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Débora Corrêa Espiña

Investigação do Efeito Antitumoral de Novos Compostos Pirimidobenzimidazóis em Câncer de Mama: uma Abordagem *in vitro*

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Orientadora: Dr[a] Jenifer Saffi

Co-orientadora: Dr[a] Cassiana Macagnan Viau

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RESUMO

A quimioterapia convencional é o principal tratamento para o câncer e beneficia os pacientes na forma de diminuição da reincidência e metástase, além de proporcionar uma maior sobrevida. No entanto, os alvos das drogas quimioterápicas não são inteiramente precisos, o que resulta em alguns efeitos secundários e menor eficiência do tratamento, podendo gerar resistência, recidiva e metástase. No presente estudo, avaliaram-se os efeitos citotóxicos e o mecanismo molecular de uma série de 3-trifluoro(oxo)pyrimido[1,2-*a*] derivados benzimidazóis (PBZ 1-9) em diferentes linhagens de células de câncer humano. A potencial atividade antitumoral dos compostos foi estudada em linhagens de células humanas de câncer de mama (MCF-7), fígado (HepG-2), bexiga (T-24) e colorretal (HCT-116, HT-29 e Caco-2), além da linhagem não tumoral HEK-293, derivada de células embrionárias de rim. Após o tratamento, a citotoxicidade foi expressa em valores de IC50 e o índice de seletividade (IS) foi calculado através da razão IC50 da HEK-293/IC50 das células tumorais. Os compostos que demostraram atividades citotóxicas mais relevantes foram submetidos aos demais ensaios biológicos e moleculares. A genotoxicidade foi analisada por eletroforese em gel de única célula (ensaio cometa). A produção de espécies reativas de oxigênio (ERO) e as alterações no potencial de membrana mitocondrial ($\Delta\Psi m$) foram estudados utilizando sondas específicas de fluorescência, DCFH(2)-DA (2',7'-diclorodiidrofluoresceinadiacetato) e rodamina-123 por citometria de fluxo. O ciclo celular foi determinado por citometria de fluxo e as alterações na expressão da proteína γ-H2AX (marcador de dano de DNA) foram examinados por Western Blot. A inibição da enzima topoisomerase I humana pelos compostos foi avaliada utilizando-se o Kit comercial TopoGEN®. Os nossos resultados sugerem que os compostos PBZ 4, 5 e 9 exerceram a maior citotoxicidade nas células MCF-7, com valores de IC50 de 15,41, 19,52 e 8,11μM, respectivamente. O composto mais promissor foi o PBZ 9. A análise de citometria de fluxo indica que esta molécula altera a progressão do ciclo celular, induz a parada em G1 e causa DNA poliplóide (> 4N). O PBZ 9 interage com a enzima Topo I, gera a produção ERO, indução de danos no DNA e fosforilação de γ-H2AX. O pré-tratamento com N-acetilcisteína (NAC) reverte a produção de ERO observada.

Os nossos resultados sugerem que os efeitos encontrados podem ser consequência da interação do PBZ 9 com a enzima Topo I (ação direta) e pela geração de ERO (ação indireta). Desta forma, o composto PBZ 9 demonstra ser bastante promissor para a continuidade de estudos que comprovem seu potencial uso para terapia do câncer de mama.

Palavras-chave: Trifluoro(oxo)pyrimido[1,2-*a*] derivados benzimidazóis, atividade citotóxica, morte celular, espécies reativas de oxigênio, terapia antitumoral.

ABSTRACT

Conventional chemotherapy is the main treatment for cancer and benefits patients in the form of decreased relapse and metastasis and longer overall survival. However, as the target therapy drugs is not wholly precise, it also results in quite a few side effects, and is less efficient in many cancer cells, which are considered the reason for chemotherapy resistance, relapse, and metastasis. In the present study, we evaluated the cytotoxic effects and the molecular mechanism of a series of substituted 3-trifluoro(oxo)pyrimido[1,2-*a*] benzimidazoles derivatives (PBZ 1-9) in different human cancer cell lines**.** The potential antitumoral activity of the compounds was evaluated in the breast cancer cell lines (MCF-7), liver (HepG-2), bladder (T-24), colorectal (HCT-116, HT-29, Caco-2), as well as in the nontumoral human embrionic kidney cells HEK-293. After the treatment the cytotoxicity was expressed as IC50 values. Selective index (SI) was calculated by the ratio IC50 of HEK-293 cells/IC50 of tumoral cells. Compounds showing cytotoxic activities were subjected to further analysis. Genotoxicity induction was analyzed by single-cell gel electrophoresis (comet assay). The production of reactive oxygen species (ROS) and changes in mitochondrial membrane potential ($\Delta\Psi m$) were studied using specific fluorescence probes, DCFH(2)-DA (2',7'-dichlorodihydrofluorescein diacetate) and rhodamine-123 by flow cytometry. The cell cycle was determined by flow cytometry and changes in protein expression (γ-H2AX) were examined by western blotting. The compounds interaction with Topo I was evaluated in a cell-free system (TopoGEN® kit). Our results suggest that PBZ 4, 5 and 9 exerted the highest cytotoxicity against MCF-7 cells, with IC50 values of 15.41, 19.52 and 8.11 μM, respectively. The most promising compound was the PBZ 9 in MCF-7 cells. Flow cytometric analysis indicates that PBZ 9 alters cell cycle progression, induces G1 arrest and causes DNA polyploidy (>4N). PBZ 9 interacts with Topo I, generates ROS and γ-H2AX phosphorylation. The pretreatment with N-acetyl cysteine (NAC) abrogates the observed ROS production. Our results demonstrate that these effects might be consequence to the putative interaction of PBZ 9 with Topo I enzyme (direct action) and ROS generation (indirect action). Therefore, PBZ 9 seems to be an interesting molecule to be further investigated in breast cancer therapy.

Keywords: Trifluoro(oxo)pyrimido[1,2-a] benzimidazoles derivatives, **cytotoxic** activity, cell death, reactive oxigen species, antitumoral therapy.

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1 INTRODUÇÃO

O câncer é um dos maiores problemas de saúde pública e uma das principais causas de morte no mundo. Segundo a Organização Mundial de Saúde (OMS), em 2012 houve cerca de 8,2 milhões de mortes decorrentes desta doença (FERLAY et al., 2015).

Conhecido por ser uma doença caracterizada pelo aumento descontrolado de células em um determinado tecido, o câncer se desenvolve a partir do surgimento de uma única célula anormal, que perdeu o seu mecanismo de regulação do crescimento e da multiplicação celular, geralmente, devido a danos no DNA (HANAHAN & WEINBERG, 2011).

Trata-se de uma doença complexa que envolve fatores moleculares, celulares e teciduais, além de presumir uma iniciação mutagênica com potencial para invadir tecidos adjacentes ou distantes do seu local de origem (CARRASSA & DAMIA, 2011). A etiologia múltipla da doença indica que os cânceres podem se desenvolver devido a fatores exógenos ou endógenos, ou ambos. De acordo com a OMS, cerca de 30% das mortes por câncer são devidos aos cinco principais fatores de riscos comportamentais e alimentares: (1) alto índice de massa corporal, (2) baixa ingestão de frutas e legumes, (3) falta de atividade física, (4) tabagismo e (5) uso de álcool (WHO, 2014).

As principais abordagens terapêuticas para o tratamento e/ou completa remoção do câncer são a cirurgia, radioterapia e quimioterapia. Entretanto, os efeitos adversos resultantes desses tratamentos são muitos, além da total ineficácia em alguns casos (HEROLD & MARCOM, 2008).

Durante as últimas décadas, o aumento do conhecimento dos mecanismos moleculares envolvidos no câncer serve de base para a procura de novas estratégias terapêuticas mais eficientes (LAURIA et al., 2010). Sendo assim, na busca por novas moléculas que apresentem mecanismos de ação inéditos, os compostos de síntese química têm sido incluídos como fonte potencial para o desenvolvimento de novos medicamentos (SIDHU et al., 2015). Tais moléculas proporcionam uma rica fonte de alvos que possam ser específicos para a inibição do crescimento de células cancerosas, abrindo oportunidades para encontrar novos agentes anticâncer com melhores características de ação (MARIN et al., 2010).

Dentre os compostos químicos que possuem atividades antitumorais, há os derivados benzimidazóis, com estrutura semelhante a purina, molécula que faz parte da estrutura do DNA e RNA. Estes compostos apresentam interessantes propriedades, despertando grande interesse na medicina (NOFAL et al., 2011; KAMAL et al., 2012). Porém, ainda é necessário um entendimento mais detalhado acerca do mecanismo de ação destes compostos, a fim de serem eficientemente administrados ao paciente como um agente único ou em combinação com outros fármacos (REDMOND et al., 2008).

Tendo em vista os altos índices de óbitos causados por essa doença e, também as resistências que os tumores vêm apresentando frente ao tratamento quimioterápico, este trabalho buscou avaliar o potencial citotóxico e genotóxico de novos compostos derivados pirimídicos, em diferentes linhagens tumorais.

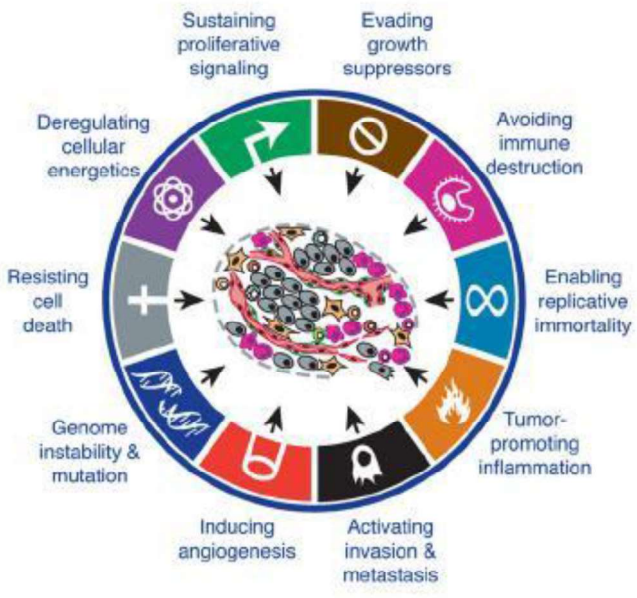
2 REFERENCIAL TEÓRICO

2.1 Aspectos Gerais do Câncer

O câncer é uma doença multifatorial, de etiologia ainda desconhecida, porém com fatores de risco significativos para o seu desenvolvimento, como as alterações genéticas, o desequilíbrio hormonal e a influência de fatores ambientais. Além disso, sabe-se que o câncer se origina de uma única célula mutada que se multiplicou e suas descendentes acumularam essas alterações genotípicas ao longo do tempo, dando origem à célula cancerosa (AMERICAN CANCER SOCIETY; HANAHAN & WEINBERG, 2011).

De acordo com Hanahan & Weinberg (2011), para o desenvolvimento da doença é necessária uma série de alterações fisiológicas que causem o desequilíbrio da homeostase celular. Estas alterações podem ser: perda da sinalização da proliferação celular; insensibilidade a sinais supressores de crescimento; habilidade de invasão e metástase; resistência celular; indução da angiogênese; potencial replicativo ilimitado; instabilidade genômica e mutação; inflamação; e mais recentemente: o papel do sistema imune na progressão do tumor; e a reprogramação da energia celular (Figura 1).

Figura 1. Principais características moleculares para o desenvolvimento do câncer.



Fonte: Hanahan & Weinberg, 2011.

O processo de carcinogênese geralmente é lento, podendo levar alguns anos até que o tumor se desenvolva e seja visível. Didaticamente, esse processo pode ser dividido em três estágios: iniciação, promoção e progressão tumoral. O estágio de iniciação é a etapa em que ocorre o dano no DNA e se acumulam as lesões genotóxicas. O estágio de promoção é quando acontece a transformação da célula normal em cancerosa. E por fim, o estágio de progressão é quando o microambiente tumoral começa a se estabelecer e aparecem as primeiras manifestações clínicas da doença (COUSSENS & WERB, 2002).

Mutações espontâneas são frequentes no DNA, entretanto, não significa que alteram o crescimento normal das células, mas podem gerar alguns fenômenos como danos oxidativos e erros na ação de enzimas. Contudo, nas células cancerosas, a característica principal é o rápido crescimento da população celular, evidenciando a perda dos mecanismos de multiplicação celular (JELONEK et al., 2010).

Várias alterações genéticas são geralmente necessárias para o desenvolvimento do tumor. Os genes que participam na formação de tumores, são os que nas células normais estão envolvidos nos processos de controle do ciclo celular, reparo de DNA e apoptose (WANG et al., 2015).

Normalmente, os proto-oncogenes ativam a proliferação celular no embrião e no feto, além de desempenharem um papel importante na regeneração de tecidos destruídos. Quando sofrem mutação, tornam-se oncogenes que codificam proteínas que alteram o controle do ciclo mitótico, gerando uma divisão desordenada. O mais frequente é o oncogene *ras*, que envia sinais constantes para a célula iniciar o processo de mitose (STAMATAKOS et al., 2010).

Mutações nos genes supressores de tumor (p53, BRCA1 e 2, APC, p16 e RB) estão relacionados com a maioria dos tumores malignos. Cerca de 50% dos tumores malignos diagnosticados apresentaram mutação ou deleção no gene p53 evidenciando a importância deste gene no ciclo celular normal (SIEGEL et al., 2014).

O ciclo celular normal apresenta alguns mecanismos de reparo de DNA, bloqueando a propagação da informação genética incorreta às células filhas. Apesar de eficiente, estes mecanismos são passíveis de erros, permitindo que a mutação seja transmitida às células filhas ou permaneça latente na célula alterada (MERKLE, 2007).

O acúmulo dessas alterações pode ocorrer em um ou mais genes e assim, provocar a instabilidade genômica, o que determina a magnilidade do tumor e sua capacidade de apresentar metástases (JELONEK et al., 2010).

As interações moleculares e celulares do microambiente tumoral demonstram a complexidade do câncer no que tange à sua origem. Sabe-se que fatores externos também têm grande influência no desenvolvimento da doença. O aumento no número de casos de câncer está diretamente relacionado às mudanças nos hábitos nutricionais e alterações hormonais (BRAY et al., 2012).

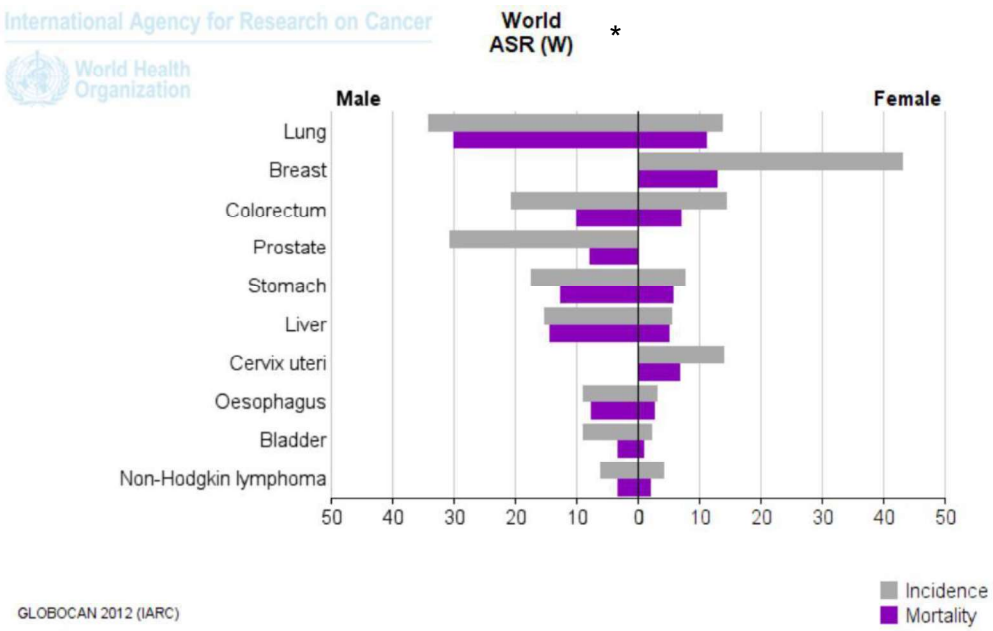
Diversos estudos epidemiológicos relacionam o desenvolvimento de câncer e a presença de recidivas após o tratamento com o uso de álcool, tabaco, excesso de sal e carne vermelha, dietas pobres em vegetais, falta de exercícios e uso de hormônios exógenos (SIEGEL et al., 2014).

2.2 Epidemiologia do Câncer

De acordo com a Organização Mundial de Saúde (OMS), no ano de 2012, os três tipos de câncer que mais causaram mortes foram o câncer de pulmão (19,4%), o câncer de fígado (9,1%) e o câncer de estômago (8,8%), respectivamente (FERLAY et al., 2015).

A estimativa da taxa de incidência de câncer da Agência Internacional de Pesquisa em Câncer (International Agency for Research on Cancer - IARC), em 2012 foi de 14,1 milhões de novos casos e os cânceres mais comumente diagnosticados foram de pulmão (13%), de mama (11,9%) e colorretal (9,7%), respectivamente, conforme demonstrado na Figura 2 (SIEGEL et al., 2014).

Figura 2. Estimativa das taxas de incidência e de mortalidade padronizadas por idade em ambos os sexos, no ano de 2012.



Fonte: IARC, 2012.

*Age-standardised rate (W): é a taxa do número de novos casos ou óbitos por 100.000 pessoas por ano. Uma taxa padronizada por idade é a taxa que a população teria se tivesse uma estrutura etária padrão. A padronização é necessária quando se comparam várias populações que diferem em relação à idade, porque a idade tem uma forte influência sobre o risco de câncer.

Países de baixa e média renda são os que apresentam o maior número de mortes e incidência, cerca de 65% (5,3 milhões) e 57% (8 milhões), respectivamente (IARC, 2012). Problemas no sistema público de saúde relacionados ao diagnóstico precoce, o custo elevado dos tratamentos, crescimento e envelhecimento da população, além do aumento da prevalência dos fatores de risco conhecidos são implicações causadas pelas diferenças socioeconômicas das regiões (PISANI, 2011; BRAY et al., 2012; TORRE et al., 2015).

