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Derivados retinóides para o tratamento de câncer de mama: estudos de mecanismos celulares e moleculares

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Dissertação de Mestrado

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Farmácia da Universidade Federal de Mato Grosso do Sul como requisito parcial para a obtenção do título de Mestre em Farmácia.

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Dedico aos meus pais que guiaram meus passos até aqui e me fizeram acreditar que o sonho era possível.

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Albert Einstein

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SUMÁRIO

RESUMO

O câncer de mama é o tipo de câncer mais comum entre as mulheres e acomete milhares de mulheres todos os anos. Sua incidência vem aumentando juntamente com seus fatores de risco. Grandes são os esforços para o desenvolvimento de novas terapias mais eficientes e menos tóxicas, sendo os estudos com derivados retinóides muito promissores nestes aspectos. Os retinóides desempenham papel em diversas funções biológicas, incluindo controle de crescimento e apoptose, e suas funções genômicas são mediadas através de ligações a receptores nucleares específicos presentes em células tumorais de mama. Tratamentos envolvendo combinação entre retinóides e outros agentes quimioterápicos estão sendo testados e apresentados como novas alternativas, a fim de obter efeitos sinérgicos no controle de proliferação e morte celular. O presente trabalho analisou os mecanismos celulares e moleculares envolvidos na resposta a duas novas moléculas retinóides denominadas de RT1 e RT3, derivadas de modificações moleculares nos compostos líderes AM80 e AM580, ligantes seletivos RARα, e sua associação aos agentes antitumorais 5-fluorouracil, gencitabina, irinotecano, metotrexato, paclitaxel e tamoxifeno, em células da linhagem de adenocarcinoma de mama MCF-7. A citotoxicidade dos compostos foi avaliada juntamente com sua capacidade de induzir apoptose e interferir na proliferação celular. Adicionalmente foram analisados os efeitos das combinações entre os compostos e diferentes agentes quimioterápicos. Os resultados mostraram que os fármacos diminuem a viabilidade celular e causam morte celular por apoptose e redução no número de células em proliferação em períodos curtos de tratamento. Além disso, quando combinados com o tamoxifeno, os retinóides potencializaram a morte celular por apoptose, bem como a redução de células proliferativas. Assim, estas observações possibilitam o desenvolvimento de novos protocolos terapêuticos que utilizem retinóides seletivos no tratamento de câncer de mama que poderão proporcionar uma menor toxicidade, melhor interação e ativação com os receptores nucleares.

Palavras-chave: câncer de mama, receptores retinóides, retinóides, quimioterapia, MCF-7

ABSTRACT

Breast cancer is the most common type of cancer among women and affects thousands of women every year. Incidence is increasing along with their risk factors. Great efforts have been done to develop new more efficient and less toxic therapies, and studies with derivatives retinoids are very promising in these aspects. Retinoids play a role in several biological functions including growth and apoptosis control, and their genomic functions are mediated through connections to specific nuclear receptors in breast tumor cells. Treatments based on retinoids combination with chemotherapeutic agents are being investigated and presented as new alternatives in order to achieve synergistic effects in cellular proliferation control and cell death. This study analyzed cellular and molecular mechanisms involved in the response to two new molecules retinoids called RT1 and RT3 derived from molecular changes to the leads compounds AM80 and AM580, selectives ligands RARα, and its association with antitumor agents 5-fluorouracil, gemcitabine, irinotecano, methotrexate, paclitaxel and tamoxifen in breast adenocarcinoma line cells MCF-7. Cytotoxicity was performed along with its ability to induce apoptosis and interfere in cell proliferation. In addition the effects of combinations of compounds and various chemotherapeutic agents were analyzed. Results showed that the drugs decreased cell viability, caused cell death by apoptosis and decrease in number of proliferating cells in short periods of treatment. Furthermore, when combined with tamoxifen, retinoids potentiated cell death by apoptosis, as well as the reduction of proliferating cells. Thus, these findings allow the development of new treatment protocols using selective retinoids in treating breast cancer that may provide a lower toxicity, better interaction and activation with nuclear receptors.

