observed between micronucleus frequencies in the lines that were treated and the negative control indicating that the type of damage caused by compound 5 was selective for tumor cells and that could mean a mechanism of induction to cell death by apoptosis due to DNA damage. This discloses that compound 5 has desirable characteristics for a possible antineoplastic drug.

Acknowledgment

The authors thank the CNPq and Foundation of Research of the State of Pernambuco FACEPE for the financial support.

Conflict of interest

The authors declare that they have no conflict of interest to disclose.

References:

Albertine, R. J., Anderson, D., Douglas, G. R. (2000). IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. *Mutat. Res*., 463: 111-172.

Albuquerque, J. F., Albuquerque, A., Azevedo, C. C., Thomasson, F., Galdino, S. L., Chantegrel, J., Catanho, M. T. J., Pitta, I. R., Luu-Duc, C. (1995). Substituted thiazolidinediones and thio-imidazolidinones: synthesis, structural study and pharmacological activity. *Pharmazie*, 50: 387–389.

Alegaon, S. G., Alagawadi, K. R. (2012). New thiazolidine-2,4-diones as antimicrobial and cytotoxic agent. *Med Chem Res*. 21: 3214–23.

Amin, K. M., Rahman, A. D. E., AL-eryani, Y. A. (2008). Synthesis and preliminary evaluation of some substituted coumarins as anticonvulsant agents. *Bioorg. Med. Chem*., 16: 5377- 88.

Aquino, I., Efeito genotóxico da artemisinina e do artesunato em células de mamíferos. (2010). 81 f. Dissertação (Mestrado) - Curso de Biologia Geral e Aplicada, Departamento de Biomoléculas, Universidade Estadual Paulista Julio Mesquita Filho, Botucatu: São Paulo.

Bruno, G., Costantino, L., Curinga, C., Maccari, R., Monforte, F., Nicoló, R., Ottanà, M., Vigorita, M.G. (2002). Synthesis and aldose reductase inhibitory activity of 5-arylidene-2,4-thiazolidinediones. *Bioorg. Med. Chem*., 10: 1077-1084.

Bzdağ-Dündar, O., Ozen Ozgen, A. M., Nurten, A., Onur, A., Engine, K., Rahimiye, E. (2007). Synthesis and antimicrobial activity of some new thiazolyl thiazolidine-2,4-dione derivatives. *Eur. J. Med. Chem*., 15: 6012-6017

Divya, G. S., Mansoor, K. P., Shebina, P. R., et al. (2013). PPARɣ agonistas: Uma estratégia eficaz para o tratamento do câncer. *J. Pharm.Inov. Cien*., 2: 1-3.

Fairbairn, D. W., Olive, P. J., O'Nell, K. L. (1995). The comet assay: a comprehensive. *Mutat Res.*, 339: 37-59.

Fenech, M., Morley, A. (1989). Kinetochore detection in micronuclei: an alternative method for measuring chromosome loss. *Mutagenesis*. 4: 98-104, 1989.

Geran, R. I., Greenberg N. H., Mcdonald, M.M., Schumacher, A.N., Abbot, B. J. (1972). Protocols for screening clinical agents and natural products against animal tumors and other biological systems.

Gouveia, F. L., Oliveira, R. M. B., Oliveira, T. B., et al. (2009). Synthesis, antimicrobial and cytotoxic activities of some 5-arylidene-4-thioxo-thiazolidine-2-ones. *Eur J Med Chem.*, 44: 2038-43.

Leite, F. H. A., da Santiago, P. B. G., Froes, T. Q., da Silva Filho, J., da Silva, S. G., Ximenes, R. M., de Faria, A. R., Brondani, D. J., de Albuquerque, J. F. C., Castilho, M. S. (2016). Structure-guided discovery of thiazolidine-2,4-dione derivatives as a novel class of Leishmania major pteridine reductase 1 inhibitors. *Eur J Med Chem*. 123: 639–648.

Lieberman, S., Dobriner, K., Hills, B.R., Fieser, L. F., Rhoads, C. P. (1948). Studies in Steroid Metabolism II. Identljication and Characterization of Ketosteroids Isolated From Urine of Healthy and Diseased Persons. *J of Bio Chem*. 172: 263-295.

Marzin, D. (1997). The position of the in vitro micronucleus test within the battery of screening for genotoxic potential determination and regulatory guidelines. *Mutat Res*. 175-181.

Pikkemaat, M. G., Linssen, A. B. M., Berendsen, H. J. C., Janssen, D. B. (2002). Molecular dynamics simulations as a tool for improving protein stability. *Prot Eng*. 15: 185–192

Reanmongkol, W., Songkram, C. (2013). In vivo anti-inflammatory, analgesic and antipyretic activities of novel thiazolidine-2, 4-dione analogs derived from some classical NSAIDs. *Songklanakarin J. Sci. Technol*. 35: 33- 40.