Em contrapartida, os países mais ricos apresentam uma maior taxa de prevalência de câncer, em torno de 16,9 milhões de casos (52%) (IARC, 2012). As transformações que os padrões de saúde-doença sofreram nas últimas décadas se deve ao crescimento da economia mundial que desencadeou uma mudança nas condições de trabalho, nutrição e consumo (KUSHI et al., 2012; TORRE et al., 2015).

Conhecida como transição epidemiológica, as transformações nos padrões de saúde-doença em cada região acompanham as variações no perfil demográfico de cada país (SANTOSA et al., 2014). Países em processos de urbanização e de industrialização, com maiores avanços nas áreas da ciência e tecnologia tendem a

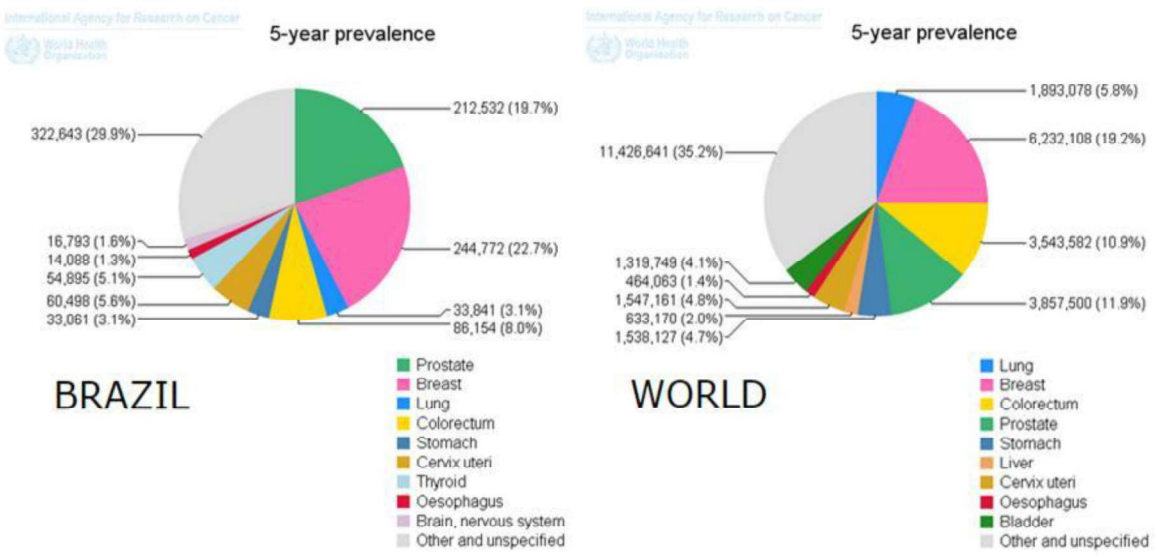
apresentar uma maior exposição a fatores de risco, bem como baixas taxas de natalidade e mortalidade (OMRAN, 2005).

O processo de transição epidemiológica fica ainda mais evidente no que diz respeito à morbimortalidade da população mundial, onde a ocorrência de doenças infectocontagiosas está diminuindo e o número de doenças crônico-degenerativas está cada vez maior (HASHIM & BOFFETTA, 2014).

Na última década, o Brasil teve um crescimento econômico alto em relação à média mundial, e consequentemente, uma mudança no estilo de vida e nos hábitos alimentares dos brasileiros. Sendo assim, a transição epidemiológica acompanhou o desenvolvimento demográfico do país, e doenças crônicas, como o câncer tiveram maior destaque e atenção dos órgãos responsáveis pela saúde pública do país (CONDE & MONTEIRO, 2014; CURADO & DE SOUZA, 2014).

Segundo a OMS (2014), os tipos de cânceres prevalentes no Brasil acompanham as estatísticas de saúde mundiais, sendo o tipo de câncer mais prevalente no país o de mama (22,7%), seguido pelo câncer de próstata (19,7%) e câncer colorretal (8%). Na escala mundial, os tipos mais prevalentes também são: o câncer de mama (19,2%), câncer de próstata (11,9%) e câncer colorretal (10,9%), respectivamente (Figura 3).

Figura 3. Estimativa das taxas de prevalência dos principais tipos de câncer no Brasil e no mundo, em ambos os sexos.



Fonte: Adapatado de GLOBOCAN, 2012.

De acordo com o Instituto Nacional do Câncer (INCA), em 2014 foram esperados cerca de 576 mil novos casos de câncer no Brasil, sendo 52% destes em homens. O câncer de próstata (22,8%) é o que mais acomete os homens; já nas mulheres, o câncer de mama (20,8%) é o mais representativo (Figura 4).

Figura 4. Distribuição proporcional dos dez tipos de câncer mais incidentes estimados para 2014 por sexo, exceto pele não melanoma*

Localização primária	casos	%			Localização primária	casos	%
Próstata	68.800	22,8%	Homens	Mulheres	Mama Feminina	57.120	20,8%
Traqueia, Brônquio e Pulmão	16.400	5,4%			Cólon e Reto	17.530	6,4%
Cólon e Reto	15.070	5,0%			Colo do Útero	15.590	5,7%
Estômago	12.870	4,3%			Traqueia, Brônquio e Pulmão	10.930	4,0%
Cavidade Oral	11.280	3,7%			Glândula Tireoide	8.050	2,9%
Esôfago	8.010	2,6%			Estômago	7.520	2,7%
Laringe	6.870	2,3%			Corpo do Útero	5.900	2,2%
Bexiga	6.750	2,2%			Ovário	5.680	2,1%
Leucemias	5.050	1,7%			Linfoma não Hodgkin	4.850	1,8%
Sistema Nervoso Central	4.960	1,6%			Leucemias	4.320	1,6%

Fonte: Estimativa 2014 – INCA.
*Números arredondados para 10 ou múltiplos de 10.

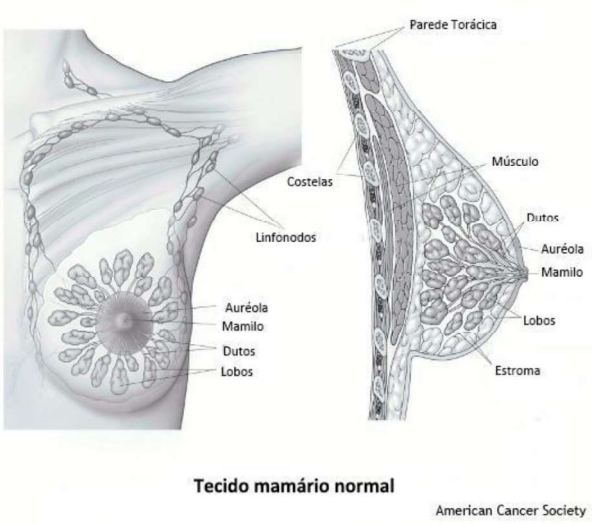
2.3 Câncer de Mama

O câncer de mama é o segundo tipo de câncer mais frequente no mundo e o primeiro entre as mulheres. No Brasil a estimativa de incidência para o ano de 2014 foi de 57.120 casos, com um risco estimado de 56,09 casos a cada 100 mil mulheres (INCA, 2014).

O risco estimado para câncer de mama no Brasil varia conforme a região: Sudeste (71,18/ 100 mil), Sul (70,98/ 100 mil), Centro-Oeste (51,30/ 100 mil) e Nordeste (36,74/ 100 mil). Na região Norte, é o segundo tumor mais incidente com 21,29 casos para cada 100 mil mulheres (INCA, 2014). Esta diferença entre regiões também se aplica em nível mundial, as maiores taxas de incidência estão na Europa Ocidental (96/ 100 mil) e as menores se encontram na África Central e Ásia Oriental (27/ 100 mil) (BRAY et al., 2015; FERLAY et al., 2015).

O tecido mamário (Figura 5) é composto basicamente por lobos (tecido glandular), por ductos e por estroma (tecido adiposo e tecido conjuntivo). Desenvolve-se sob a ação de hormônios esteróides ovarianos (progesterona e estrógeno) e fatores de crescimento durante a puberdade e ainda no período de gravidez e lactação, sendo que na menopausa o tecido glandular se atrofia (MILLS et al., 2011).

Figura 5. Tecido Mamário Normal.



Fonte: American Cancer Society.

Os mecanismos pelos quais o câncer de mama se desenvolve ainda não estão totalmente elucidados, porém alguns fatores de risco importantes estão geralmente relacionados à eventos hormonais e reprodutivos. A idade também é um dos principais fatores de risco para o desenvolvimento da doença. De acordo com a Sociedade Americana de Câncer, as taxas de incidência e mortalidade aumentam com a idade, 79% dos novos casos e 88% das mortes ocorreram em mulheres com 50 anos ou mais (DIETERICH et al., 2014; AMERICAN CANCER SOCIETY).

A predisposição genética é outro evento conhecido na etiologia do câncer de mama. Estima-se que entre 5-10% do total de casos são resultados de mutações hereditárias, incluindo mutações nos genes BRCA1, BRCA2 e p53 (SCHWARTZ et al., 2009; FASCHING et al., 2013).

São diversos os tipos e subtipos de câncer de mama. Alguns critérios são importantes para o diagnóstico como: tipo histológico, avaliação imunohistoquímica, estadiamento e o caráter invasivo do tumor. De acordo com o caráter invasivo, o câncer de mama é dividido em dois grupos: o câncer de mama *in situ* é do tipo não invasivo, ou seja, as células tumorais permanecem no seu local de origem; e o câncer de mama invasivo, que atinge além do local de origem do tumor, os tecidos adjancentes circundantes ou distantes (COCQUYT & VAN BELLE, 2005).

O diagnóstico precoce do câncer de mama influencia diretamente no tratamento e na cura. Em países desenvolvidos, a sobrevida média após cinco anos de tratamento é cerca de 85%, enquanto em países em desenvolvimento é de 60%.

A diferença provavelmente se deve ao diagnóstico precoce e acesso aos tratamentos (YOULDEN et al., 2012).

A rapidez e precisão na análise dos tumores também influenciam na diminuição das taxas de mortalidade. Conhecer o tamanho da massa tumoral, grau de diferenciação, marcação para proteínas receptoras de estrógeno (RE) e de proteínas receptoras de progesterona (RP), superexpressão de HER2, Bcl-2 ou p53, mutação em BRCA1 e BRCA2 são informações relevantes para o diagnóstico e posterior tratamento (AMERICAN CANCER SOCIETY; SOTIRIOU & PUSZTAI, 2009).

A detecção de RE e RP através de imunohistoquímica indica o prognóstico da doença e quanto maior o número de marcações, melhor o tempo de sobrevida. Por serem receptores de hormônios, a presença de RE e RP na imunohistoquímica também indica o tratamento hormonal com moduladores seletivos. Um exemplo é o tamoxifeno, que compete com o estradiol pela ligação ao RE evitando a proliferação das células tumorais em resposta ao hormônio (DROOG et al., 2013).

Outro marcador importante utilizado na imunohistoquímica é o receptor de fator de crescimento epidermal 2 (HER2). A sua superexpressão é observada em aproximadamente 30% dos tumores de mama e indica um pior prognóstico com uma resposta menor ao tratamento, além de um aumento no potencial metastático (SOTIRIOU & PUSZTAI, 2009).

Baseados nesses biomarcadores, o câncer de mama pode ser classificado em: receptores hormonais positivos, HER2 positivo (com RE e RP positivos ou negativos) ou triplo negativo (RE, RP e HER2 negativos). As marcações positivas ou negativas para as proteínas receptoras hormonais dentro da imunohistoquímica podem diagnosticar diferentes estágios da doença, ou seja, tumores RE positivos e RP negativos ou ambos positivo são diferentes biologicamente e clinicamente. Geralmente, RE+ e RP- apresentam mau prognóstico e são tumores mais agressivos em relação ao tumores RE+ e RP+ (CHIOREAN et al., 2013).

Diferenças clínicas também são observadas nos tumores hereditários. Mutações no gene BRCA1 são geralmente notadas no carcinoma ductal invasivo e triplo negativo, já o câncer de mama ligado ao BRCA2 geralmente apresenta morfologia semelhante ao câncer de mama esporádico. Estes genes são muito importantes para a estabilidade genômica, pois estão envolvidos em processos de

reparo de DNA e *checkpoint* do ciclo celular. Portanto, é compreensível que as formas mutadas destes genes estejam diretamente envolvidos na tumorigênese do câncer de mama (KITAGISHI et al., 2013; SINN et al., 2013).

2.4 Quimioterapia

De forma geral, o tratamento padrão para os casos de câncer é cirurgia, radioterapia e quimioterapia. Porém, em alguns casos, o sucesso desses tratamentos é limitado, uma vez que os tumores podem apresentar metástases ou ainda resistência aos medicamentos (KROL et al., 2010).

Existem outros tipos de abordagens terapêuticas para o câncer, como por exemplo, a imunoterapia, a terapia alvo-específica e o transplante de células tronco, porém a quimioterapia ainda é a abordagem mais utilizada, seja de forma isolada ou em combinações com outros tratamentos (AMERICAN CANCER SOCIETY).

Os medicamentos antineoplásicos podem ser utilizados isolados ou em combinações, dependendo do tipo do tumor e do avanço da doença. A quimioterapia pode ser neoadjuvante (primeira intervenção terapêutica, antes mesmo da cirurgia), adjuvante (logo após a cirurgia) ou paliativa, onde o objetivo é amenizar os sintomas no paciente, já que não há possibilidade de cura (NEIDLE & THURSTON, 2005).

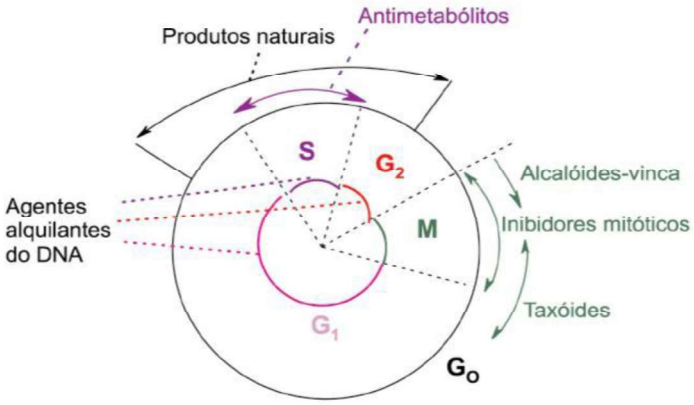
Em muitos casos a terapia neoadjuvante tem a finalidade de reduzir o tamanho do tumor antes da remoção cirúrgica e assim facilitar a completa ressecção, uma vez que as taxas de sobrevida de pacientes são maiores com a total remoção do tumor primário. Ainda assim, alguns pacientes apresentam recidiva local ou metástase mesmo após a completa ressecção do tumor primário (DOVAL et al., 2013).

Apesar dos efeitos adversos resultantes da quimioterapia, visto que os medicamentos não diferenciam as células normais das tumorais, o uso de diferentes drogas com mecanismos de ação diversos faz com que esta seja uma das principais abordagens de tratamento para o câncer. Isto se dá pela grande variabilidade dos tipos de tumores, nos quais as características morfológicas, genotípicas e fisiológicas são distintas (SHAIKH & SHIH, 2012).

Conforme demonstrado na Figura 6, os quimioterápicos podem ser divididos em duas classes: ciclo celular específicos, onde exercem sua ação sobre as células

que encontram-se em divisão; e ciclo celular não específicos, que podem atuar nas células tumorais independente de estarem em divisão ou em G0.

Figura 6. Fases do ciclo celular em que atuam os agentes quimioterápicos.



Fonte: Almeida et al., 2005.

A classificação dos agentes quimioterápicos disponíveis para o tratamento de diversos tipos de câncer se dá de acordo com o seu respectivo mecanismo de ação. Na tabela 1, encontra-se um esquema com as principais classes de quimioterápicos.

Tabela 1: Principais classes de quimioterápicos, mecanismo de ação e exemplos de diferentes medicamentos antineoplásicos.

Classe	Mecanismo de ação	Exemplos
Agentes Alquilantes	Interagem quimicamente com o DNA, gerando lesões que interferem nos processos de replicação e síntese de proteínas. Atuam em todas as fases do ciclo celular de modo inespecífico.	Mostardas nitrogenadas, agentes platinados, nitrossuréias e sulfonatos de alquila.
Agentes Antimetabólitos	Bloqueiam bioquimicamente a síntese de DNA, portanto, atuam na fase S do ciclo. São extremamente tóxicos uma vez que atuam como análogos de	5-fluorouracil, metotrexato, citarabina, 6-mercaptopurina e floxuridina.

	base, substituindo a base hidrogenada do ácido nucléico, levando a DNA polimerase ao erro.	
Antraciclinas	Estão inseridos no grupo de antibióticos anti-tumorais. Atuam como agentes intercalantes de DNA e interferem nas enzimas envolvidas na sua replicação.	Doxorrubicina, daunorrubicina, idarrubicina.
Inibidores de Topoisomerase	Inibem as topoisomerases através do envenenamento da enzima ou da inibição catalítica. As topoisomerases são diferenciadas em tipos I e II de acordo com seus mecanismos e propriedades físicas.	Camptotecina, irinotecano, topotecano, etoposídeo e teniposídeo.
Inibidores Mitóticos	Atuam durante à divisão celular ligando-se nas proteínas microtubulares do fuso mitótico que resulta no bloqueio da replicação e perda da viabilidade celular.	Alcalóides de vinca (vimblastina e vincristina) e taxanos (paclitaxel).
Derivados de Platina	Transferem o grupamento platino na posição N7 da guanina e da adenina no DNA através de pontes mono e bifuncionais que impedem a replicação e síntese do DNA.	Cisplatina, carboplatina e oxaliplatina.

2.4.1 Resistência Tumoral

Após o tratamento com agentes quimioterápicos, alguns pacientes que recidivam apresentam o desenvolvimento de resistência a múltiplas drogas (RMD) dificultando, assim, a terapêutica (KROL et al., 2010).

A resistência a drogas antitumorais é caracterizada pela ausência de resposta ao tratamento em alguns tipos de tumores, sendo um dos maiores obstáculos para o sucesso da quimioterapia. A habilidade dos tumores de sobreviverem à exposição aos agentes citotóxicos em doses máximas toleradas pelos tecidos normais configura esta resistência, que se encontra em cerca de 90% dos casos de câncer metastático, onde são reconhecidos dois tipos de resistência: a intrínseca, quando não há resposta já no primeiro ciclo do tratamento, e a adquirida, quando aparece após o segundo ciclo da quimioterapia (YARDLEY, 2013).