Keywords: breast cancer, retinoids receptors, retinoids, chemotherapy, MCF-7

LISTA DE ABREVIATURAS E SIGLAS

APL - Leucemia promielocítica aguda

ATRA - Ácido trans-retinóico

BRCA 1 - gene Câncer de mama 1

BRCA 2 - gene Câncer de mama 2

BrdU - (5-bromo-2-desoxiuridina) – Bromodesoxiuridina

CI - Índice de combinação

IC_{50} - Concentração à qual um composto possui a capacidade de inibir o crescimento celular em 50%

HER2 - Receptor de crescimento epidermal humano 2

MTT - brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difeniltetrazólio

MTX: Metotrexato

PBS - Tampão Fosfato-Salino

PFA - Paraformaldeído

RA - Ácido retinóico

RAR - Receptor de ácido retinóico

RE - Receptor de estrogênio

RP - Receptor de progesterona

RT1 - 4-[4-(5,5,8,8-tetrametil-5,6,7,8-tetrahidronaftalen-2-il)-1*H*-1,2,3-triazol-1-il]ácido benzóico

RT3 - 4-[4-(5,5,8,8-tetrametil-5,6,7,8-tetrahidronaftalen-2-il)-1*H*-1,2,3-triazol-1-il] anilina

RXR - Receptor X retinóide

SBF - Soro Bovino Fetal

TAM: Tamoxifeno

5-FU: 5-Fluorouracil

9-cis-RA - Ácido 9-cis-retinóico

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Capítulo I

1. Revisão da literatura

1.1. Câncer

Câncer é o nome dado a uma coleção de doenças nas quais ocorre o crescimento rápido e desenfreado de células anormais, podendo afetar negativamente as células saudáveis e levar à morte do paciente (AL-SHEDDI et al., 2015). Estas células anormais podem se proliferar em diferentes tipos de tecidos, que incluem ossos, músculos ou cartilagens, podendo se espalhar para outras regiões do corpo formando metástases (INCA, 2017a). O processo de carcinogênese está relacionado com diversos fatores, tais como: desregulação da angiogênese; alterações genéticas herdadas; danos no DNA induzidos por agentes físicos, químicos e biológicos; e o envelhecimento (WANG et al., 2015). Entretanto, as chances de cura são muito prováveis quando detectado precocemente e tratado de maneira adequada (IBCC, 2017).

Somente no ano de 2012, cerca de 14 milhões de novos casos surgiram no mundo todo e foram reportadas 8,8 milhões de mortes relacionadas à doença no ano de 2015, sendo que 70% do total de mortes ocorreram na África, Ásia e América Central e do Sul. Nas próximas duas décadas é esperado um aumento em torno de 70%, de 14 milhões para 22 milhões, de novos casos (WHO, 2017). Segundo estimativas do INCA (2016) para o Brasil, biênio 2016-2017, ocorrerão cerca de 600 mil novos casos de câncer e o perfil epidemiológico observado é semelhante ao da América Latina e do Caribe.

1.2. Câncer de mama

Dentre os diversos tipos de câncer, o câncer de mama é o tipo mais comum entre as mulheres e sua incidência está aumentando na maioria dos países e, para os próximos 20 anos, está projetado para aumentar ainda mais, mesmo com todos os esforços atuais para prevení-lo (ECCLES et al., 2013; ARNOLD et al., 2013; RAHIB et al., 2014; COLDITZ & BOHLKE, 2014; HOWELL et al., 2014). Este tipo de câncer foi responsável por 571.000 mortes em 2015, no mundo (WHO, 2017), e 14.388 mortes em 2013, no Brasil, sendo estimado o surgimento de 57.960 novos casos, apenas em 2016 (INCA, 2017b).

Estudos sugerem que milhares de genes podem contribuir com as fisiopatologias do câncer de mama quando desregulados por meio de alterações genômicas e epigenéticas (NEVE et al., 2006). O aumento de sua incidência tem sido relacionado com o aumento no número dos fatores de risco como: menstruação precoce; primeira gravidez tardia; menos gravidezes; menores ou nenhum período de amamentação; menopausa tardia; aumento da obesidade; consumo de álcool; inatividade; terapia de reposição hormonal (TRH) (COLDITZ & BOHLKE, 2014; HOWELL et al., 2014); e mutações em genes de reparo de DNA BRCA 1 e BRCA 2 (SAMAVAT & KURZER, 2016).