Ribeiro, L. R., Salvadori, D. M. F., Marques, E. K. (2003). Mutagênese Ambiental. Editora da ULBRA, Canoas, 356p.

Romagnoli, R., Baraldi, P. G., Salvador, M. K., et al. (2013). Anticancer activity of novel hybrid molecules containing 5-benzylidene thiazolidine-2,4-dione. *Eur J Med Chem***.** 63: 544-57.

Singh, N. P., Mccoy, M.T., Tice, R. R., Schneider, E. L., (1988). A simple technique for the quantification of low levels of DNA damage in individual cells. *Exp Cell Res*. 175:184-191.

Tice, R. R. et al. (2000). Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ. Mol. Mutagen*. 35: 206–221.

Verma, A., Saraf, S. K. (2008). 4- Thiazolidinone- A biologically active scaffold. Eur J Med Chem. 43: 897-905.

Vicini, P.; Geronikaki, A.; Anastasia, K.; Incerti, M.; Zani, F. (2006). Synthesis and antimicrobial activity of novel 2-thiazolylimino-5-arylidene-4- thiazolidinones. *Bioorg. Med. Chem*. 14: 3859–3864.

Figures Captions

Fig. 1: Scheme of synthesis of compound 5.

Fig. 2: Levels of damage analyzed by the comet assay.

Fig. 3: Damage index (ID) obtained by the Comet assay peripheral blood mononuclear cells (PBMC).

Fig. 4: Damage index (ID) obtained by the Comet assay adeno breast carcinoma cells (MCF-7).

Fig. 5: Index of damage (ID) obtained by the Comet assay in promyelocytic leukemia cells (HL-60).

Fig. 6: Presence of micronuclei.

Fig. 1: Scheme of synthesis of compound 5 (d) Derivative (5- (2-Bromo-5-methoxybenzylidene) thiazolidine-2,4-dione).

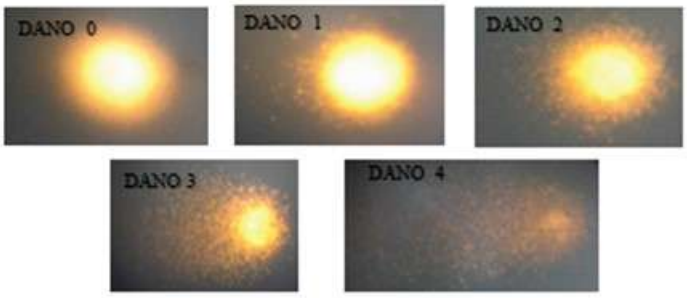

Fig. 2: Levels of damage analyzed by the comet assay. Damage levels ranging from 0 to 4 obtained from breast cancer cells (MCF-7) after treatment for 48 hours with compound 5. Level 0 = no damage (<5%); level 1 = low levels of damage (5-20%); level 2 = medium level of damage (20-40%); level 3 = high levels of damage; 4 = maximum damage (≥ 95%).

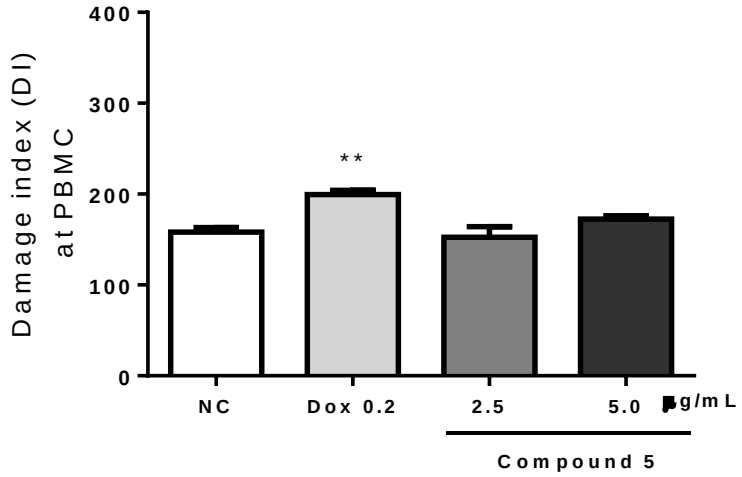

Fig. 3: Damage index (ID) obtained by the Comet test. Mononuclear cells of peripheral blood (PBMC) isolates from healthy human volunteers treated with DMEM 0,5% DMSO (NC); Doxorubicin 0.2 µg / mL as positive control (Dox) and compound 5 for 48 hours. Values (damage index of 100 cells in 2 slides, n = 2) corresponds to the mean ± SD of two independent experiments. * $P < 0.05$ compared to negative control (NC) and analyzed by ANOVA followed by Dunnett's posttest.