No caso, a RMD é do tipo adquirida, na qual não se observa resposta do tumor às drogas utilizadas no início da quimioterapia, tanto quanto nas que são implementadas ao longo do tratamento (VADLAPATLA et al., 2013).

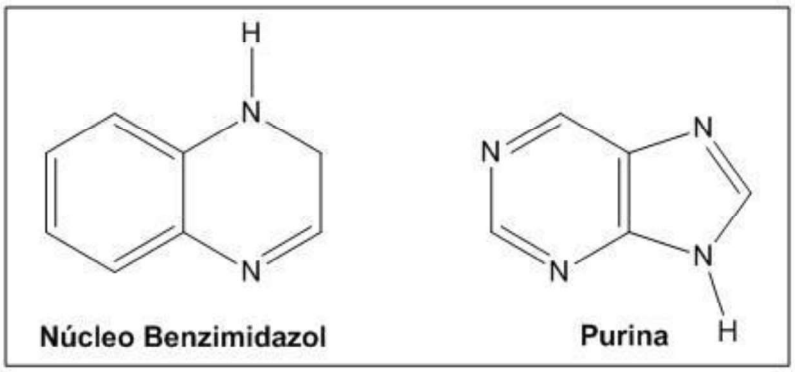
Os mecanismos envolvidos na RMD são diversos e ainda não totalmente compreendidos, pois este tipo de resistência atua de forma simultânea em diferentes sítios de ação, contra uma variedade de agentes citotóxicos com estruturas químicas distintas. Inclusive, esta é uma das principais dificuldades em se compreender a RMD, os medicamentos quimioterápicos utilizados não têm analogia estrutural ou farmacológica, como, por exemplo, a vimblastina, que é um alcaloide bisindólico, e a doxorrubicina, que é uma antraciclina (KROL et al., 2010; MARQUETTE & NABELL, 2012).

2.5 Derivados Benzimidazóis

São muitas as frentes de atuação farmacológica de moléculas derivadas benzimidazóis. Em uma completa revisão sobre a ação terapêutica dessas moléculas, Bansal & Silakari (2012) utilizam o termo "*Master Key*" para denominar o núcleo benzimidazol como importante agente terapêutico para diferentes alvos biológicos.

Na década de 40, os primeiros estudos acerca do potencial terapêutico do núcleo benzimidazol (Figura 7) começaram a ser publicados e especulava-se que o amplo espectro de atuação se devia, principalmente, à similaridade da estrutura do núcleo benzimidazol com a estrutura das purinas (heterocíclicos constituintes do DNA e RNA) (WOOLLEY, 1943; BRINK & FOLKERS, 1949).

Figura 7. Comparativo entre a estrutura do núcleo benzimidazol e a estrutura da purina.



Fonte: Adaptado de Amaral, 2005.

A partir desses estudos, a pesquisa constante e o desenvolvimento de novas moléculas contendo o núcleo benzimidazol demonstraram um importante avanço na descoberta de novas drogas (BANSAL & SILAKARI, 2012).

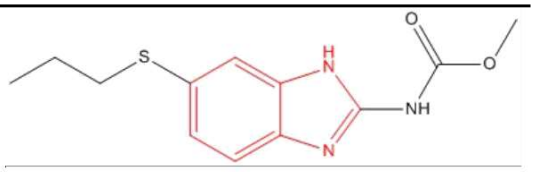
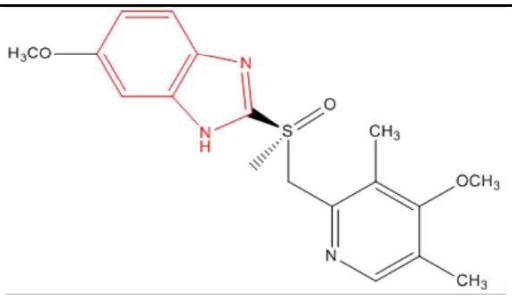
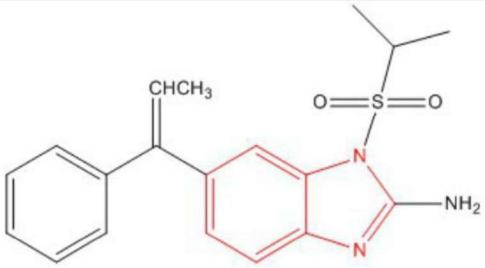
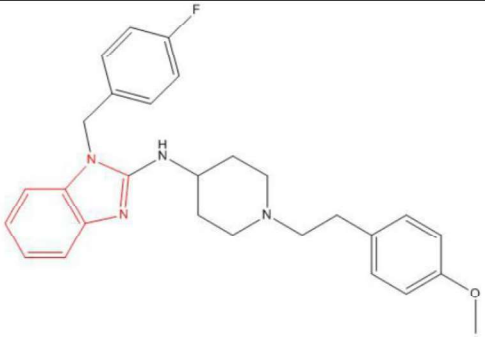
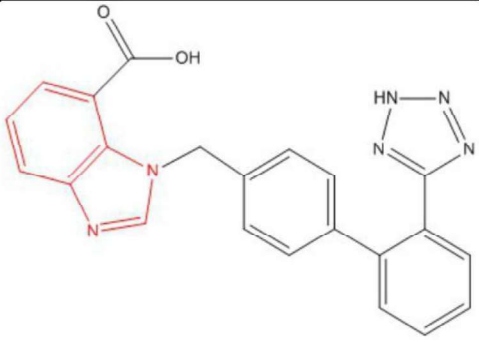
As ações biológicas dos derivados benzimidazóis descritas na literatura científica são variadas e entre as principais estão: anti-inflamatória, analgésica, anti-histamínico, antimicrobiana, anti-helmíntica, antiviral, anti-hipertensiva, antioxidante, antiúlcera, antibacteriana, antifúngica, antitumoral, e outras (BOIANI & GONZALEZ, 2005; BANSAL & SILAKARI, 2012; BAROT et al., 2013; GABA et al., 2014).

Os compostos heterocíclicos nitrogenados, como os derivados benzimidazóis, apresentam também um importante papel no desenvolvimento de agrotóxicos (fungicidas) e antiparasitários veterinários. Heterocíclicos nitrogenados são mais abundantes na natureza que os que contêm oxigênio ou enxofre, provavelmente pela ampla distribuição de ácidos nucléicos envolvidos em quase todos os processos de plantas e animais (DANAHER et al., 2007).

Todas as sete posições do núcleo benzimidazol podem ser substituídas por diferentes radicais químicos, embora a maior parte dos compostos biologicamente ativos são os que apresentam os grupos funcionais nas posições 1, 2 e/ou 5 ou 6 (YADAV & GANGULY, 2015).

Tamanha versatilidade das moléculas derivadas benzimidazol resultou em diferentes medicamentos utilizados na clínica, conforme demonstrado na tabela 2:

Tabela 2: Alguns exemplos de medicamentos derivados benzimidazol.

Medicamento	Ação	Molécula
Albendazol	**Anti-helmíntico**	
Omeprazol	**Antiúlcera**	
Enviradine	**Antiviral**	
Astemizol	**Anti-histamínico**	
Candesartana	**Anti-hipertensivo**	

2.5.1 Atividade Antitumoral

Sendo o núcleo benzimidazol um isóstero das bases purínicas dos ácidos nucléicos, apresentando uma estrutura que permite a ampla atividade biológica,

estas moléculas são importantes para o desenho de novas drogas antitumorais (YADAV & GANGULY, 2015).

Skibo e colaboradores desenvolveram o pyrrolo[1,2-α]benzimidazol, um potencial agente anticâncer que atua clivando as bases A e G, além de promover a alquilação do DNA (SCHULZ et al., 1995).

Uma série de compostos sintetizados a partir da base de uma droga antitumoral em estudo clínico (SNS-314), derivados de 2-aminobenzimidazol, se mostraram potentes inibidores de Aurora quinase em linhagens de câncer colorretal (HCT-116) e em camundongos. Essas quinases são expressas na fase G2/M do ciclo celular e são importantes na regulação da mitose (ZHONG et al., 2009).

Ni e colaboradores desenvolveram uma série de inibidores de quinase envolvidas em *checkpoint*, como o 4-(aminoalquilamino)-3-benzimidazol-quinolinone e observaram o efeito sinérgico desta molécula com a camptotecina, importante agente citotóxico, em células de melanoma (MDA-MB-435) (NI et al., 2006).

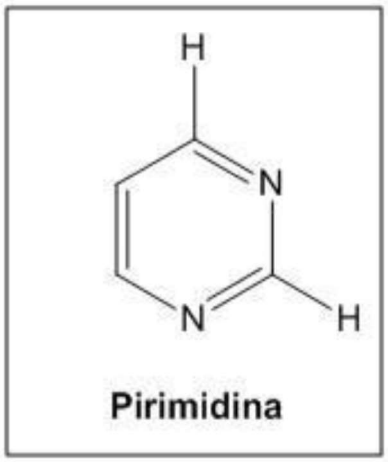
Bis-benzimidazóis e seus derivados apresentaram atividade anticâncer em diferentes linhagens celulares. Mostraram-se inibidores de topoisomerase I e II em células de câncer de mama (MDA-MB 231), além de uma significativa atividade contra linhagens que expressaram multirresistência da glicoproteína P (BIELAWSKI et al., 2004; STYSKALA et al., 2008).

Como dito anteriormente, o núcleo benzimidazol é muito versátil e por isso um importante modelo para o desenho de novas drogas. Complexos de benzimidazóis combinados com metais de transição, principalmente o cobre, demonstraram a ação intercalante da dupla hélice do DNA, promovendo assim, uma alta citotoxicidade em linhagens tumorais (ZHOU & YANG, 2006).

2.5.2 Derivados Pirimidobenzimidazóis

As pirimidinas são compostos heterocíclicos, encontrados nos ácidos nucléicos, que contém dois átomos de nitrogênio nas posições 1 e 3 do anel (Figura 8).

Figura 8. Estrutura geral de uma pirimidina.



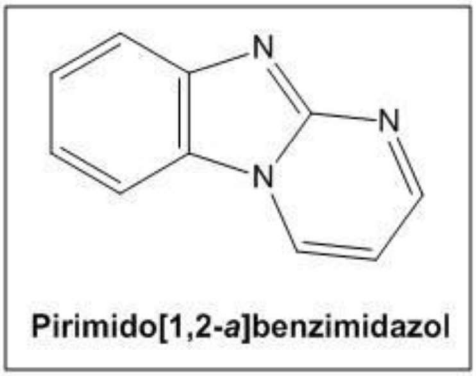
Fonte: Amaral, 2005.

Alguns estudos com compostos derivados pirimidínicos revelaram uma série de atividades farmacológicas, incluindo a atividade antitumoral (ZHAO et al., 2012). Os nitrogênios que se encontram nos anéis pirimidínicos são nucleofílicos, podendo ser alquilados e é esta alquilação que determina as potenciais atividades antitumorais, anti-inflamatórias, antivirais, entre outras (BRONDANI, 2008).

Em um estudo realizado por Zanatta et al. (2009), os compostos derivados de 3-trifluormetilpirimido[1,2-a] benzimidazóis e de 3-oxo-pirimido[1,2-a] benzimidazóis apresentaram efeito inibitório da topoisomerase I destacando assim o potencial uso destas moléculas para terapia antitumoral.

No presente trabalho foram estudados compostos derivados de pirimidobenzimidazóis (Figura 9), sintetizados e gentilmente cedidos pela professora Simone Schneider Amaral da Universidade de Ciências da Saúde de Porto Alegre (UFCSPA).

Figura 9. Estrutura principal dos compostos estudados.



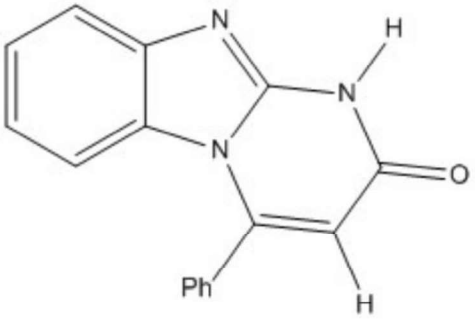
Fonte: Amaral, 2005.

A síntese dessas moléculas foi obtida através de reações de ciclocondensação. A nomenclatura se dá conforme a posição em que os radicais se encontram nos anéis heterocíclicos (mono, di ou tri) (AMARAL, 2005).

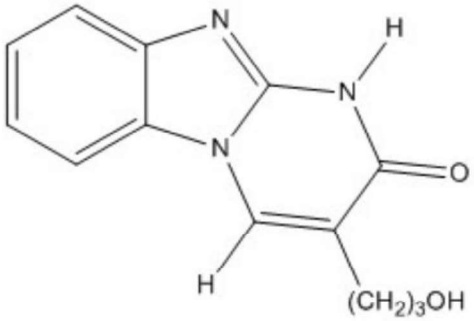
A estrutura e a nomenclatura dos compostos denominados PBZ (pirimidobenzimidazóis) e numerados de 1 a 9, exceto os números 7 e 8 (Figura 10).

Figura 10. Estrutura dos compostos PBZs estudados.

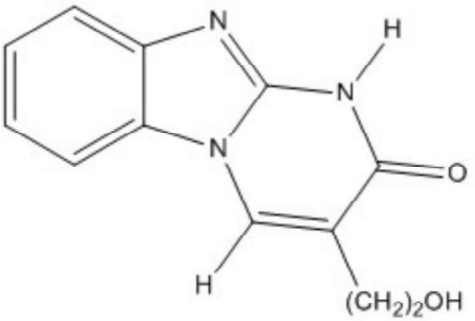
PBZ 01 - $C_{16}H_{11}N_3O$



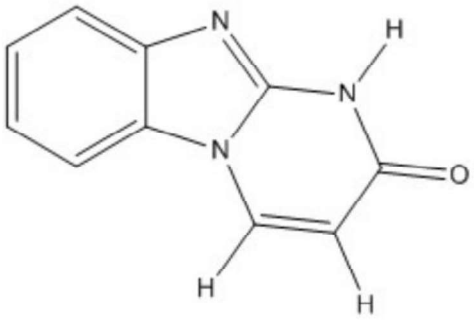
PBZ 02 - $C_{13}H_{13}N_3O_2$



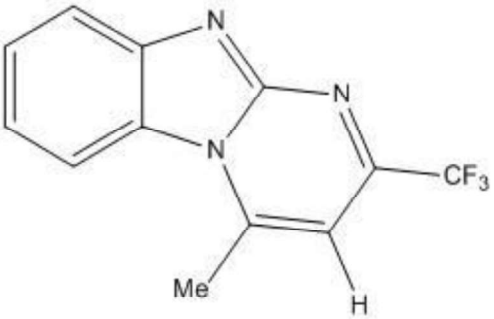
PBZ 03 - $C_{12}H_{11}N_3O_2$



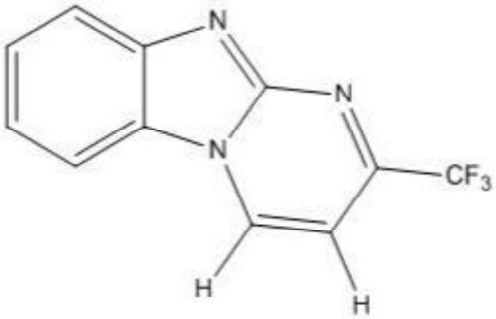
PBZ 04 - $C_{10}H_7N_3O$



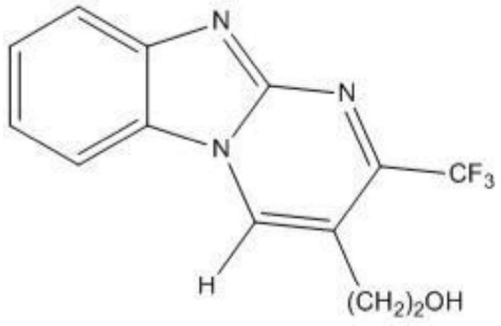
PBZ 05 - $C_{12}H_8F_3N_3$



PBZ 06 - $C_{11}H_6F_3N_3$



PBZ 09 - $C_{13}H_{10}F_3N_30$



Fonte: Amaral, 2005.

Alguns estudos demonstraram a potente citotoxicidade de derivados pirimidobenzimidazóis em diferentes linhagens celulares. A estrutura planar destes análogos pode facilitar a inserção no DNA, resultando na quebra da molécula (ABDEL-MOHSEN et al., 2010; NEOCHORITIS et al., 2011; BANSAL & SILAKARI, 2012;)

Diversos são os estudos acerca do mecanismo de ação de medicamentos que contenham em sua estrutura o núcleo benzimidazol. Entretanto, os medicamentos estudados já estão estabelecidos no mercado, sendo que são poucas as moléculas inéditas em suas estruturas, principalmente, em moléculas que combinam o anel pirimidínico com o núcleo benzimidazol. Diante do exposto, buscamos neste trabalho elucidar os efeitos citotóxicos e genotóxicos das moléculas PBZs contribuindo, desta forma, para o estudo do design de novos fármacos.

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3 OBJETIVOS

3.1 Objetivo Geral

Avaliar o potencial citotóxico e genotóxico de compostos derivados pirimidobenzimidazóis (PBZs) em células tumorais humanas e buscar estabelecer um possível mecanismo de ação das moléculas neste modelo de estudo.

3.2 Objetivos Específicos

- Verificar o potencial citotóxico dos diferentes compostos PBZs nas linhagens tumorais MCF-7 (adenocarcinoma de mama), HCT-116 (carcinoma colorretal), HepG-2 (carcinoma hepatocelular), HT-29 (adenocarcinomacolorretal),T-24 (câncer de bexiga) e Caco-2 (adenocarcinomacolorretal) e na linhagem não tumoral HEK-293 (células de rim de embrião humano), pelo ensaio de MTT. A partir dos resultados de IC50 e índice de seletividade (IS), realizar demais ensaios biológicos com os compostos mais promissores, como:
    - Avaliar a indução de danos no DNA dos compostos, utilizando o ensaio cometa alcalino, bem como a indução de fosforilação da histona H2AX, por western blot;
    - Analisar a progressão de ciclo celular das células tratadas com os compostos PBZs por citometria de fluxo, com marcação por iodeto de propídeo;
    - Medir a produção intracelular das espécies reativas de oxigênio pelo ensaio DCFH-DA, por citometria de fluxo;
    - Determinar a frequência de células apoptóticas induzidas pelos compostos, através da medição de anexina V, por citometria de fluxo;
    - Verificar o potencial transmembrânico da mitocôndria, através da retenção mitocondrial da rodamina 123, por citometria de fluxo;
    - Analisar a inibição da enzima topoisomerase I por ensaio de relaxamento de plasmídeo (TopoGEN®).