Tumores de mama em estágio inicial exigem abordagens multifatoriais de tratamento para erradicação residual e prevenção de possível recorrência da doença. Sendo assim, compreender as vias que promovem ou sustentam o crescimento e invasão das células tumorais é essencial para um tratamento eficaz (LIN & RUGO, 2007; VOGT et al., 2007; SCHLOTTER et al., 2008). Segundo a *American Cancer Society* (ACS, 2014b), a doença pode ter início em diferentes partes da mama, tais como nos ductos galactóforos que levam o leite ao mamilo; nas glândulas que secretam o leite materno; e em tecidos da mama. Por apresentarem diferentes localizações e características, seus tipos são diversos: o carcinoma ductal *in situ* ou carcinoma intraductal (DCIS) considerado não invasivo; o carcinoma ductal invasivo (IDC) iniciado nos ductos e capaz de desenvolver metástase; o carcinoma lobular invasivo (ILC) encontrado nas glândulas produtoras de leite (lóbulos); o câncer de mama inflamatório (IBC) um tipo invasivo raro; e o angiosarcoma encontrado em células que revestem os vasos sanguíneos ou vasos linfáticos (ACS, 2014a).

O estrogênio, hormônio esteróide derivado do colesterol C27 (SAMAVAT & KURZER, 2016), desempenha um papel importante no desenvolvimento e progressão de cânceres de mama (BUZDAR & HOWELL, 2001; DOWSETT, 2003; BUZDAR, 2009). Segundo Winer (2005), três quartos de todos os tumores de mama invasivos são receptores de estrogênio positivo (RE+) ou receptores de progesterona positivo (RP+), tornando o receptor de estrogênio (RE) um importante alvo para terapias endócrinas (hormonais) que visam o bloqueio de ação do estrogênio. Estudos moleculares de terapias-alvo envolvendo os REs e o tamoxifeno (modulador seletivo RE), e o receptor de crescimento epidermal humano 2 (*HER2*) e o trastuzumabe (anticorpo monoclonal) mostram melhorias consideráveis em taxas

de cura e prevenção do câncer de mama (SCHLOTTER et al., 2008). Infelizmente, muitas vezes, existem resistências aos tratamentos que podem limitar os benefícios oferecidos pelas terapias. Estas informações nos levam a estudar novas estratégias para terapias voltadas à indução de apoptose e interferências na transdução do sinal, angiogênese e ciclo celular.

1.3. Vitamina A e retinóides

A vitamina A (retinol) é o composto de origem natural e protótipo dos retinóides, que estão presentes em todos os organismos vivos (DI MASI et al., 2015). Ela desempenha papel crucial nos processos biológicos, incluindo crescimento e diferenciação celular, desenvolvimento, metabolismo e imunidade (ORTIZ et al., 2002). Seus derivados, os metabólitos ativos do ácido retinóico ou retinóides (classe de análogos da vitamina A), podem ser de moléculas de origem natural, como os compostos ácido trans-retinóico (ATRA) e ácido 9-cis-retinóico (9-cis-RA) ou sintéticas, tais como o TTNPB e o bexaroteno (BOEHM et al., 1994).

Retinóides são naturalmente de baixo peso molecular, isoprenóides insaturados solúveis em gordura, exercem as suas funções biológicas principalmente pela regulação da expressão do gene (BUSHUE & WAN, 2010) e desempenham um papel essencial em vários aspectos da fisiologia e desenvolvimento dos mamíferos, incluindo a espermatogênese, fertilização, manutenção da gravidez, morfogênese, organogênese, crescimento fetal, perinatal (DI MASI et al., 2015), além de ajudar a manter a homeostase e mediar a proteção contra doenças como o câncer (DAS et al., 2014). No adulto, eles regulam a reprodução, função imune, visão, proliferação celular, diferenciação, a ativação de genes supressores de tumores (ALIZADEH et. al., 2014), e são necessários para o bom funcionamento da pele, pulmão, medula óssea, fígado e sistema neuronal (DI MASI et al., 2015).