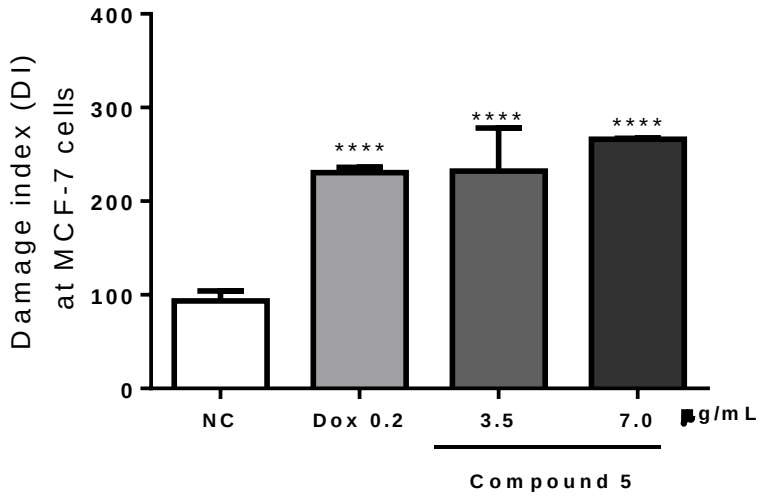

Fig. 4: Damage index (ID) obtained by the Comet test. Adeno breast carcinoma cells (MCF-7) treated with DMEM 0,5% DMSO (NC); Doxorubicin 0.2 µg / mL as positive control (Dox) and compound 5 for 48 hours. Values (damage index of 100 cells in 2 slides, n = 2) corresponds to the mean ± SD of two independent experiments. * $P < 0.05$ compared to negative control (NC) and analyzed by ANOVA followed by Dunnett's posttest.

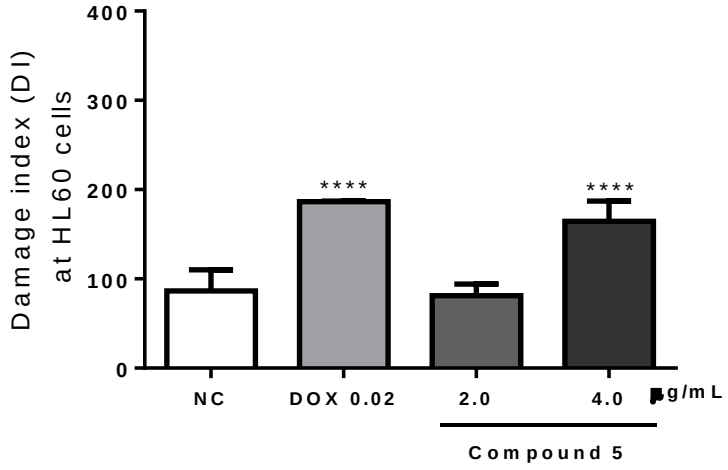

Fig. 5: Damage index (ID) obtained by the Comet test in promyelocytic leukemia cells (HL-60) treated treated with DMEM 0,5% DMSO (NC); Doxorubicin 0.2 µg / mL as positive control (Dox) and compound 5 for 48 hours. Values (damage index of 100 cells in 2 slides, n = 2) corresponds to the mean ± SD of two independent experiments. * $P < 0.05$ compared to negative control (NC) and analyzed by ANOVA followed by Dunnett's posttest.

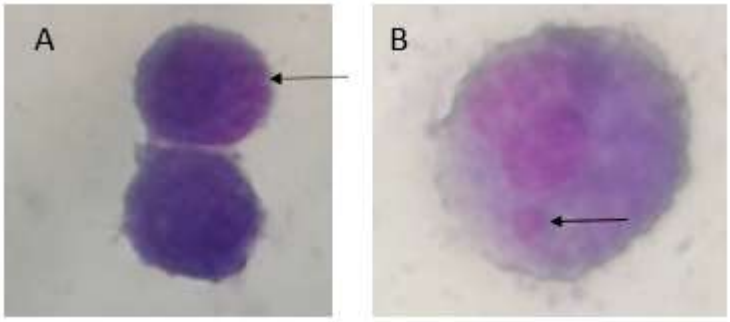

Fig. 6: Presence of micronuclei.
Image made in Optical microscope with magnification of 400x. Micronuclei (arrows) (A) in PBMC cells and in (B) MCF-7 cells after 48 hour treatment with compound 5.

Table 1: Evidence of micronuclei.

Compound 5µg/mL	Cell Lineages					
	MCF-7		HL-60		PBMC	
	CM	FMN	CM	FMN	CM	FMN
CN	13	0,013	10	0,010	1	0,001
CI_{50}	18	0,018	16	0,016	4	0,004
$2CI_{50}$	19	0,019	14	0,014	6	0,006
Dox	23	0,023	21	0,021	19	0,019

CMN (Micronucleated cells) and FMN (Micronucleus frequency) observed in tumor lines and normal (PBMC) concentrations after exposure for 48 hours at different concentrations of compound 5 (IC50 and 2xCl50) in µg / ml.