4 ARTIGO CIENTÍFICO

Os resultados e a discussão estão apresentados na forma de um artigo científico, que será submetido à revista *Investigational New Drugs*.

Para facilitar a leitura, as figuras estão inseridas ao longo do texto (diferente das normas exigidas pela revista).

CYTOTOXIC ACTIVITY AND CELL CYCLE DISTURBANCES INDUCED BY PYRIMIDOBENZIMIDAZOLE DERIVATIVES ON HUMAN CANCER CELL LINES

Cassiana Macagnan Viau[1], Débora Correa Espiña[1], Simone Schneider Amaral[2], Natália Alice Berwig[1], Renata Bartolomeu Staub[1], Josiane Moraes dos Santos[3], Nilo Zanatta[3], Jenifer Saffi[1*]

[1]Department of Basic Health Sciences - Laboratory of Genetic Toxicology – Federal University of Health Sciences of Porto Alegre (UFCSPA), Porto Alegre, RS – Brazil

[2]Department of Pharmacosciences - Federal University of Health Sciences of Porto Alegre (UFCSPA), Porto Alegre, RS – Brazil

[3]Chemical Core Heterocycles (NUQUIMHE), Chemistry Department, Federal University of Santa Maria, Santa Maria (UFSM), RS – Brazil

*Corresponding author: Dr. Jenifer Saffi

Departamento de Ciências Básicas da Saúde. Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), RS-Brasil. Rua Sarmento Leite 245 - CEP: 90150-170.

FAX +55 51 33038861

e-mail: jenifers@ufcspa.edu.br

Abstract

INTRODUCTION: Conventional chemotherapy is the main treatment for cancer and benefits patients in the form of decreased relapse and metastasis and longer overall survival. However, as the target therapy drugs is not wholly precise, it also results in quite a few side effects, and is less efficient in many cancer cells, which are considered the reason for chemotherapy resistance, relapse, and metastasis. In the present study, we report the biologic evaluation and molecular mechanism of a series of substituted 3-trifluoro(oxo)pyrimido[1,2-*a*] benzimidazoles derivatives (PBZ 1-9) against the growth of several human cancer cell lines.

METHODS: The potential antitumoral activity of the compounds was evaluated in vitro by examining the cytotoxic effects against human cancer cell lines (MCF-7, HepG-2, T-24, HCT-116, HT-29 and Caco-2) and a nontumoral cell HEK-293. After the treatment the cytotoxicity was expressed as IC50 values, i.e. the concentrations of the test agent inducing 50% reduction in cell numbers compared with control cultures. Selective index (SI) was calculated by IC50 in HEK-293 cells/IC50 in tumoral cells. The well-known drug mitoxantrone (MXT) was used as reference compound. Compounds showing cytotoxic activities were subjected to others tests.

Genotoxicity induction was analyzed by single-cell gel electrophoresis (comet assay). The production of reactive oxygen species (ROS) and changes in mitochondrial membrane potential (ΔΨm) were studied using specific fluorescence probes, DCFH(2)-DA (2',7'-dichlorodihydrofluorescein diacetate) and rhodamine-123 by flow cytometry. The cell cycle was determined by flow cytometry and changes in protein expression (γH2AX) were examined by western blotting. Topo I activity were evaluated in a cell-free system.

RESULTS: Our results suggest that PBZ 04, 05 and 09 exerted the highest cytotoxicity against MCF-7 cells, with an IC50 values of 15.41, 19.52 and 8.11 μM, respectively. The most promising compound was the PBZ 09 in MCF-7 cells.

Flow cytometric analysis indicates that PBZ 09 alters cell cycle progression, induces G1 arrest and causes DNA polyploidy (>4N). PBZ 09 generates ROS and γH2AX phosphorylation. The pretreatment with N-acetyl cysteine (NAC) abrogates PBZ 09-induced ROS production. Relaxation of the plasmid was inhibited only by PBZ 09.

CONCLUSION: The present work demonstrates that specially PBZ 09 promotes a

reduction in cell viability by necrosis and apoptosis. According to our results, these events might be consequence to the putative interaction of PBZ 09 with Topo I enzyme (direct action) and ROS generation (indirect action). There is an increased DNA damage with increased levels of γH2AX (known to be a marker of DNA damage), leading to cell cycle arrest and cell death induction. Our data indicate that PBZ 09 is a small molecule that may potentially be used for future clinical management of breast cancer.

Keywords: Trifluoro(oxo)pyrimido[1,2-a] benzimidazoles derivatives, citotoxic activity, cell death, reactive oxigen species, antitumoral therapy.

Introduction

Cancer is the second worldwide cause of death, exceeded only by cardiovascular diseases [1]. It is characterized by uncontrolled cell proliferation and an absence of cell death that, except for hematological cancers, generates an abnormal cell mass or tumor [2]. This primary tumor grows thanks to new vascularization and, in time, acquires metastatic potential and spreads to other body sites, which causes metastasis and finally death [3]. Cancer is caused by damage or mutations in the genetic material of the cells due to environmental or inherited factors [1,2]. While surgery and radiotherapy are the primary treatment used for local and non-metastatic cancers, anti-cancer drugs (chemotherapy, hormone and biological therapies) are the choice currently used in metastatic cancers [1].

Chemotherapy is based on the inhibition of the division of rapidly growing cells, which is a characteristic of the cancerous cells, but unfortunately, it also affects normal cells with fast proliferation rates, such as the hair follicles, bone marrow and gastrointestinal tract cells, generating the characteristic side effects of chemotherapy [4]. The indiscriminate destruction of normal cells, the toxicity of conventional chemotherapeutic drugs, as well as the development of multidrug resistance [5,6], support the need to find new effective targeted treatments based on the changes in the molecular biology of the tumor cells.

Heterocycles are a class of compounds very well-documented as important antitumor agents [7]. Among them, heterocyclic isosteres of purine based nucleic acid such as benzimidazoles, an important scaffold in various biologically active molecules, are widely explored for development of anticancer agents [8-11]. In this context, the goal of this research was to investigate a possible antitumor action of seven pyrimido[1,2-*a*] benzimidazoles in MCF-7, HepG-2, T-24, HCT-116, HT-29 and Caco-2 tumoral cells in comparison with HEK-293 non tumoral cell.

Materials and Methods

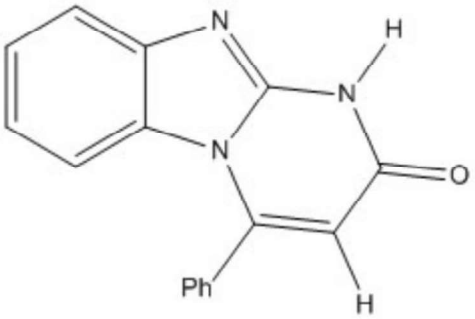
Chemicals

Dulbecco's modified Eagle's medium (DMEM), low-melting-point agarose (LMP), high-melting-point agarose (HMP), phosphate-buffered saline (PBS; Na_2HPO_4, KH_2PO_4 and KCl, pH 7.4), propidium iodide (PI), mitoxantrone (MXT), hydrogen peroxide (H_2O_2), amino acids and nitrogenated bases were purchased from

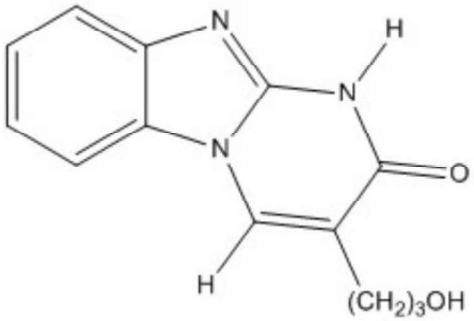
Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) and penicillin/streptomycin were obtained from Gibco-BRL (Grand Island, NY, USA). Primary antibody (anti-p-histone H2AX and anti-β-actin) and secondary antibody (anti-rabbit and anti-mouse) were obtained from Santa Cruz Biotechnology (California, USA). Annexin V-Phycoerythrin (PE) and 7-Amino-Actinomycin (7-AAD) were purchased from BD Biosciences (San Diego, CA). All other reagents were of analytical grade.

A series of seven diferent pyrimidobenzimidazoles (Fig. 1) **PBZ01** – 4-phenyl-pyrimido[1,2-*a*]benzimidazol-2(1*H*)-one, **PBZ02** – 4-(3-hydroxypropyl)pyrimido[1,2-*a*]enzimidazol-2(1*H*)-one, **PBZ03** – 2,3,4,3a,hexahidro-5,11a-4-oxo-furano[2',3',6,5]pyrimido[1,2-*a*]benzimidazol-2(1*H*)-one, **PBZ04** pyrimido[1,2-*a*]benzimidazol-2(1*H*)-one, **PBZ05** – 4-methyl-2-(trifluoromethyl)pyrimido[1,2-*a*]benzimidazole, **PBZ06** – 2-(trifluoromethyl)pyrimido[1,2-*a*]benzimidazole, **PBZ09** – 3-(1-hydroxyethyl)-2-(trifluoromethyl)-pyrimido[1,2-*a*]benzimidazole were prepared as described previously [12].

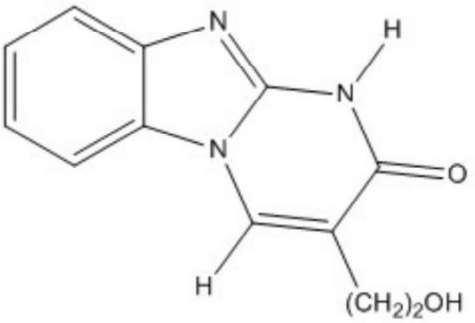
PBZ 01 - $C_{16}H_{11}N_3O$



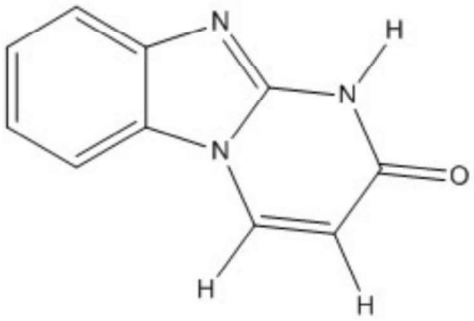
PBZ 02 - $C_{13}H_{13}N_3O_2$



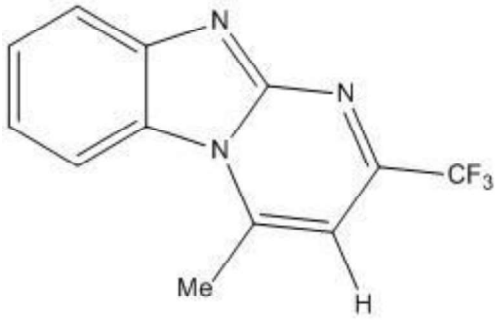
PBZ 03 - $C_{12}H_{11}N_3O_2$



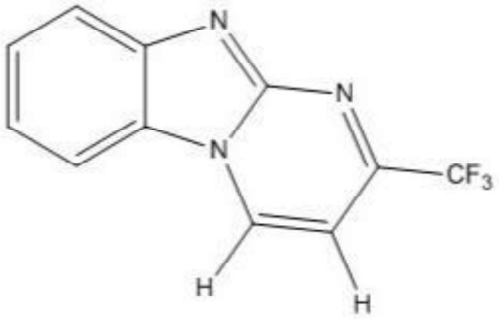
PBZ 04 - $C_{10}H_7N_3O$



PBZ 05 - $C_{12}H_8F_3N_3$



PBZ 06 - $C_{11}H_6F_3N_3$



PBZ 09 - $C_{13}H_{10}F_3N_30$

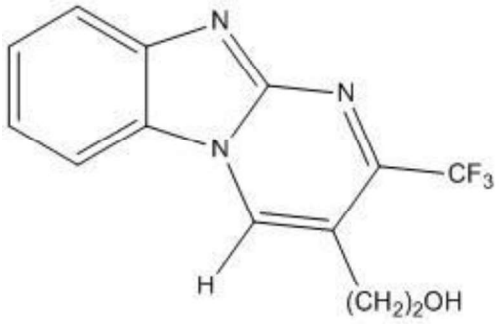


Figure 1. Chemical structure of pyrimidobenzimidazoles **PBZ01** – 4-phenyl-pyrimido[1,2-*a*] benzimidazol-2(1*H*)-one, **PBZ02** – 4-(3-hydroxypropyl)pyrimido[1,2-*a*]benzimidazol-2(1*H*)-one, **PBZ03** – 2,3,4,3a,hexahidro-5,11a-4-oxo-furano[2',3',6,5]pyrimido[1,2-*a*]benzimidazol-2(1*H*)-one, **PBZ04** - pyrimido[1,2-*a*]benzimidazol-2(1*H*)-one, **PBZ05** – 4-methyl-2- (trifluoromethyl)pyrimido[1,2-*a*]benzimidazole, **PBZ06** – 2-(trifluoromethyl)pyrimido[1,2-*a*]benzimidazole, **PBZ09** – 3-(1-hydroxyethyl)-2-(trifluoromethyl)-pyrimido[1,2-*a*]benzimidazole.

For cell treatments, stock solutions of the PBZs were prepared freshly prior to use, using dimethylsulfoxide (DMSO) as solvent. The appropriate concentrations were obtained by diluting the stock solution in sterile distilled water, and the final concentration of DMSO in the incubation mixture never exceeded 0.1%. Control samples were always treated with the same amount of DMSO (0.1% v/v) as used in the corresponding experiments.

Culture conditions

The human cell lines MCF-7 (breast adenocarcinoma), HepG-2 (hepatoma), T-24 (bladder cancer), Caco-2 (colorectal adenocarcinoma) and HEK-

293 (human embryonic kidney) were obtained from the Rio de Janeiro Cell Bank (Rio de Janeiro, RJ, Brazil). The HCT-116 cell (colorectal carcinoma), and HT-29 cell (colorectal adenocarcinoma) were kindly provided by Dr. Annette K. Larsen (Institut National de la Santé et de la Recherche Médicale - INSERM, Paris, França). All cell lines except MCF-7 were grown in DMEM supplemented with 10% or 20% (for Caco-2) FBS, 100 units.mL^{-1} penicillin and 100 µg.mL^{-1} streptomycin at 37°C in a humidified atmosphere of 5% CO_2. MCF-7 cells were maintained in RPMI-1640 supplemented with 20% FBS at the same conditions described above.

Cell viability assay

The cytotoxic potential of the **PBZs** were evaluated by the MTT assay in human tumor cell lines as well as in HEK-293 normal cells. Cells (1 × 10^4 cells – for 24 h; and 3 × 10^3 cells – for 72 and 120 h) were seeded on 96-well plates in growth medium and incubated overnight. Afterwards, **PBZs** (0.1, 0.5, 2.5, 5.0, and 10 µM) was added to each well and incubated for 24, 72, and 120h. Mitoxantrone, a cytostatic anthracenedione that intercalates in DNA and increases the incidence of double-strand breaks by stabilizing the cleavable complex of topoisomerase II and DNA, was used as positive control [13]. At the end of each treatment, cytotoxicity was assessed by means of the thiazolyl blue tetrazolium bromide [14]. Absorbance was measured with a SpectraMax reader (Bio-Rad, USA) at a test wavelength of 540nm. The absorbance of negative control cells was set as 100% viability, and the values for treated cells were calculated as a percentage of the control.

All other experiments (cell cycle comet, apoptosis, ROS production, rhodamine 123, protein expression assays and DNA relaxation assays) were conducted in the breast cancer cell line MCF-7 using **PBZ04**, **PBZ05** and **PBZ09** (IC50 = 15.41 µM, 19.52 µM, and 8.11 µM, respectively), as determined by MTT assay. Cells (2 × 10^5 cells/mL) were seeded on 6-well tissue culture plates, and grown for 1 day up to 70–80% confluence before treatment with the test substance. The **PBZs 04**, **05** and **09** were added to medium with FBS to obtain the different concentrations, and cells were treated at 37ºC for 24 h in humidified atmosphere containing 5% CO_2.

Cell cycle distribution by flow cytometric analysis

Cell cycle distribution determination analysis was performed as previously described [15]. After treatment, cells were trypsinized, centrifuged and resuspended in ice-cold 70% ethanol, and left for 24 h at 4°C. Ethanol-fixed cells were centrifuged, washed with PBS and resuspended in buffer containing 0.2 mg/mL RNAse, 50 µg/mL PI and 0.1% Triton X-100, and incubated for 30 min at room temperature. Samples were analyzedona FACS Calibur flow cytometer (Becton-Dikinson, San Fransisco, CA) using the CellQuest software. A total of 10,000 events were measured per sample.The data were analyzed to determine the percentage of cells at each phase of the cell cycle (sub-G1, G1, S and G2/M).

Assessment of apoptosis by flow cytometric analysis

Annexin V-PE was used in conjunction with a vital dye, 7-AAD, to distinguish apoptotic (Annexin V-PE positive, 7-AAD negative) from necrotic (Annexin V-PE positive, 7-AAD positive) cells. After treatment, cells were trypsinized, collected and resuspended in 40 µL of binding buffer with 2 µL Annexin V-PE. Cells were incubated for 15 min in the dark at room temperature. After incubation, 160 µL of binding buffer and 2 µL of 7-AAD were added. Cells were incubated for 5 min and additional 200 µL of binding buffer were added. Before analysis, cells were filtered through a cell strainer cap fitted to a polystyrene round bottom flow cytometric tube. Data were collected and analyzed by a FACS Calibur flow cytometer with CellQuest software, in a total of 10,000 events per sample; fluorescence was measured and the percentage of viable, early apoptotic, late apoptotic and necrotic cells was determined.