As funções genômicas dos retinóides são mediadas através das suas ligações a receptores retinóides nucleares específicos de DNA que podem estar presentes em tumores de mama (DAS et al., 2014). Por esta razão, os retinóides têm sido estudados em pacientes com carcinoma de mama (ALSAFADI et al., 2013) a fim de inibir a proliferação das células cancerosas e induzir a diferenciação de células malignas.

Com a finalidade de obter aumento da potência e diminuição dos efeitos adversos e toxicidade desses fármacos, uma série de modificações moleculares foram realizadas em agentes retinoidais e os novos análogos sintetizados mostraram um grande potencial contra uma série de malignidades como: câncer de ovário, mama, próstata, leucemia, entre outras (SIMONI et al., 2001). O grande desafio para os químicos que sintetizam novos retinóides é projetar e sintetizar ligantes de receptores de subtipos específicos (DAS et al., 2014).

1.4. Receptores retinóides e mecanismos de ação

Os receptores retinóides pertencem à superfamília dos receptores dos esteróides, dos hormônios tireoidianos e da vitamina A (COSTA, 2004). Duas classes distintas de receptores retinóides têm sido identificadas: os receptores do ácido retinóico (RAR) e receptores de retinóide X (RXR). Cada classe de receptor contém três subtipos - α, β, e γ, suas diferentes isoformas e localizações cromossômicas. Esses subtipos são produtos de genes diferentes e cada um apresenta um perfil de expressão espaço-temporal diferente durante o desenvolvimento embrionário, que varia em função do tipo celular (MANGELSDORF et al., 1994; CHAMBON, 1996; BASTTIEN & ROCHETTE-EGLY, 2004; COSTA, 2004). O RAR pode ser ativado pelo ATRA e 9-cis-RA, enquanto que o RXR é ativado exclusivamente por 9-cis-RA e podem formar homo- e heterodímeros com outros receptores (BUSHUE & WAN, 2010).

Os retinóides exercem suas ações principalmente através da ligação aos receptores que são reguladores homeostáticos e de transcrição (CONNOLLY et al., 2013). Desde 1991 estudos apontam os efeitos dos retinóides sobre a proliferação e a diferenciação celular mostrando que tais efeitos são possíveis a partir da indução ou repressão de genes que codificam os fatores de crescimento (SPORN & ROBERTS, 1991) e componentes da matriz extracelular (VASIOS et al., 1991). Os receptores retinóides regulam a transcrição de genes através do recrutamento de co-repressores e coativadores (DAS et al., 2014).

1.5. Retinóides e sua aplicação na terapia antitumoral

Os retinóides têm sido investigados extensivamente pela sua utilidade na prevenção e tratamento do câncer. Eles podem induzir a diferenciação e/ou apoptose, mostram atividade antiproliferativa e anti-oxidante, apresentando grande

potencial como quimioterapêuticos ou agentes quimiopreventivos (DAS et al., 2014). Estudos com pacientes com tumores sólidos apresentaram sensibilidade ao ácido retinóico (ARRIETA et al., 2010; BRYAN et al., 2011; ALSAFADI et al., 2013), sugerindo sua eficácia para alguns subconjuntos. O sucesso também tem sido alcançado com a sua utilização no tratamento de subtipos de leucemia abrigando translocações cromossômicas (CONNOLLY et al., 2013). Eles também têm sido relatados para evitar vários tipos de cânceres, incluindo o carcinoma hepatocelular (SHIOTA & KANKI, 2013; ALIZADEH et al., 2014).

A tretinoína é um exemplo de retinóide utilizado em ensaios clínicos para o tratamento de diversos tipos de câncer como o linfoma, a leucemia, melanoma, câncer do pulmão, câncer cervical, câncer do rim, neuroblastoma e glioblastoma (BUSHUE & WAN, 2010).