Table 2: P-values relative to the micronucleate cell frequency comparisons.

Cell Lineages	Concentrations in µg / mL (Compound 5) Negative Control - NC		
	CI_{50}	**$2CI_{50}$**	**Dox 0,2**
PBMC	**>0.05**	**>0,05**	**< 0,0001****
MCF-7	**>0.05**	**>0.05**	**> 0,05**
HL-60	**>0.05**	**>0.05**	**< 0,05 ***

Obtained in the different cell lines with their respective negative controls after treatment by 48 hours with the compound (5), obtained by the chi-square test (X2). Significance level 0.05 * Values significant ** highly significant values

7 CONCLUSÕES

Os derivados das 5- (benzileno- tiazolidina-2,4-diona) analisados são citotóxicos para as linhagens de células tumorais: cólon (HT-29), pulmão (NCIH-292), mama (MCF-7) e leucemia (HL-60). O composto 5 - (2-bromo – 5 - methoxybenzylidene) - thiazolidine-2, 4-dione mostrou resultados condizentes com processo de morte celular por apoptose nos estudos de mecanismos de ação para as células NCI-H292 de câncer de pulmão.

A citotoxicidade do derivado 5 - (2-bromo – 5 - methoxybenzylidene) - thiazolidine-2, 4-dione foi mais evidente contra as células de câncer de pulmão NCI- H292.

A ação citotóxica dos compostos ocorre por mecanismos mais específicos, pois não causam hemólise em eritrócitos humanos e nem foram citotóxicos quando testados nas células normais PBMC.

Os derivados das 5- (benzileno- tiazolidina-2,4-diona) analisados possuem potentes atividades antimicrobianas frente a cepas de bactérias Gram-positivas.

O composto 5 - (2-bromo – 5 - methoxybenzylidene) - thiazolidine-2, 4-dione apresentou genotoxicidade apenas para as células tumorais revelando uma ação mais específica deste composto pois não foi genotóxico em células normais PBMC.

REFERÊNCIAS

AFZAAL, S.; WAHAB, A. Thiazolidinediones: Beyond Glycemic Control. Medicine Update, v. 58, p. 277-280, 2005.

ALBERTINE, R. J.; ANDERSON, D.; DOUGLAS, G. R. IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. Mutat. Res., v. 463, p. 111-172, 2000.

ALBUQUERQUE, J. F.; ALBUQUERQUE, A.; AZEVEDO, C. C.; THOMASSON, F.; GALDINO, S. L.; CHANTEGREL, J.; CATANHO, M. T. J.; PITTA, I. R.; LUU-DUC, C. Substituted thiazolidinediones and thio-imidazolidinones: synthesis, structural study and pharmacological activity. Pharmazie, v. 50, p. 387–389, 1995.

ALEGAON, S. G.; ALAGAWADI, K. R. New thiazolidine-2,4-diones as antimicrobial and cytotoxic agent. Med Chem Res., v. 21, p. 3214–23, 2012.

ALMEIDA, V. L.; LEITÃO, A.; REINA, L. D. C. B.; MONTANARI, C. A. DONNIC, C. L.; Câncer e agentes antineoplásicos ciclo celular específicos e ciclo celular não específico que interagem com o DNA: uma introdução. Quim. Nova, v. 28, 118-129. 2005.

AMIN, K. M.; RAHMAN, A. D. E.; AL-ERYANI, Y. A. Synthesis and preliminary evaluation of some substituted coumarins as anticonvulsant agents. Bioorg. Med. Chem., v. 16, p. 5377- 88, 2008.

AQUINO, I. Efeito genotóxico da artemisinina e do artesunato em células de mamíferos. 81 f. 2010. Dissertação (Mestrado) - Curso de Biologia Geral e Aplicada, Departamento de Biomoléculas, Universidade Estadual Paulista Julio Mesquita Filho, Botucatu: São Paulo, 2010.

BERNARD, W.; STEWART; CHRISTOPHER P. WILD. Word Cancer Research 2014, Internacional Agency for Research on Câncer-World Câncer Research.

BERNARDI, A.; JACQUES-SILVA, M. C.; LENZ, G. Abordagem molecular no desenvolvimento de fármacos antitumorais. Infarma, v. 15, n. 9-10, p. 61-63, 2003.

BLANQUICETT, C.; ROMAN, J.; HART, C. M. Thiazolidinediones as anti-cancer agentes Cancer Ther. v. 6, p. 25–34, 2008.