Alkaline Comet assay

The alkaline comet assay was performed as previously described [16]. Briefly, 10 µL of cell suspension (1×10^4 cells) treated with **PBZs 04**, **05** and **09** were mixed with 90 µL LMP agarose, spread on a normal agarose precoated microscope slide, and placed at 4°C for 5 min to allow for solidification. Cells were lysed in high concentration of salt and detergent (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris with 1% Triton X-100 and 10% DMSO freshly added) for 2 h. Slides were removed from lysing solution and washed three times with PBS. Subsequently, cells were exposed to alkali conditions (300 mM NaOH/1 mM Na_2EDTA, pH >13, 30 min, 4°C) to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted

for25 min at 25 V and 300 mA (94 V/cm). After electrophoresis, the slides were neutralized and silver stained [17]. One hundred cells were scored visually according to the tail length and the amount of DNA present in the tail. Each comet was given an arbitrary value of 0–4 (0, undamaged; 4, maximally damaged), as described by Collins *et al.* [18]. Damage score was thus assigned to each sample and can range from 0 (completely undamaged: 100 cells X 0) to 400 (with maximum damage: 100 cells X 4). International guidelines and recommendations for the comet assay consider that visual scoring of comets is a well-validated evaluation method, as it is highly correlated with computer-based image analysis [18,19].

Reactive oxygen species (ROS) detection by flow cytometric analysis

Levels of intracellular ROS were estimated following treatment with **PBZs 04**, **05** and **09** using 2',7'-dichlorofluorescein diacetate (H_2DCFDA, Sigma) as a fluorescent probe. Detection of oxidative stress was done by incubating the cells with 20 µM of H_2DCFDA for 20 min at 37°C. Cells were then detached by trypsinization and washed twice with PBS. After filtration through cell strainer cap, cells were analyzed using a FACSCalibur flow cytometer with CellQuest software in accordance with Bass *et al.* [20]. A total of 10,000 events were measured per sample. DCF fluorescence intensity was shown in arbitrary units. In recovery experiments, cells were pre-treated with the antioxidante*N*-acetyl-cysteine (NAC) (2 mM) for 2 h and then the PBZs were added.

Assay of the electrochemical potential

After treatment cells were incubated with 2 mM Rh123 (rhodamine 123) for 30 minutes at 37° C, washed, and then resuspended in 100 µL PBS. Mitochondrial electrochemical potential is correlated to the fluorescence intensity of Rho123 (with decreased fluorescence signifying loss of the mitochondrial electrochemical potential) [21]. Flow cytometry was performed using a FACS Calibur (Becton Dickinson, San Jose, California, United States) with excitation at 488nm and emission read using a 525–550nm filter (FL1).

Western blotting

The MCF-7 cells (1 x10^7 cells/mL) were treated with **PBZs 04**, **05** and **09** at IC50 for 24 h, after which they were washed twice with PBS and resuspended in lysis buffer (20 mMTris/acetate, pH 7.5, 270 mM sucrose, 1 mM EDTA, 1 mM

EGTA, 1% Triton X-100, 1 mM ortovanadate, 1 mM sodium glycerophosphate, 5 mM sodium fluoride, 1 mM sodium pyrophosphate, 5 mMβ-mercaptoethanol, 1 mM bezamidine, 35 µg/mL PMSF, 5 µg/ mL leupeptine). The samples were homogenized and incubated on ice for 20 min before being centrifuged at 12,000 g for 15 min. The supernatants were assayed for protein concentration.

For western-blot analyses, a 30 µg sample of total proteins was separated on 15% SDS-polyacrylamide gel and transferred to a polyvinylidenedifluoride membrane. The membranes were blocked by incubation in TBS buffer containing 0.1% Tween-20 and 5% dried milk for 1 h at room temperature and washed with TBS buffer containing 0.1% Tween-20. They were then blotted overnight at 4°C with primary antibodies [rabbit polyclonal anti-p-histone H2AX (1/1000 dilution) and mouse monoclonal anti-β-actin (1/1000 dilution)]. The blots were washed 3 times with TBS-0.1% Tween and developed with peroxidase-linked secondary antibodies (1/3000). All blots were developed by ECL Western Blotting Detection Kit Reagent and detected using a ChemiDoc (Bio-Rad) imaging system. In recovery experiments, cells were pre-treated with the antioxidante NAC as described above.

DNA relaxation assay

The inhibitory effects of **PBZs 04**, **05** and **09** on human Topo I were measured using Topo I Drug Screening Kit (TopoGEN, Inc, Port Orange, Florida, USA). Supercoiled (pHOT-1) plasmid DNA (250 ng) was incubated with Topo I (4 units) at 37°C for 30 minin relaxation buffer (10 mMTris buffer pH 7.9, 1 mM EDTA, 0.15 M NaCl, 0.1% BSA, 0.1 mM spermidine and 5% glycerol) in the absence or presence of compounds. 100 µM of campothecin (CPT) was used as positive control. The reaction was stopped by addition of 10% SDS (2 µL). Proteinase K (50 µg/mL) was added and incubated at 37°C for 30 min. DNA samples were subjected to electrophoresis on a 1% agarose gel for 90 min. at room temperature and visualized with 0.5 mg/mL ethidium bromide [22].

Statistical analysis

The IC50 values and their 95% confidence intervals (CI 95%) were obtained by nonlinear regression using GraphPad Prism v5 program (Intuitive Software for Science, San Diego, CA, USA). Selective index (SI) was calculated by

IC50 in HEK-293 cells/IC50 in tumoral cells. All experiments were independently repeated at least three times, with triplicate samples for each treatment. Results are expressed as means ± standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA), and means were compared using Tukey test, with $P \leq 0.05$ considered as statistically significant.

Results

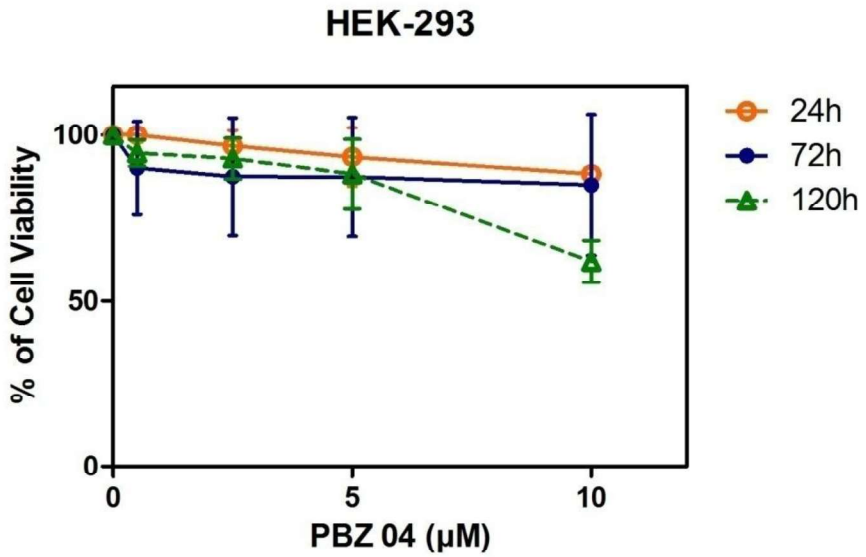
Effects of PBZs on tumoral cells

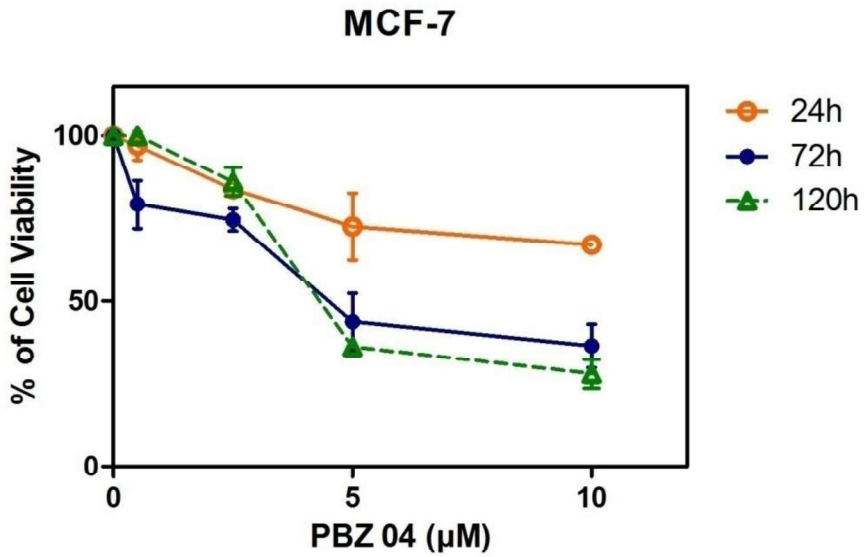
Table 1 shows the cytotoxic effects of **PBZs** on cell lines. The antiproliferative effects were quantified in terms of IC50 values. The lower the IC50 value, the higher the antiproliferative activity [23]. The ratio between the cytotoxic parameters found in HEK-293 cells and those observed in tumoral cell lines can be considered as a measurement of compound selectivity index (SI). The SI value is also shown in Table 1 (superscript). Each cell line had a different sensitivity and selectivity to **PBZs**, as evidenced by IC50 and SI values. Interestingly, our results suggest that **PBZ 04**, **05** and **09** exerted the highest cytotoxicity against MCF-7 cells, with IC50 values of 15.41, 19.52 and 8.11 μM, respectively. In addition, **PBZ04** also showed a significant inhibitory activity in human cancer cell line T-24 and HepG-2 (Table 1) with IC50 values of 6.90 and 17.67 μM after 24 h of treatment and potencies of four and two fold greater in those cells than in normal cells (HEK-293), respectively. When we compared all **PBZs**, the most resistant cell line was Caco-2, which showed an IC50 of 44.9 μM, while the MCF-7 cell line was found to be the most sensitive which an IC50 of 8.11 μM. Considering that breast cancer is an entity that becomes more prominent day by day, with increasing importance [24], we chosed the MCF-7 cells for further experiments to verify the cytotoxic mechanisms of **PBZ 04**, **05** and **09** in this cell lines. Our results demonstrated that treatment with **PBZ 04**, **05** and **09** reduced cell viability in a dose- and time-dependent manner (Fig. 2). The MTT assay confirmed that MCF-7 cells have a higher sensitivity to **PBZ-09** at 24, 72 and 120h (Table 1 and Fig. 2). Mitoxantrone, used in this study as a positive control, demonstrated IC50 values < 4 μM in human cell lines (Table 1).

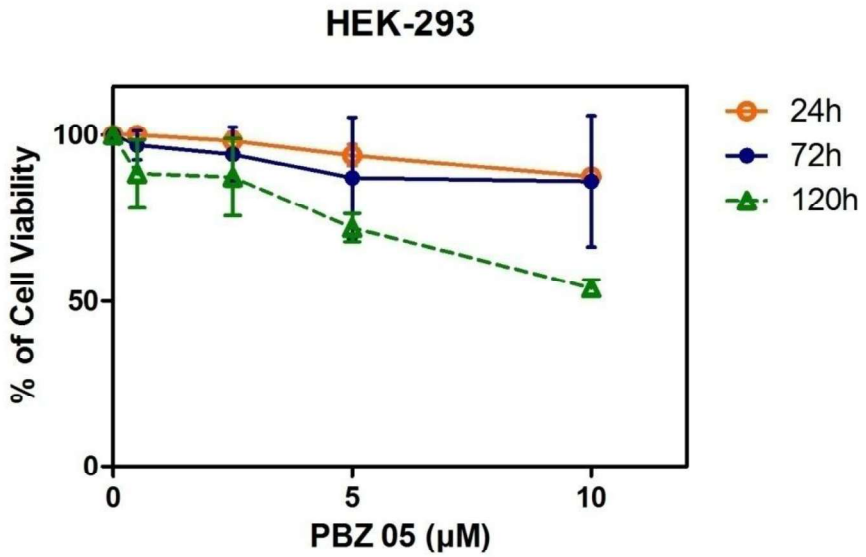
Table 1. Antiproliferative activity of PBZs on human cell lines

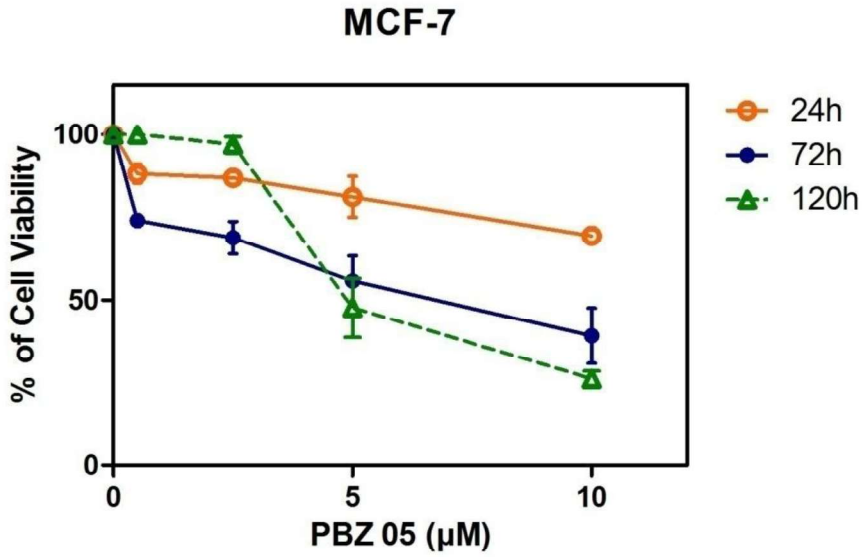
	IC50 (μM)[a] Selectivity index (SI)[b]/superscript value						
Compounds	**HEK-293**	**MCF-7**	**HepG-2**	**T-24**	**HCT-116**	**HT-29**	**Caco-2**
PBZ01	33.0	34.88 ^{MR}	>50	>50	37.0 ^{MR}	>50	>50
PBZ02	25.11	26.22 ^{MR}	37.24 ^{MR}	>50	>50	43.0 ^{MR}	44.9 ^{MR}
PBZ03	23.05	>50	19.0	22.09	42.44 ^{MR}	>50	>50
PBZ04	28.75	15.41 ^{2X}	17.67 ^{2X}	6.90 ^{4X}	20.25	>50	>50
PBZ05	27.86	19.52	>50	>50	22.66	>50	>50
PBZ06	31.99	42.66 ^{MR}	>50	>50	>50	34.99 ^{MR}	>50
PBZ09	21.21	8.11 ^{2X}	14.62	23.88	>50	>50	>50
MXT[c]	3.88	0.87	3.5	2.50	0.61	0.88	2.4

[a]Drug concentration required to inhibit the cell growth by 50% after 24 h of incubation. [b]Selectivity index (*in vitro*): IC50 in HEK-293 cells/IC50 in tumoral cells. [c]Mitoxantrone (MXT) was used as positive control. MR: more resistant in tumoral cells.


HEK-293
% of Cell Viability
100
50
0
0
5
10
PBZ 04 (µM)
24h
72h
120h


MCF-7
% of Cell Viability
100
50
0
0
5
10
PBZ 04 (µM)
24h
72h
120h


HEK-293
% of Cell Viability
100
50
0
0
5
10
PBZ 05 (µM)
24h
72h
120h


MCF-7
% of Cell Viability
100
50
0
0
5
10
PBZ 05 (µM)
24h
72h
120h

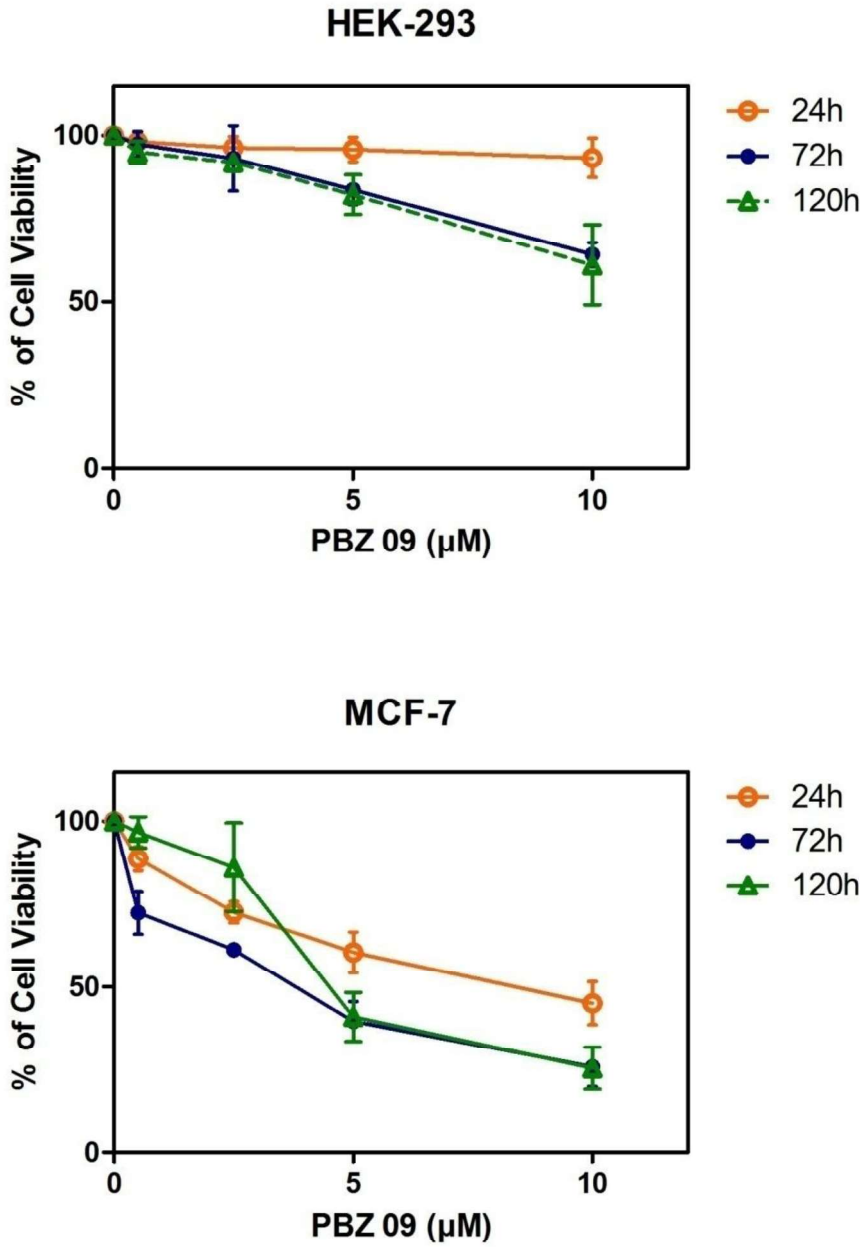
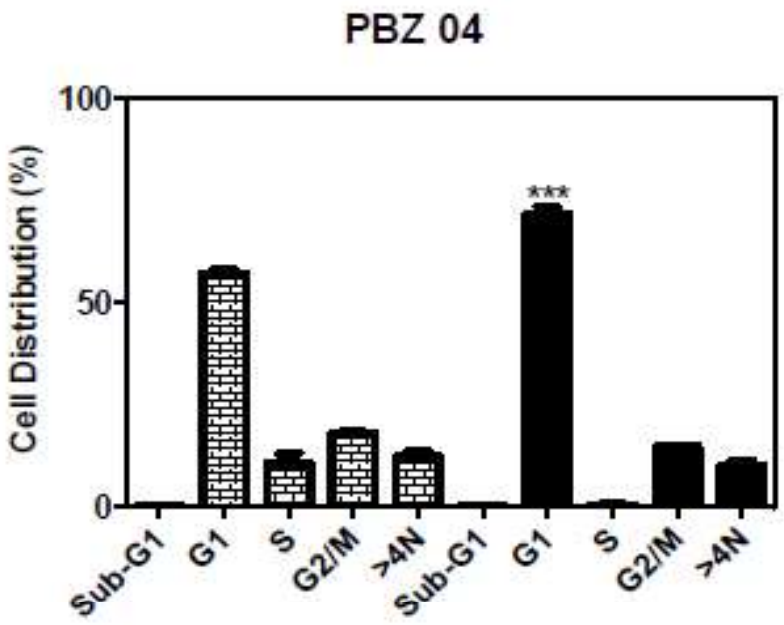


Figure 2. Cell viability in each HEK-293 and MCF-7 cell lines was assessed by MTT assay. Cells were treated with increasing concentrations of **PBZs 04**, **05** and **09** for 24, 72 and 120 h. Data are expressed as the percentage of the control cells and are the means ± standard deviation (SD).