Para aumentar a potência e diminuir os efeitos adversos e a toxicidade desses fármacos, uma série de modificações moleculares foram realizadas nos agentes retinoidais, e seus análogos obtidos mostram um grande potencial contra uma série de malignidades como: câncer de ovário, mama, próstata, leucemia, entre outras (BOEHM et al., 1994; BENBROOK et al., 1997; LI et al., 1998; CLIFFORD et al., 1999; KIKUCHI et al., 2000; SIMONI et al., 2000; SIMONI et al., 2001; SIMONI et al., 2005; GARCIA et al., 2012). Segundo estudos de Gianni et al. (2000), em linhagens celulares NB4 de leucemia promielocítica aguda (APL), os retinóides ATRA, 9-cis-RA, TTNPB e AM580, induzem a inibição do crescimento, diferenciação granulocítica e apoptose. No entanto, o tratamento com o derivado retinóide AM580 se mostrou mais ativo do que o ATRA ou 9-cis-RA na indução da maturação de granulócitos e apoptose, causando redução de células NB4.

Novos agentes retinóides quimiopreventivos também foram testados em modelos pré-clínicos como terapias-alvo das vias de sinalização não endócrinas, sendo identificados os RXRs como agentes mais indicados para prevenção de tumores de mama RE- por se mostrarem mais eficazes (WU et al., 2000; WU et al., 2002; WU et al., 2002; MAZUMDAR et al., 2012).

Tratamentos que combinam o uso de retinóides e outras drogas são novas alternativas que podem conduzir efeitos sinérgicos sobre o controle do crescimento ou indução de apoptose (ALTUCCI et al., 2007). As combinações de drogas fazem com que se utilizem baixas concentrações de retinóides ou drogas já estabelecidas, o que possibilita manter a eficácia terapêutica, além de reduzir efeitos secundários

ou probabilidades de resistência aos tratamentos (ALTUCCI et al., 2007;BUSHUE & WAN, 2010;CONNOLLY et al., 2013; DI MASI et al., 2015).

1.6. Retinóides e o tratamento anticâncer de mama

Os principais retinóides apresentam potencial antitumoral em linhagens de câncer de mama e, por este motivo, estão sendo realizados ensaios clínicos utilizando protocolos que incluem a combinação com antagonistas de estrogênio para tratar ou prevenir a progressão da doença (VERONESI et al., 2006; ZANARDI et al., 2006; RECCHIA et al., 2009; BUSHUE & WAN, 2010).

Vários retinóides naturais e sintéticos inibem o crescimento de células de câncer e o desenvolvimento de tumores mamários induzidos por agentes carcinogênicos (MOON et al., 1983; FONTANA et al., 1988; DI MASI et al., 2015), além de modular a sinalização por *HER2* e RE (ALIZADEH et. al., 2014).

Segundo Mazumdar et al. (2012) alguns resultados sugerem que a prevenção unilateral de ambos câncer de mama RE+ e RE- podem necessitar de uma terapia de combinação contando com os benefícios preventivos individuais obtidos através do tratamento tanto com um agente anti-estrogênio, quanto com um retinóide.

Em muitas células de câncer da mama os receptores RARα, RARγ, RXRα e RXRβ são expressos em condições basais (SHEIKH et al., 1993; PARONI et al., 2012), sendo os níveis de RARα mais elevados em tumores RE+ do que em tumores RE- (TERAO et al., 2011; GARATTINI et al., 2014), uma vez que as células RE+ exibem maior sensibilidade a receptores de ácido retinóico (RA) (SHEIKH et al., 1993; RUBIN et al., 1994; HAN et al., 1997; TOMA et al., 1998; PRAKASH et al., 2001; RIBEIRO et al., 2014). Segundo Terao et al. (2011) os agonistas RARα inibem o crescimento de linhagens celulares RE+ e os retinóides e antiestrógenos bloqueiam a progressão do ciclo celular na fase G1 (RIBEIRO et al. 2014).

O sistema experimental mais empregado para o estudo de tumores de mama positivos para o REα utiliza a linhagem celular de câncer de mama humano MCF-7. Elas possuem os receptores REα e RARα/RARγ que têm em comum regiões de ligação no DNA e possibilitaram a oportunidade de comparar efeitos genômicos da sinalização do ácido retinóico (RA) e do estrogênio (LEVENSON & JORDAN, 1997; HUA et al., 2009).

1.7. Retinóides derivados de 1,2,3-triazólicos retinoidais

Segundo Aleixo et al. (2017), o planejamento estrutural dos novos agentes 1,2,3-triazólicos retinoidais foi inspirado em resultados positivos de uma série de análogos preparados como agentes anticâncer arotinoidais apresentados por autores como Simoni et al. (SIMONI et al., 2000; SIMONI et al., 2001; SIMONI et al., 2005) e Nagai et al. (KIKUCHI et al., 2000c).