BONINI, A. L.; MOURA, L. A. R.; FRANCO, M.; Revisão: Apoptose em Glomerulopatias J Bras Nefrol; v. 22(2), p.70-7, 2000.

BRUSICK, D.J. Principles of genétic toxicology. 2ed. New York: Plenum Press. 284p. 1987.

BOZDAĞ-DÜNDAR, O.; OZEN OZGEN, A. M.; NURTEN, A.; ONUR, A.; ENGINE, K.; RAHIMIYE, E. Synthesis and antimicrobial activity of some new thiazolyl thiazolidine-2,4-dione derivatives. Eur. J. Med. Chem., v. 15, p. 6012-6017, 2007.

BRUNO, G.; COSTANTINO, L.; CURINGA, C.; MACCARI, R.; MONFORTE, F.; NICOLÓ, R.; OTTANÀ, M.; VIGORITA, M. G. Synthesis and aldose reductase inhibitory activity of 5-arylidene-2,4-thiazolidinediones. Bioorg. Med. Chem., v. 10, p. 1077-1084, 2002.

COTTER, T. G. Apoptosis and cancer: the genesis of a research field. Nature Reviews Cancer, v. 9; p. 501-507, 2009.

COSTA, A. I. S.; CHAVES, M. D. Pain in cancer patients under chemotherapy. Rev Dor, v. 13, p. 45-9, 2013.

COUTO, J. A. Novos Compostos 5-(4-metil-sulfonil-benzilideno)-imidazolidínicos e tiazolidínicos: Síntese, Elucidação Estrutural e Atividade Antiinflamatória. 2006. (Mestrado em Biotecnologia de Produtos Bioativos) Departamento de Antibióticos, Universidade Federal de Pernambuco. Recife. Brasil.

DEGTEREV, A.; LUGOVSKOY, A.; CARDONE, M.; MULLEY, B.; WAGNER, G; MITCHISON, T.; YUAN, J. Identification of small- molecule inhibitors of interaction between the BH3 domain and Bcl-xL. Nature Cell Biology, v. 3, p. 173-182, 2001.

DIVYA, G. S.; MANSOOR, K. P.; SHEBINA, P. R.; KUMA, A.; PPAR GAMMA AGONIST: An effective strategy for cancer treatment. Journal of Pharmaceutical e Inovação Científica www.jpsionline.com Artigo de revisão, v. 2, p. 1-3, 2013.

DUARTE, R. L. M.; PASCHOAL, M. E. M. Marcadores moleculares no câncer de pulmão: papel prognóstico e sua relação com o tabagismo. J. bras. Pneumol. v. 32, n. 1, p. 56-65, 2006.

FAIRBAIRN, D. W.; OLIVE, P. J.; O'NELL, K. L. The comet assay: a comprehensive. Mutation Research, v. 339, p. 37-59, 1995.

FENECH, M. The in vitro micronúcleos techinique. Mutat. Res., v. 455, p. 81-95, 2000.

FENECH, M. Cytokinesis-block micronucleus assay evolves into a "cytome" assay of chromosomal instability, mitotic dysfunction and cell death. Mutation Research, v. 600, p.58-66, 2006.

FENECH, M. Cytokinesis-block micronucleus cytome assay. Nat. Protocols, v. 2, p. 1084 –1104, 2007.

FERREIRA-MACHADO, S. C., RODRIGUES, M. P., NUNES, A. P. M., DANTAS, F. J. S., MATTOS, J. C. P., SILVA, C. R., MOURA, E. G., BEZERRA, R. J. A. C., CALDEIRA-ARAÚJO, A. Genotoxic potentiality of aqueous extract prepared from *Chrysobalanus icaco* L. leaves. Toxicol. Lett. 151, 481-487, 2004.

FOSTER, I. Cancer: A cell cycle defect. Radiography, v.14, p.144-149, 2008.

GOMES, M., B.; Glitazonas e Resistência Insulínica, Arq Bras Endocrinol Metab, v. 50 nº 2, p. 271-280, 2006.

GONZALES, L.; SANDERSON, B.J.S.; VOLDERS, M.K. Adaptations of the *in vitro* MN assay for the genotoxicity assessment of nanomaterials. Mutagenesis, v. 26, n. 1, p. 185-191, 2011.

GERAN, R. I., GREENBERG N. H., MCDONALD, M.M., SCHUMACHER, A.N., ABBOT, B. J. Protocols for screening clinical agents and natural products against animal tumors and other biological systems, 1972.

GOUVEIA, F. L., OLIVEIRA, R. M. B., OLIVEIRA, T. B., SILVA, I. M., NASCIMENTO, S. C., SENA, K. X. F. R., ALBUQUERQUE, J. F. C. Synthesis, antimicrobial and cytotoxic activities of some 5- arylidene-4-thioxo-thiazolidine-2-ones. European Journal of Medicinal Chemistry, v. 44, n. 5, p. 2038–2043, 2009.