Cell cycle distribution by flow cytometric analysis

In order to identify the mechanisms underlying **PBZ 04**, **05** and **09**-induced sensitivity of MCF-7 cells, we examined the effect of them on cell cycle distribution. After treatment with IC50 values for 24h, cells were analyzed and as shown in Fig. 3A-B, **PBZ 04**, **05** and **09** significantly provoked cell cycle arrest leading to the accumulation of cells in G1 phase and cells at S/G2 phases were markedly decreased. These results indicated that these compounds increased sensitivity of MCF-7 cells through inducing G1 phase arrest rather than promoting apoptosis.



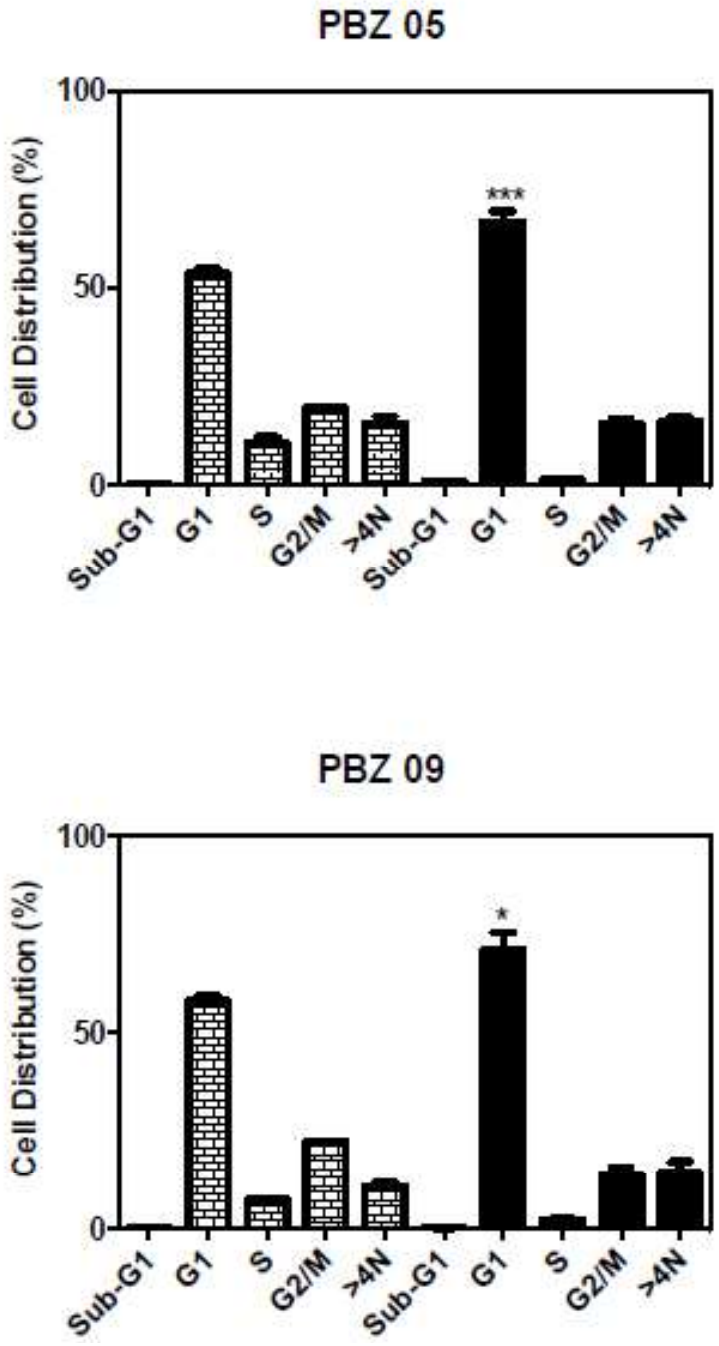


Figure 3A. Effect of **PBZs** treatment on the cell cycle distribution. Statistical analysis was applied for comparing control non-treated cells with treated cells (black bars). Data are expressed as mean ± SD of three independent experiments.

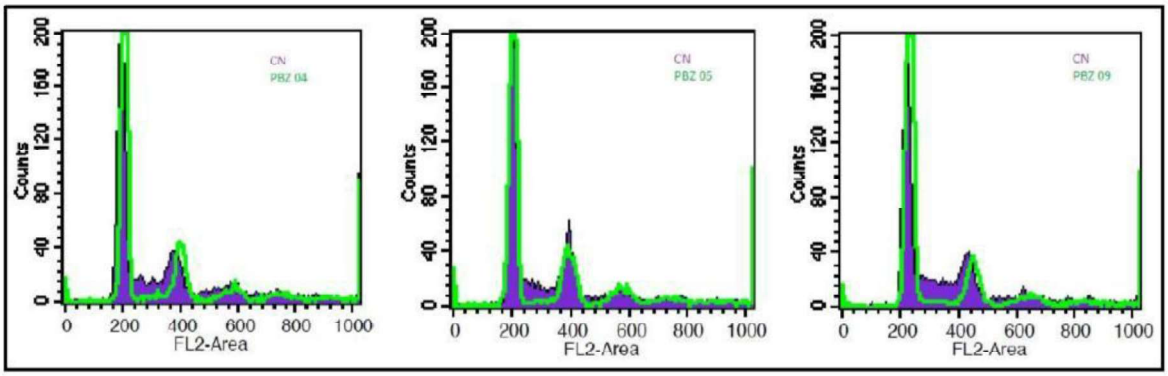


Figure 3B. Cell cycle profile of MCF-7 cells after 24 hours treatment with **PBZs 04**, **05** and **09**. Purple histogram: control and green line: PBZs treatment.

Cell death

The morphology of treated MCF-7 cells was analyzed using annexin V-PE/7-AAD staining and the percentages of viable, apoptotic and necrotic cells were calculated. After 24 h of **PBZ 04**, **05** and **09** treatment, approximately 7.18%, 9.49% and 6.13% MCF-7 cells were in late apoptosis (labeled with both annexin V-PE and 7-AAD), and a remarkable decrease was observed in the viable cells population (unlabeled with either annexin-V or 7-AAD) compared with the untreated control cells (Fig. 4A-B). The percentage of necrotic cells (labeled with 7-AAD) were high for all three compounds (16.75%, 19.55% and 28.55% for **PBZ 04**, **05** and **09**, respectively). So, the dominant cell death type was late apoptosis and necrotic cells.

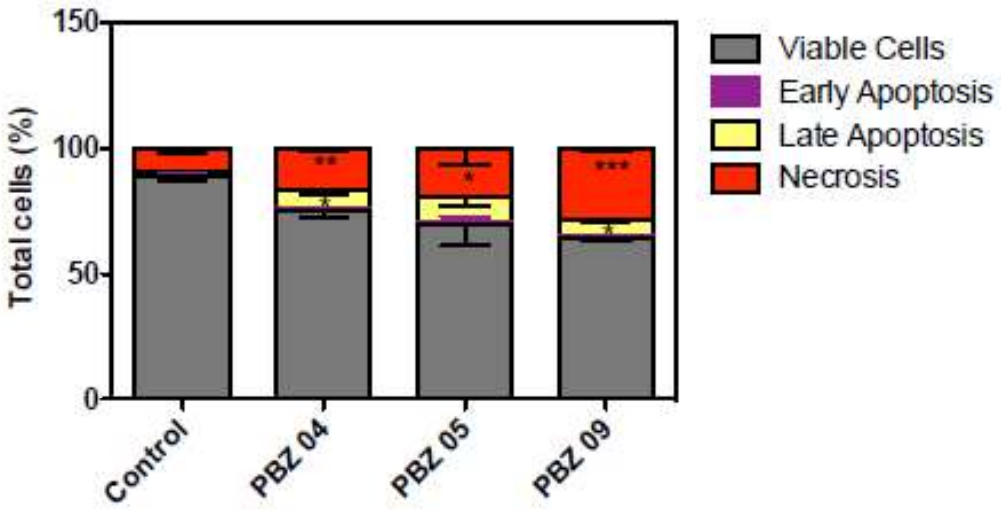


Figure 4A. Annexin V assays analyzing in MCF-7 cells treated with IC50 **PBZs**. The sum of the percentages of Annexin V and 7-AAD-PE-positive cells was calculated. Three independent experiments were pooled and analyzed as a combined data set. Results are expressed as means ± standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA), and means were compared using Tukey test, with $P \leq 0.05$ considered as statistically significant.

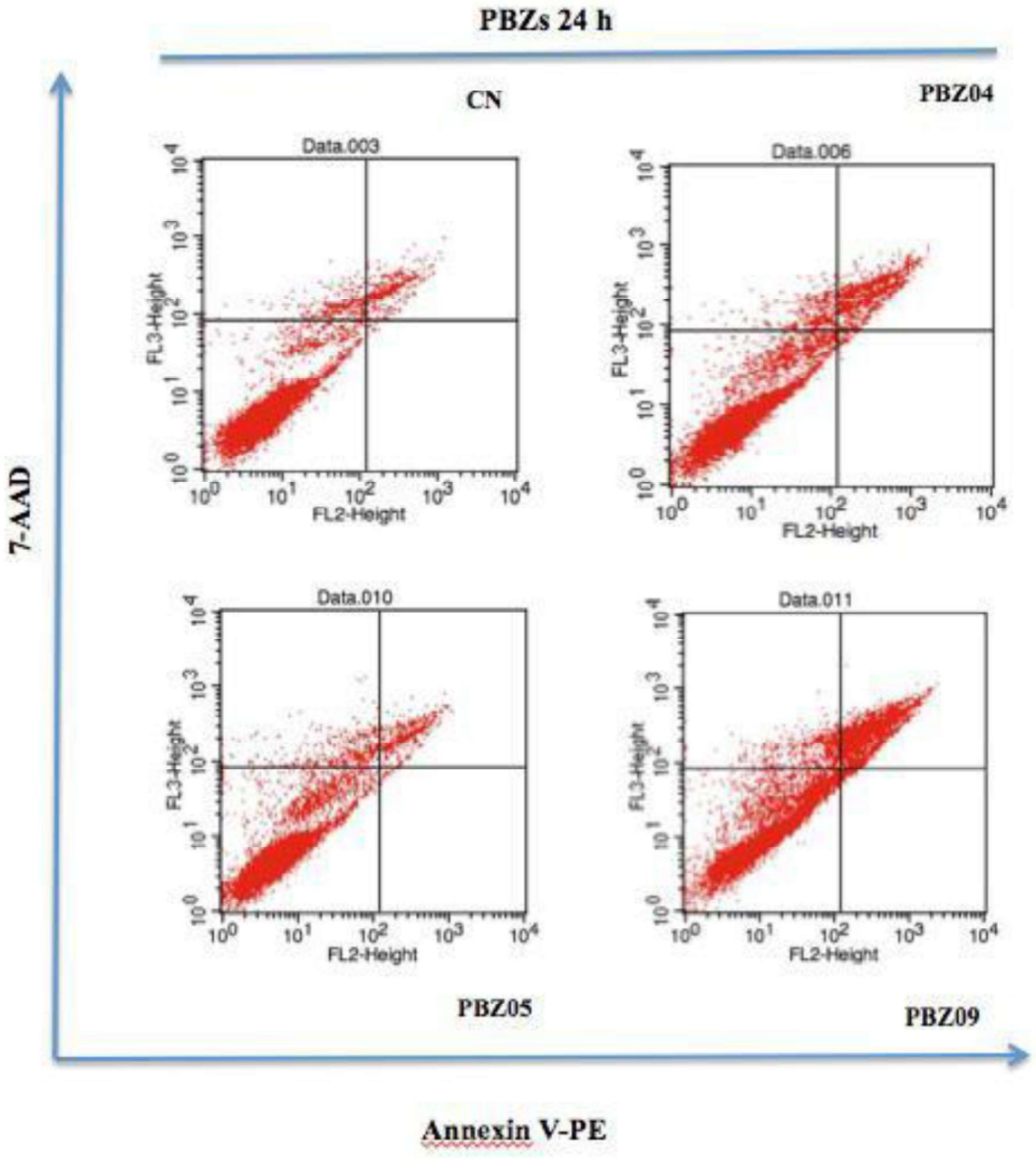


Figure 4B. Apoptosis induction in MCF-7 cells. **PBZs 04**, **05** and **09**-induced apoptosis as shown in the representative example of Annexin V flow cytometry analysis with the x axis showing Annexin V staining and the y axis 7-amino-actinomycin D (7-AAD) and phycoerythrin (PE) staining. The percentage of annexin-V-positive cells was determined in the whole-cell population (10,000 cells) by FACSCalibur flow cytometry and CELLQuest software. Percentage of cells in each quadrant, LL: Viable cells (Annexin V −/ PE −), LR: early apoptotic cells (Annexin V +/ PE −), UL: necrotic cells (Annexin V −/ PE +) and UR: late apoptotic/necrotic cells (Annexin V +/ PE +).

Genotoxicity induction

The alkaline (pH > 13) comet assay detects DNA strand breaks and alkali-labile sites. Our results showed that **PBZ04** does not generate DNA-strand breaks. However, **PBZ 05** and **09** induce signifcant DNA damage (Fig. 5). MMS (80 µM) was used as positive control.

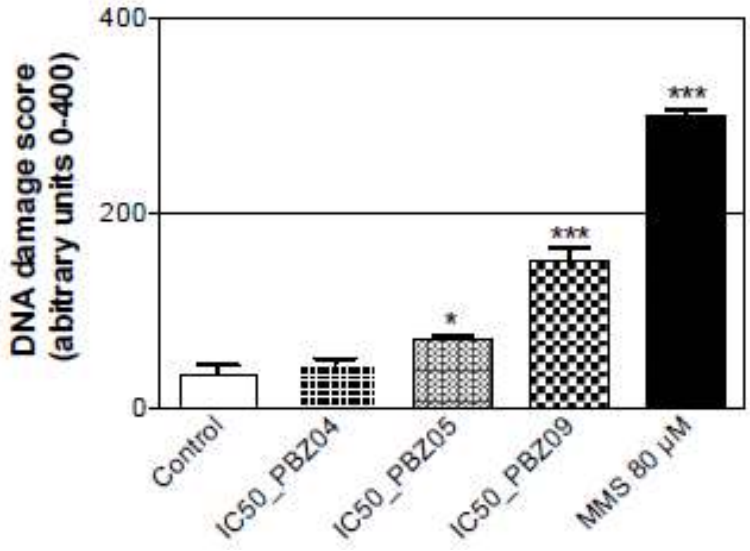


Figure 5. Effect of **PBZs 04**, **05** and **09** after 24 h of treatment on DNA damage index using MCF-7 cells, determined by comet assay. Control (untreated) or treated cells with **PBZs** at concentrations IC50 were used. Data are expressed as mean ± SD of three independent experiments. The alkylating agent methyl methane sulphonate (MMS) was used as the positive control.

ROS detection, mitochondrial depolarization and expression of γH2AX

Many compounds may sensitize cancer cells through ROS generation [23,25-26]. In addition, ROS have also been reported to be important mediators of apoptosis and also to be related to caspase activation [23]. Consequently, we investigated whether **PBZ 04**, **05** and **09** antitumoral activities in MCF-7 cells were initiated through ROS generation. Results shown in Fig. 6A revealed that both **PBZ04** and **PBZ09** induce an increase in intracellular ROS production in MCF-7 cells, as measured by flow cytometry. The intensity of the fluorescence probe increased in both **PBZs** (Fig. 6B).

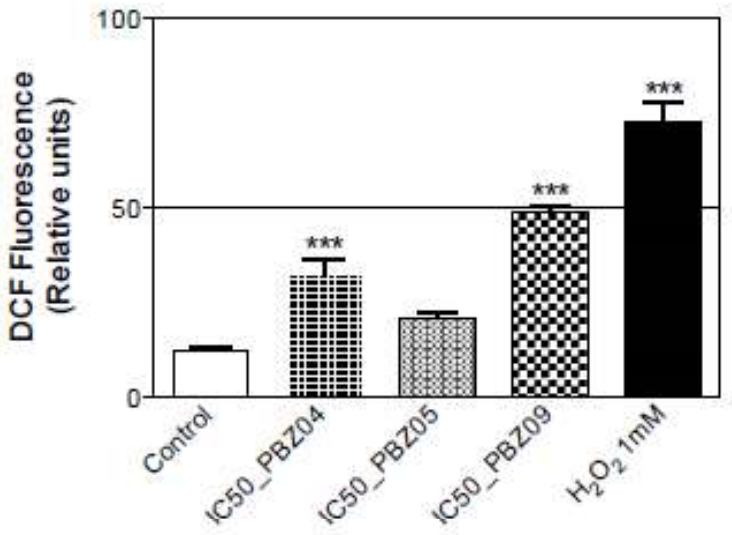


Figure 6A. Flow cytometry detection of reactive oxygen species in MCF-7 cells challenged with **PBZs 04**, **05** and **09**. Cells were treated with IC50s after 24 hours's treatment, and 1mM H_2O_2 (hydrogen peroxide). Mean data for DCF fluorescence. Results are expressed as means ± standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA), and means were compared using Tukey test, with $P \leq 0.05$ considered as statistically significant.