GUZMÁN-RODRÍGUEZ, J. J.; OCHOA-ZARZOSA, A.; LÓPEZ-GÓMEZ, R.; LÓPEZ-MEZA, J. E. Plant Antimicrobial Peptides as Potential Anticancer Agents. BioMed Research International, v. 2015, article ID 735087, doi:10.1155/2015/735087, 2015.

GRIVICICH, I.; REGNER, A.; ROCHA, A. B. Morte Celular por Apoptose. Revista Brasileira de Cancerologia, v. 53, n. 3, p. 335-343, 2007.

HANAHAN, D.; WEINBERG, R. A. The halmarks of cancer. Cell, v. 100, p. 57-70, 2000.

HANAHAN, D.; WEINBERG, R. A. Hallmarks of cancer: The Next Genration, Cell, v. 144, p. 646- 674, 2011.

HARRINGTON, K. J. Biology of Cancer. Medicine, v. 36, n.1, p.1-4, 2007.

HARTMANN, A.; AGURELL, E.; BEEVERS, C.; BRENDLER-SCHWAAD, S.; BURLINSON, B.; CLAY, P.; COLLINS, A.R.; SMITH, A., SPEIT, G.; THYBAUD, V.; TICE, R. R. Recommendations for conducting the *in vivo* alkaline comet assay. Mutagenesis, v. 18, p. 45-51, 2003.

HEDDLE, J.A.; HITE, M.; KIRKHART, B.; MAVOURNIN, K.; MACGREGOR, J.T.; HOUTGRAAF, J. H., VERSMISSEN, J., VAN DER GIESSEN, W. J. A concise review of DNA damage checkpoints and repair in mammalian cells. Cardiovasc. Revasc. Med., v. 7, p. 165-172, 2006.

INSTITUTO NACIONAL DO CÂNCER (Brasil). Estimativas 2012: incidência de câncer no Brasil. Disponível em <http://www.inca.gov.br> acessado em 24/09/2012.

INSTITUTO NACIONAL DO CÂNCER (Brasil). Estimativas 2014/2015: Incidência de câncer no Brasil. Disponível em <http://www.inca.gov.br> acessado em 14/10/2015.

ISLAMI, F.; TORRE, L. A.; JEMAL, A. Global trends of lung cancer mortality and smoking prevalence, Transl Lung Cancer Res., v. 4, p. 327-38, 2015.

JAIN, V. S.; VORA, D. K.; RAMAA, C. S. Thiazolidine-2, 4-diones: Progress towards multifarious applications. Bioorganic & Medicinal Chemistry v. 21, p. 1599–1620, 2013.

JUNG, K. Y.; SAMADANI, R.; CHAUHAN, J.; NEVELS, K.; YAP, J. L.; ZHANG, J. et al. Structural Modifications of (*Z*)-3-(2-aminoethyl)-5-(4-ethoxybenzylidene) thiazolidine-2,4-dione that Improve Selectivity for the Inhibition of Melanoma Cells Containing Active ERK Signaling. Org Biomol Chem. v. 11(22), p. 3706–3732, 2013.

KALAITZIDIS, R. G.; SARAFIDIS, P. A.; BAKRIS, G. L. Effects of Thiazolidinediones Beyond Glycaemic Control. Current Pharmaceutical Design, v. 15, n. 5, p. 529-536, 2009.

KERR, J. F. R.; WYLLIE, A.H.; CURRIE, A. R.; Apoptosis: a basic biological phenomenon with wideranging implications in tissue kinetics Br. J. Cancer v.26, p. 239,1972.

KIRSCH-VOLDERS, M.; VANHAUWAERT, A.; EICHENLAUB-RITTER, U.; DECODIER, I. Indirect mechanisms of genotoxicity. Toxicol. Lett. 140-141: 63-74, 2003.

KROEMER, G.; REED, J.C. Mitochondrial control of cel death. Nat. med. v. 6: 513-519. 2000.

KROEMER, G.; GALLUZZI, L.; VANDENABEELE, P.; ABRAMS, J.; ALNEMRI, ES.; BAEHRECKE, EH, et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death, Cell Death Differ v. 16 (1), p. 3–11, 2009.

LEITE, F. H. A.; DA SANTIAGO, P. B. G.; FROES, T. Q.; DA SILVA FILHO, J.; DA SILVA, S. G.; XIMENES, R. M.; DE FARIA, A. R.; BRONDANI, D. J.; DE ALBUQUERQUE, J. F. C.; CASTILHO, M. S. Structure-guided discovery of thiazolidine-2,4-dione derivatives as a novel class of Leishmania major pteridine reductase 1 inhibitors. Eur J Med Chem., v. 123, p. 639–648, 2016.

LIEBERMAN, S.; DOBRINER, K.; HILLS, B. R.; FIESER, L. F.; RHOADS, C. P. Studies in Steroid Metabolism II. Identljication and Characterization of Ketosteroids Isolated From Urine of Healthy and Diseased Persons. J of Bio Chem., v. 172, p. 263-295, 1948.

MAGGS, D. G.; BUCHANAN, T. A.; BURANT, C. F.; CLINE, G.; GUMBINER, B.; HSUEH, W. A. Metabolic effects of troglitazone monotherapy in type 2 diabetes

mellitus. A randomized, double blind, placebo-controlled trial. Ann Intern Med v.128, p.176-85, 1998.

MANTOVANI, A. Inflaming metastasis. Nature, v. 457, p. 36-37, 2009.

MARZIN, D. The position of the in vitro micronucleus test within the battery of screening for genotoxic potential determination and regulatory guidelines. Elsevier: Mutation Research, Lille, p. 175-181, 1997.

MOHANTY, S.; REDDY, G. S.; & KARMAKAR, A.C. Synthesis of New 5-Substituted – Aminomethylene-Thiazolidine-2, 4-dione derivatives as potential antibacterial agents journal of applicable chemistry v. 3, p. 82-90, 2014.

MOTA, T.C.; CARDOSO, P.C.; GOMES, L.M.; VIEIRA, P.C.; CORREA, R.M.; SANTANA, P.D.; MIRANDA, M.S.; BURBANO, R.M.; BAHIA, M.O. In vitro evaluation of the genotoxic and cytotoxic effects of artesunate, an antimalarial drug, in human lymphocytes. Environ Mol Mutagen. v.52, p. 590-4, 2011.

NEWELL, G.W. The induction of micronuclei as a measure of genotoxicity: A report of the U.S. environmental protection agency Gene-Tox program. Mutation Research/Reviews in Genetic Toxicology, v. 123, p. 161–118, 1983.

PANCHAGNULA, R.; THOMAS, N. S. Biopharmaceutics and pharmacokinetics in drug research. International Journal of Pharmaceutics, v. 201, p. 131-150, 2000.

PANIKAR, V.; CHANDALIA, H.B.; JOSHI, S.; FAFADIA, A.; SANTVAN, C.; Beneficial Effects of Triple Drug Combination of Pioglitazone with Glibenclamide and Metformin in Type 2 Diabetes Mellitus Patients on Insulin Therapy. Japi, v. 51, p. 1061-1064, 2003.

PATIL, V.; TILEKAR, K.; MEHENDALE-MUNJ, S.; MOHAN, R.; RAMAA, C. S. Synthesis and primary cytotoxicity evaluation of new 5-benzylidene-2, 4-thiazolidinedione derivatives. European Journal of Medicinal Chemistry, v.45, n. 10, p. 4539-4544, 2010.

PEREIRA, A. C.; CARMO, E. D.; SILVEIRA, V. A. S.; AMADEI, S. U.; ROSA, L. E. B. O Papel das MMP-2 e -9 no desenvolvimento do carcinoma epidermoide. Revista Brasileira de Oncologia v. 3 p. 257-262 2006.

PIKKEMAAT, M. G.; LINSSEN, A. B. M.; BERENDSEN, H. J. C.; JANSSEN, D. B. Molecular dynamics simulations as a tool for improving protein stability. Prot Eng., v.15, p. 185–192, 2002.

RAMIREZ, A.; SALDANHA, P. H. Micronucleus Investigation of Alcoholic Patients with Oral Carcinomas. Genet. Mol. Res., v. 1, p. 246-260, 2002.

RIBEIRO, L.R.; SALVADOR, D. M. F.; MARQUES, E. C. Mutagênese Ambiental. Ed. Ulbra, p. 345, 2003.

REANMONGKOL, W.; SONGKRAM, C. In vivo anti-inflammatory, analgesic and antipyretic activities of novel thiazolidine-2, 4-dione analogs derived from some classical NSAIDs. Songklanakarin J. Sci. Technol., v. 35, p. 33- 40, 2013.

RIBEIRO, L. R.; SALVADORI, D. M. F.; MARQUES, E. K. Mutagênese Ambiental. Editora da ULBRA, Canoas, 356p, 2003.

RICCI, M.S; ZONG, W.X. Chemotherapeutic for targerting cell death pathways. The Oncologist., v. 11, p. 342-357, 2006.

ROBERTS, P. J.; DER, C. J. Review: Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer Oncogene. Nature Publishing Group. v. 26 p. 3291–3310, 2007.
ROMAGNOLI, R., BARALDI, P. G., SALVADOR, M. K. (2013). Anticancer activity of novel hybrid molecules containing 5-benzylidene thiazolidine-2,4-dione. Eur J Med Chem**.,** v. 63, p. 544-57, 2013.