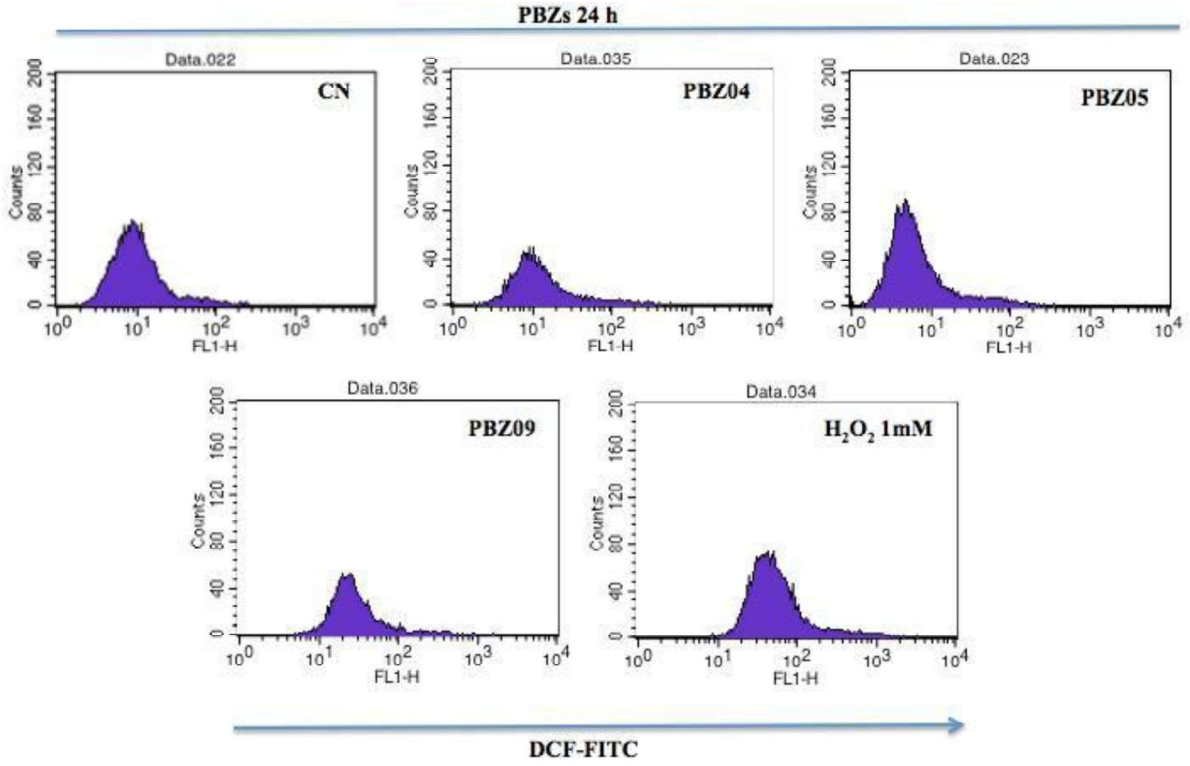


Figure 6B. Representative histograms: number of cellular events versus fluorescence intensity. FL1-H: relative DCF fluorescence intensity. Cells were treated with vehicle negative control, **PBZs 04**, **05** and **09** after 24 hours's treatment, and 1mM H_2O_2 (hydrogen peroxide).

In order to understand the implications of these findings with regards to indirect effect of PBZs on ROS production in MCF-7 cells, we treated cells with NAC, a ROS scavenger, should abrogate ROS effect. The intensity of the fluorescence probe decreased in both PBZs (Fig. 7).

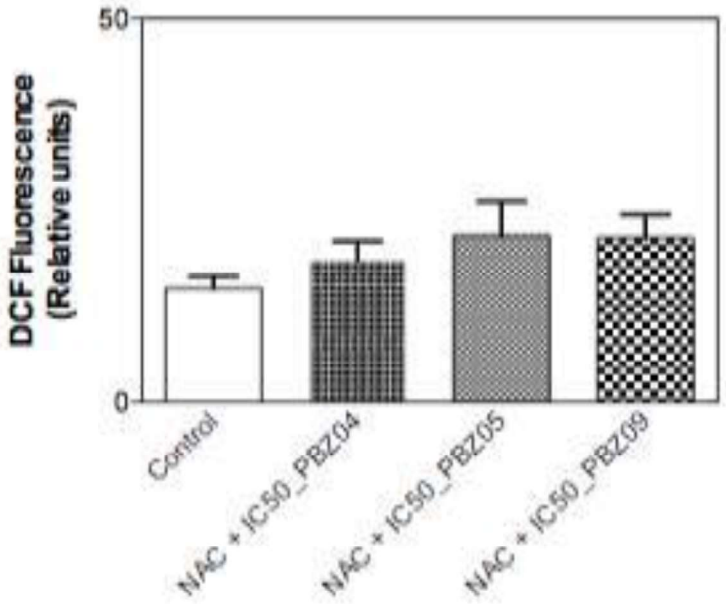


Figure 7A. PBZ04, 05 and **09**-induced 2′,7′-dichlorofluoroscein (DCF) fluorescence is blocked by antioxidants. Cells were pretreated with 2 mM *N*-acetyl-l-cysteine (NAC) for 2 h.

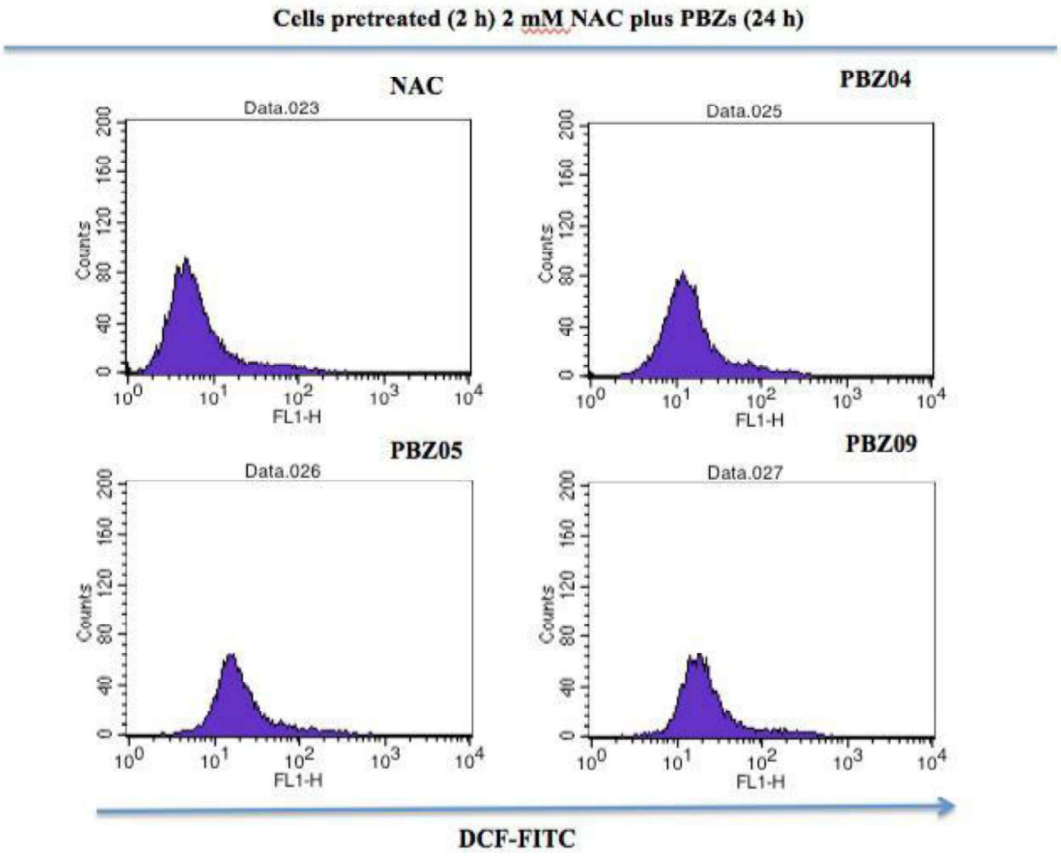


Figure 7B. Representative histograms: number of cellular events versus fluorescence intensity. FL1-H: relative DCF fluorescence intensity.

Mitochondrial electrochemical potential is correlated to the fluorescence intensity of Rho123 (with decreased fluorescence signifying loss of the mitochondrial electrochemical potential) [21]. Our flow cytometry results with MCF-7 cell showed that after 24 h treatment **PBZ 04**, **05** and **09** induce significant mitochondrial depolarization (Fig. 8).

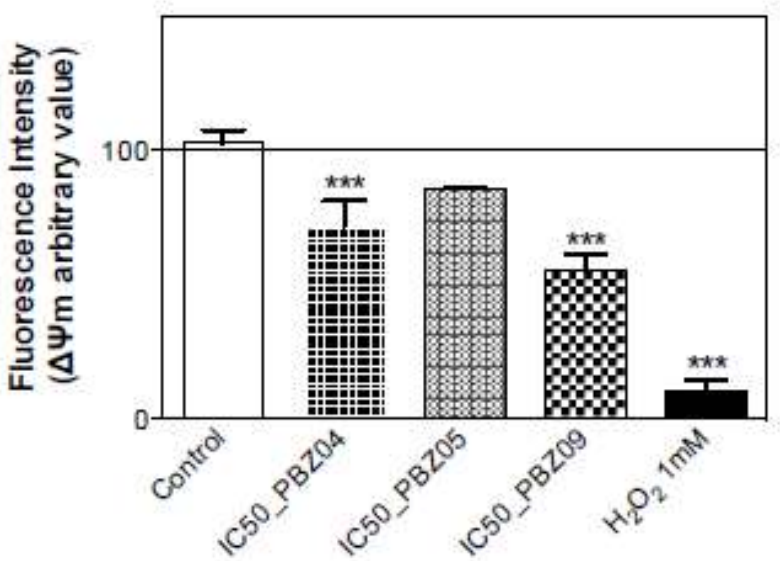


Figure 8. Decreased of mitochondrial electrochemical potential, with decreased fluorescence signifying loss of the mitochondrial electrochemical potential. Mean data for Rho-123 fluorescence. Results are expressed as means ± standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA), and means were compared using Tukey test, with $P \leq 0.05$ considered as statistically significant.

ROS are known to induce DNA damage, therefore we evaluated the expression of the phosphorylated form of H2AX (γH2AX), a well-known marker of double strand DNA breaks (DSBs) [26]. The protein γH2AX was induced after **PBZ 04**, **05** and **09** treatment and returned to the baseline levels of untreated cells after treatment with NAC (Fig. 9). These results suggested that ROS generation promotes DNA damage in MCF-7 cells treated with **PBZ 04**, **05** and **09** and therefore contributes to cell death induction.

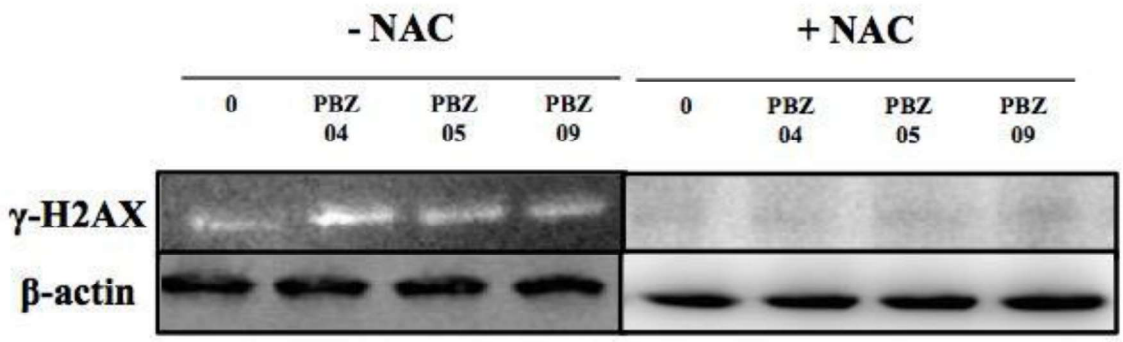


Figure 9. The level of γ-H2AX protein induction was analyzed by Western blotting. Cells were treated with **PBZs 04**, **05** and **09** for 24 h with or without NAC (2 mM). The data shown represent three independent experiments with compareble outcomes.

TOPO I inhibition

In addition, the effects of **PBZ 04**, **05** and **09** on Topo I activity were evaluated in a cell-free system. Purified human DNA Topo I was incubated with **PBZ 04**, **05** and **09** in the presence of supercoiled plasmid DNA. Relaxation of the plasmid was inhibited only by **PBZ09** (Fig. 10). The Topo I inhibitor CPT was used as positive control.

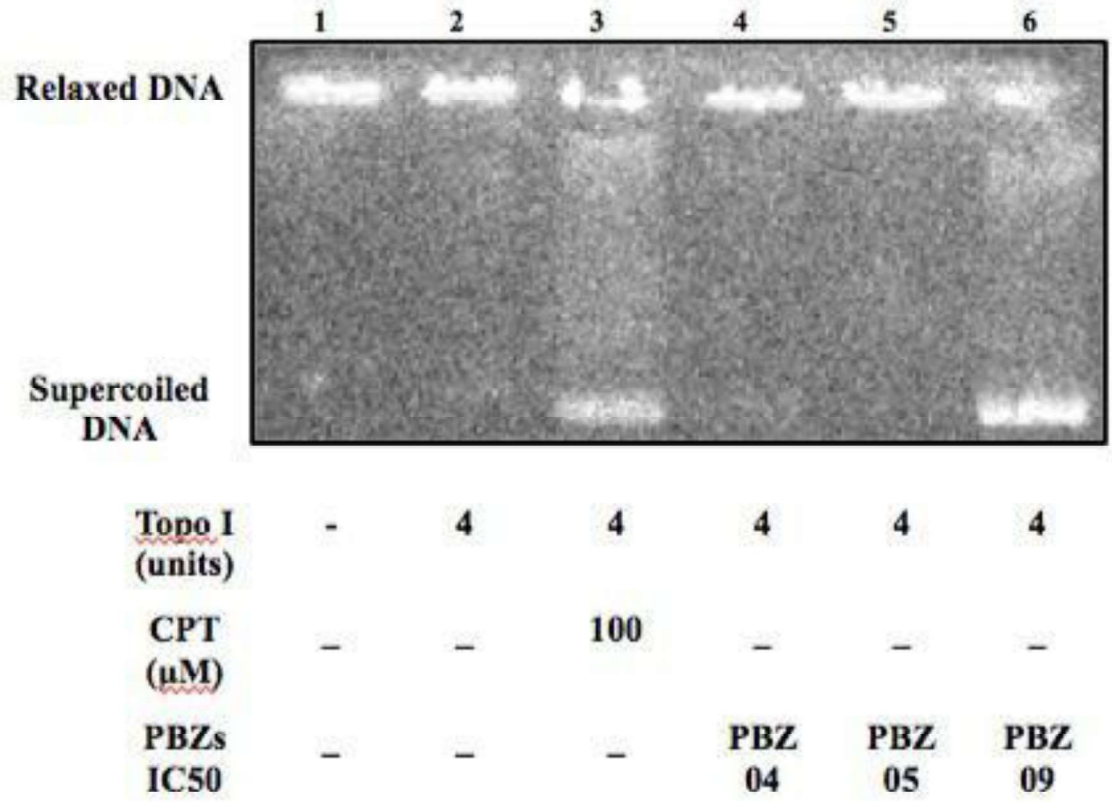


Figure 10. Inhibition of topoisomerase I-mediated plasmid relaxation in the presence of **PBZs**. Line 1 of agarose gel contains marker relaxed DNA. In the negative control, Topo I enzyme was incubated with the vehicle used for substance dilution (Line 2). Campothecin (CPT) served as positive control (Line 3). The supercoiled DNA was incubated with Topo I in the presence of **PBZs 04**, **05** and **09** at IC50. (Lines 4-6). In the negative control, Topo I enzyme was incubated with the vehicle used for substance dilution (Line 4). The DNA was analysed by electrophoresis using a 1% agarose gel. The gel was stained with ethidium bromide and photographed under UV light.

Discussion

Lack of selectivity of most cancer chemotherapeutics remains a major limiting factor and standard cancer drugs such as doxorubicin [28], cisplatin [29], and

5-fluorouracil [30] have very limited efficacy. The development of new specific therapies is necessary. In the present study we tested the effect that compounds derived from pyrimidobenzimidazoles (PBZs) cause against a panel of six human tumoral cell lines: MCF-7, HepG-2, T-24, HCT-116, HT-29, Caco-2 (Table 1), aiming to contribute with new therapeutic alternatives.

For this investigation, an initial screening with seven different **PBZs** in acute treatment was done, in a tentative to select only the **PBZs** capable to cause great impact on cell viabilitty. Subsequently, selected **PBZs 04**, **05** and **09**were used for long time treatments, in a sub chronic (72 h) and chronic (120 h) treatment just only in MCF-7 cells (Fig. 2).

Considering the cytotoxic results and selectivity index, we chosed the 24 h treatment to verify the **PBZs 04**, **05** and **09** cytotoxic mechanisms in tumoral cell line MCF-7 cells. To determine whether the decrease in cell viability was related to apoptosis and/or necrosis induction, Annexin V assay was performed in MCF-7 cells treated with **PBZs 04**, **05** and **09** (IC50 values) for 24 h. Surprisingly, the three compounds increased the apoptotic and a great number of necrotic cells. These agents appear to act rapidly on the mitochondria (Fig. 8), resulting in necrotic cell death (Fig. 4A-B). The cell data support a great selectivity of **PBZ09** for cancer cells at low drug concentration (8.11 μM), without any noticeable toxicity against human nontumoral cells. When energy demand is greater than supply as it is seen with necrosis, cells encounter a bioenergetic catastrophe that leads to necrotic cell death with a rapid ATP depletion [31-33]. Recently, the Food and Drug Administration approved anticancer agent topotecan, shown to result in necrotic cell death, increased levels of major histocompatibility complex class I proteins in breast cancer cells, leading to increased cytokine release, immune stimulation, and cancer death [34].

Many compounds may sensitize cancer cells through ROS generation [23]. Elevated ROS levels cause extensive DNA lesions, PARP1 hyperactivation, and severe NAD+/ATP depletion that stimulate Ca^{2+}-dependent programmed necrosis [35]. On the basis of this evidence and our observations, we ascertained the effect of **PBZs 04** and **09** on ROS production (Fig. 6A-B), suggesting that cell death might be due to the presence of high level of ROS. ROS are highly reactive molecules that can

oxidize macromolecules, such as DNA [36]. The comet assay is sensitive and valuable technique to detect primary (repairable) single and double-strand DNA breaks, as well asalkali-labile sites in the alkaline version. The results indicate that the three **PBZs** induced significant DNA strand breaks in MCF-7 cells (Fig. 5).

The cell cycle analysis also showed a significant increase in the fraction of G0/G1 phase in the treated cells when compared to control cells (Fig. 3A-B). Induction of G0/G1 growth arrest and DNA polyploidy (>4 N) by a variety of drugs were associated with increase in DNA damage response and checkpoint proteins like γH2AX. Level of γH2AX, known to be a marker of DNA damage [37-39], were increased after **PBZs 04**, **05** and **09** treatment and level in treatment cells returned to baseline after treatment with NAC (Fig. 7A-B; Fig. 9).

Planar fused benzimidazole analogs have the potential to get inserted into the space between the base pairs of DNA resulting in DNA cleavage [7]. Since PBZs also display a planar structure, we decided to investigate if PBZs could interact with the TopoI enzyme. Consistently, just **PBZ09** inhibited human Topo I activity in vitro (Fig. 10). A key function of topoisomerase I enzyme is relaxation of DNA supercoiling ahead of the replication and transcription machinery, and during DNA repair and chromatin remodelling [40,41]. Inhibitors of topoisomerase I block the second transesterification reaction, which prevents the DNA religation step and stabilizes the cleavage complex with topoisomerase I. Thus, transient reversible topoisomerase I-cleavage complex intermediates can be converted into irreversible DNA lesions upon collision with the DNA replication or transcription machinery. The DNA replication fork collision with the drug-stalled topoisomerase I-cleavage complex on the leading strand produces a replication-dependent DNA double-strand end, resulting in so-called replication fork run-off [42]. On the whole, we consider that **PBZ09** is a potent Topo I inhibitor and acts as a kind of Topo I suppressor. **PBZ09** can insert into DNA base pairs, resulting in DNA structure distortion, and then inhibiting the binding of DNA to Topo I, finally affecting the catalytic activity of Topo I.

Reported literature has shown the various substituted derivatives of benzimidazole nucleus exhibiting remarkable biological activity as antitumor/antiproliferative/anticancer activity [43]. Based on this activity, a benzimidazo[1,2-*a*]quinoline derivative has exerted potent activity on all cell lines

tested with IC50 of 0.8-30 μM and showed a special selectivity toward HeLa cells [43]. More fused planar benzimidazole derivatives have been reported to exhibit potent cytotoxicity. The examplaries include pyrimido[1,2-*a*]benzimidazole-3(4*H*)-one [45] and 1,3-diarylpyrazino[1,2- *a*] benzimidazole [46]. Bis-benzimidazoles are another class of compounds exploited in the discovery of an effective anticancer agent. The Hoechst-33342 is the openers of this series exhibiting in vitro antitumor as well as DNA topoisomerase I inhibitory activities [47]. *N*-aminomethyl-1*H*-benzimidazole-5-carboxylic acid derivatives showed potent growth-inhibitory activity against tumoral cell lines and inhibited topoisomerase II activity at 10 times lower concentration than etoposide in relaxation assay [48].

Why particular **PBZ09** exhibit enhanced cytotoxicity in MCF-7 cancer cell lines compared with others cancer cells and normal cells, and why this compound exhibited increased cytotoxicity compared with **PBZ04** and **05**, it is not obvious from comparing the chemical structures of these molecules. However, some correlations of **PBZ04**, **05** and **09**structure and activity can be made. For example, the compound with a methyl group at the position 4 of the pyrimidine ring (**PBZ05**, Fig.1) was less active than the compounds with hydrogen at the same position (structure **PBZ 04** and **09**, Fig.1). In addition, the introduction of a hydroxyethyl group at the position 3 of the pyrimidine ring (**PBZ09**) seems to enhance cytotoxicity. This may be associated with the nucleophilicicity of such group which can contribute to its increased activity in MCF-7 cells. Even taking these features into account, the cytotoxicity of individual **PBZ09** most probably represents a multi-factorial interplay between innate chemical properties and the metabolic pathways that are active in the different cell lines.

Conclusions

The present work, demonstrated that especially **PBZ09** promotes a reduction in cell viability by necrosis and apoptosis. According to our results, summarized in Figure 11, these events might well be consequence to the putative interaction of **PBZ09** with Topo I enzyme (direct action) and ROS generation (indirect action). There is an increased DNA damage with increased levels of γH2AX (known to be a marker of DNA damage), leading to cell cycle arrest and cell death induction.

Our data indicate that **PBZ09** is a small molecule that may potentially be used for future clinical management of breast cancer.

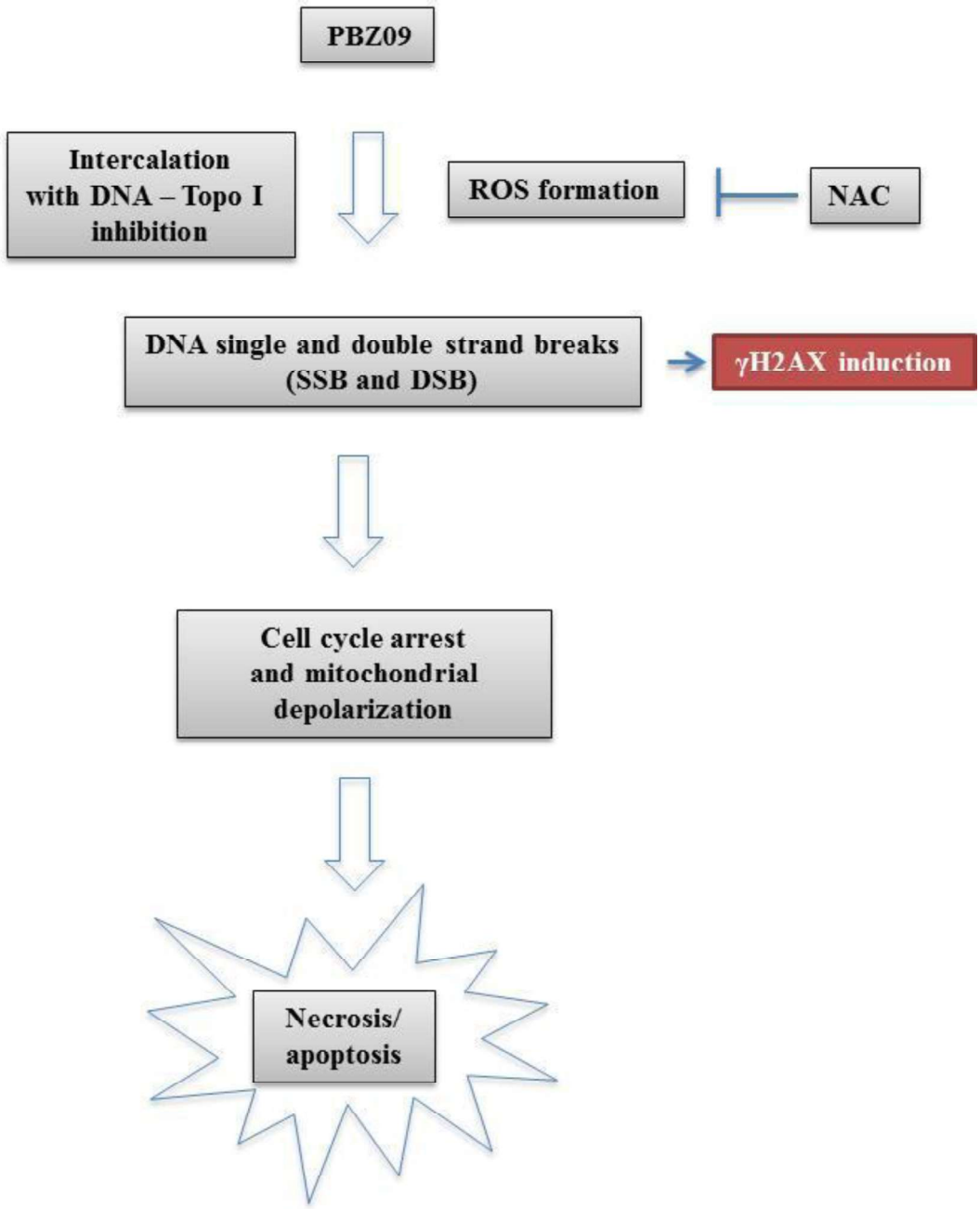


Figure 11. Schematic representation of the plausible molecular mechanism proposed for the induction of cytotoxicity by **PBZ09**. The induction of single (SSB) and double (DSB) strand breaks originated by topoisomarese I inhibition and/or ROS formation as the main mechanisms involved in cell death.

Acknowledgments

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Authors' details

[1]Department of Basic Health Sciences - Laboratory of Genetic Toxicology - Federal University of Health Sciences of Porto Alegre (UFCSPA), Porto Alegre, RS – Brazil

[2]Department of Pharmacosciences - Federal University of Health Sciences of Porto Alegre (UFCSPA), Porto Alegre, RS - Brazil

[3]Chemical Core Heterocycles (NUQUIMHE), Chemistry Department, Federal University of Santa Maria, Santa Maria, RS - Brazil

Authors'contributions

CMV, DCE, NAB, RBS carried out all assays relating to the study and participated in the drafting of the manuscript. SSA and JMS participated evenly in all assays relating to the study. JS and NZ conceived the study, were responsible for the design and coordination of this study and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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5 CONSIDERAÇÕES FINAIS

O conjunto de dados gerados neste trabalho evidencia que uma nova classe de compostos pirimidobenzimidazóis (PBZs) possui potencial citotóxico para o tratamento do câncer, especialmente para o câncer de mama. Dentre os sete compostos testados, os PBZs 04, 05 e 09 foram capazes de inibir o crescimento da linhagem tumoral MCF-7 na faixa do IC50 de 20 μM, sendo o PBZ 09 o mais efetivo na citotoxicidade nesta mesma linhagem (8,11 μM) e com a seletividade de 3x maior em comparação as células não tumorais (HEK-293).

A interação direta do composto PBZ 09 com enzima topoisomerase I, além da sua capacidade de induzir a formação de espécies ativas de oxigênio e o consequente aumento de danos no DNA, parecem ser os principais responsáveis pela indução da morte celular observada.

Para corroborar se de fato houve o aumento de espécies reativas de oxigênio, as células foram previamente tratadas, durante 2 h, com N-acetilcisteína (2 mM). A N-acetilcisteína é um antioxidante que atua diretamente como sequestrador de radicais livres, além de ser um precursor da glutationa importante antioxidante presente no organismo. De fato, a N-acetilsisteína inibiu a produção de ERO causada pelos PBZs 04 e 09, conforme demonstrado pela diminuição da intensidade relativa do fluoróforo DCF e na expressão da proteína fosforilada γH2AX.

Portanto, o PBZ 09 parece ser uma molécula promissora para futuros estudos *in vivo*, uma vez que foi bastante seletiva para a linhagem tumoral MCF-7 e não afetou a linhagem normal, situação ideal que se busca para os esquemas quimioterápicos.

6 PERSPECTIVAS

• Realizar ensaios de viabilidade, ciclo e morte celular em tempos mais longos de exposição com os PBZs 04, 05 e 09, utilizando também outras linhagens de câncer de mama;

• Avaliar o envolvimento de caspases sinalizadoras e efetoras na resposta celular induzida pelos compostos PBZs 04, 05 e 09;

• Verificar a expressão de genes de reparo de DNA antes e após os tratamentos;

• Utilização de um modelo *in vivo* de câncer de mama para averiguar a ação dos PBZs 04, 05 e 09.

ANEXO A - REGISTRO DO PROJETO NA COMISSÃO DE PESQUISA

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COMISSÃO DE PESQUISA

Atestado

Atestamos, para o fim de inscrição de projeto de pesquisa no processo seletivo de iniciação científica e iniciação tecnológica e inovação da UFCSPA, que os projetos de pesquisa abaixo listados estão registrados na Comissão de Pesquisa:

Pesquisador Responsável	Título do projeto de pesquisa	Número de registro
Jenifer Saffi	Bioprospecção sistemática de extratos vegetais para a busca de novos agentes antineoplásicos	008/2014
Jenifer Saffi	Prospecção de novos compostos sintéticos pirimídicos como potenciais medicamentos para a terapia do câncer	014/2014
Josiane Somariva Prophiro	Estudo da atividade tripanocida e imunomoduladora de Photorhabdus luminescens e Xenorhabdus nematophilia (Enterobacteriaceae)	015/2014
Kellen Cristhinia Borges de Souza	Padronização e avaliação da ação antimicrobiana de extrato de folhas de Psidium cattleianum (araçá) como matéria prima para formulações de higiene oral	004/2013
Kellen Cristhinia Borges de Souza	Desenvolvimento tecnológico e avaliação da ação antimicrobiana de extrato de Acca sellowiana	005/2013
Kellen Cristhinia Borges de Souza	Determinação de parâmetros de qualidade e estudo preliminar da estabilidade de tintura de Passiflora edulis (maracujá)	006/2013
Luiz Carlos Rodrigues Junior	Efeitos imunobiológicos de nanocápsulas poliméricas contendo o epitopo SSIEFARL do Herpex Simplex Virus-1 em células dendríticas murinas	047/2015
Maiquidieli Dal Berto	Efeito antitumoral e na modulação de genes da apoptose de extratos de alimentos cafeinados em células DU145 de câncer de próstata	026/2014
Márcia Rosângela Wink	Avaliação de marcadores de célululas iniciadoras de tumores originadas de linhagens de câncer cervical humano	045/2015
Maria Ismenia Zulian Lionzo	Preparação e caracterização de sistemas matriciais de quitosana	023/2014
Maria Ismenia Zulian Lionzo	Estudo da interação de conjugados peptídeo-lipossoma	024/2014

Porto Alegre, 06 de abril de 2015.

Paulo Ricardo Gazzola Zen
Coordenador Geral da Pesquisa
UFCSPA

ANEXO B: NORMAS DA REVISTA *INVESTIGATIONAL NEW DRUGS*

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Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

SCIENTIFIC STYLE

Genus and species names should be in italics.

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Citation

Reference citations in the text should be identified by numbers in square brackets.
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2. This result was later contradicted by Becker and Seligman [5].
3. This effect has been widely studied [1-3, 7].

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- Book

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- Book chapter

  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257
- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007
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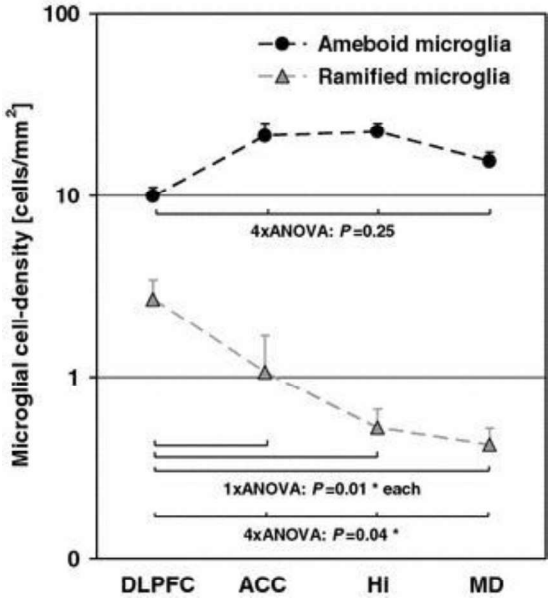

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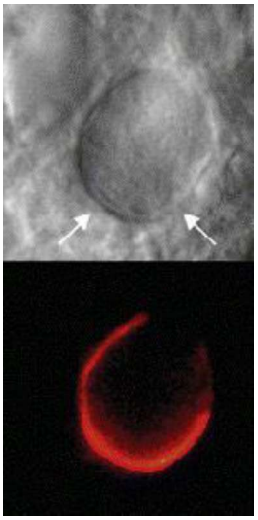

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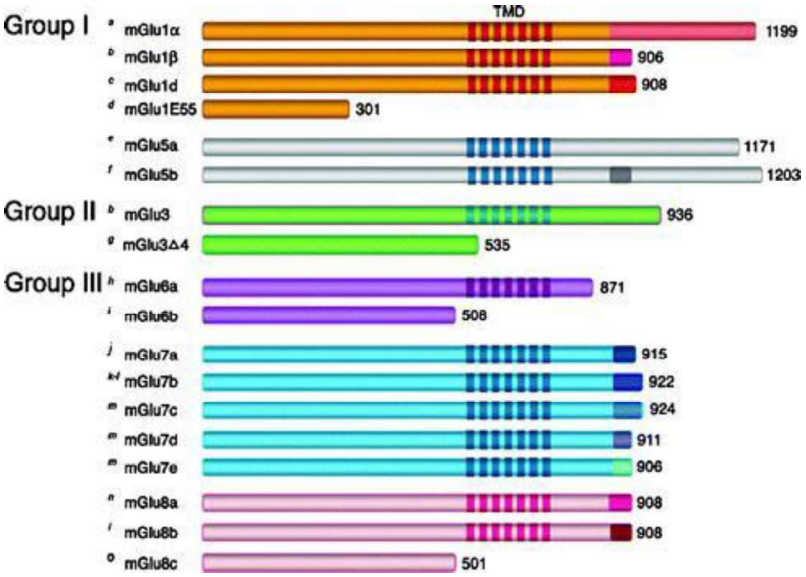

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