SANTOS, NQ. A resistência bacteriana no contexto da infecção hospitalar. Texto contexto - enferm. Florianópolis, v. 13, n. spe, 2004.

SCHMID, W. The Micronucleus Test. Mutat. Res., v. 31, p. 9-15, 1975.

SEIXAS, R. J.; KESSLER, A.; FRISON, V. B. Atividade Física e Qualidade de Vida em Pacientes Oncológicos durante o período de tratamento Quimioterápico. Revista Brasileira de Cancerologia. v. 56 p. 322, 2010.

SILVA, I. M.; SILVA FILHO, J.; SANTIAGO, P.B.G. S.; EGITO, M. S.; Souza, C. A.; Gouveia, F.L.; XIMENES, R. M. et al. Synthesis and Antimicrobial Activities of 5-Arylidene-thiazolidine-2,4-dione Derivatives. Biomed Research International Hindawi Publishing Corporation, p. 1-8, 2014.

SINGH, N.P.; MCCOY, M.T.; SCHNEIDER, E.L. A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Reseach, v. 175, n. 1, p. 184–191, 1988.

SPELLBERG, B.; GUIDOS, R.; GILBERT, D.; BRADLEY, J.; BOUCHER, H. W.; SCHELD, W. M.; BARTLETT, J. G.; EDWARDS, J. Jr.; INFECTIOUS DISEASES SOCIETY OF AMERICA. The epidemic of antibiotic-resistant infections: a call to action for the medical community from the Infectious Diseases Society of America. Clinical Infectious Diseases, v. 46, n. 2, p. 155-164, 2008.

STEWART, B.W.; WILD, C.P. World cancer report 2014, International Agency for Research on Cancer. Distributed by World Health Organization.

TERRADAS, M.; MARTIN, M.; TUSELL, L.; GENESCA, A. Genetic activities in micronuclei: Is the DNA entrapped in micronuclei lost for the cell? Mutation Research, v. 705, p. 60-67, 2010.

TICE, R.R., AGUERRÍ, E., ANDERSON, D., BURLINSON, B., HARTMANN, A., KOBAYASHI, H., MIYAMAE, Y., ROJAS, E., RYU, J.C., SASAKI, Y. Single cell gel/comet assay: guidelines for in Vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen., v. 35, p. 206-221, 2000.

TOLBERT, P. E.; SHY, C. M.; ALLEN, J. W. Micronuclei and Other Nuclear Anomalies in Buccal Smears: Methods development. Mutat. Res., v. 271, p. 69-77, 1992.

TOMASIC, T.; ZIDAR, N.; MUELLER-PREMRU, M.; KIKELJ, D.; MASIC, L. P. Synthesis and antibacterial activity of 5-ylidenethiazolidin-4-ones and 5-benzylidene-4, 6-pyrimidinediones. European Journal of Medicinal Chemistry, v. 45, p. 1667–1672, 2010.

TONETO, M.G.; SEHN, A.A.; LISOT, B.C. History of gastric cancer treatment: the pioneers, early misconceptions and the current guidelines. Scientia Medica, v. 22, n. 4, p. 216-223, 2012.

VAN LOO, G.; DEMOL, H.; VAN GURP, M.; HOORELBEKE, B.; SCHOTTE, P.; BEYAERT, R. et al. A matrix-assisted laser desorption ionization post-source decay (MALDI-PSD) analysis of proteins released from isolated liver mitochondria treated with recombinant truncated Bid. Cell Death Diff., v. 9, p. 301-308, 2002.

VENUGOPAL, D. V. R.; YARLA, N. S.; UMADEVI, P. Synthesis, of Novel Piperine Analogs of Dipeptidyl Boronic Acid as Antimicrobial and Anticancer Agents, Medicinal Chemistry, n. 4, p. 606-610, 2014.

VERMA, A.; SARAF, S. K.; 4-Thiazolidinone-A biologically active scaffold. European Journal of Medicinal Chemistry, v. 43, p. 897-905, 2008.

VICINI, P.; GERONIKAKI, A.; ANASTASIA, K.; INCERTI, M.; ZANI, F. (2006). Synthesis and antimicrobial activity of novel 2-thiazolylimino-5-arylidene-4-thiazolidinones. Bioorg. Med. Chem., v. 14, p. 3859–3864.

WOJEWÓDZKA, M.; GRÀDZKA, BURACZEWSKA, I. Modified neutral comet assay for human lymphocytes. Nukleonika, v. 47, p. 1-5, 2002.

ANEXO A

NORMAS PARA SUBMISSÃO - ARTIGO 1- Author Guidelines - PHARMACOLOGICAL REPORTS

PHARMACOLOGICAL REPORTS

AUTHOR INFORMATION PACK

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