A síntese dos análogos triazólicos foi realizada via *Click Chemistry*, utilizando como ferramenta o bioisosterismo, uma estratégia de modificação molecular baseada na troca de determinados fragmentos moleculares de compostos protótipos por átomos ou grupo de átomos que possuem propriedades físico-químicas similares (BARREIRO & FRAGA, 2008). Os compostos AM80 e AM580, retinóides sintéticos e agonistas transcricionais seletivos RARα (DELESCLUSE et al., 1991; BOSCH et al., 2012; TENG et al., 1996; ALTUCCI et al., 2007; BARNARD et al., 2009; ÁLVAREZ et al., 2014; TANABE et al., 2014; OKUNO et al., 2004; VIVAT-HANNAH & ZUSI, 2005; SHUDO et al., 2009; KAGECHIKA et al. 1988; AMANO et al., 2013; NAU & BLANER, 1999; OSTROWSKI et al., 1998; KAGECHIKA & SHUDO, 2005; KIKUCHI et al., 2000a; KIKUCHI et al., 2000b; LE MAIRE et al., 2012; DESPHANDE et al., 2013; ALEIXO et al., 2017) com atividade antitumoral significativa em modelos animais de câncer de mama (LU et al., 2010; RIBEIRO et al., 2014), foram utilizados como moléculas líderes. Seu grupamento amida é bioisóstero do anel triazólico existente nos compostos arotinoidais, sugerindo comportamento similar na ligação aos receptores biológicos e respostas biológicas parecidas. Foram realizadas trocas no *linker* e na parte carboxílica dos compostos AM80 e AM580, com a finalidade de desenvolver análogos mais potentes, menos tóxicos, com nenhum ou pouco efeito adverso, e características físico-químicas, farmacocinéticas e farmacodinâmicas mais aprimoradas (Figura 1).

Diante dos fatos expostos, o presente trabalho analisou os aspectos celulares envolvidos na resposta ao tratamento de células de adenocarcinoma de mama humano MCF-7 a duas novas moléculas retinóides denominadas RT1 e RT3, desenvolvidas a partir de modificações moleculares nos compostos AM80 e AM580, ligante seletivo RARα, com a finalidade de diminuir a toxicidade, melhor a interação e ativação do receptor, bem como, proporcionar o aprimoramento das características físico-químicas, farmacocinéticas e farmacodinâmicas da molécula, podendo ser importantes na terapia do câncer.

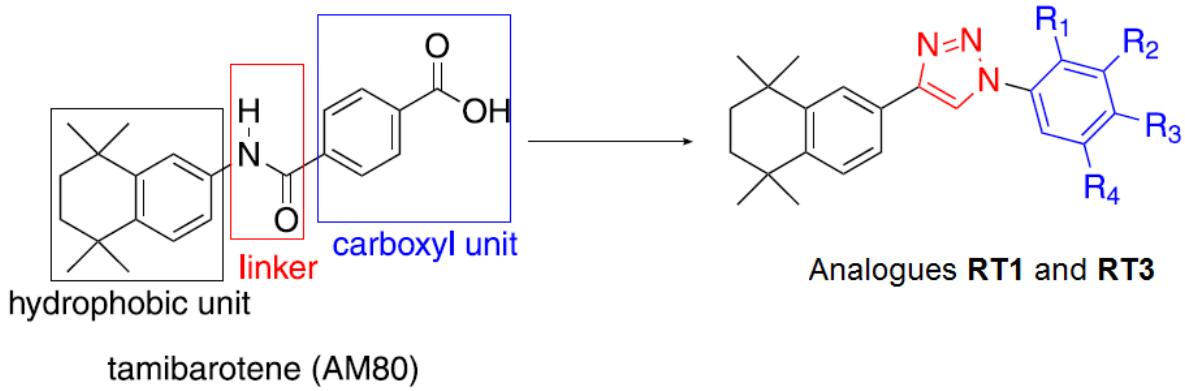

Figura 1. Planejamento sintético e estrutura química do precursor AM80 e seus derivados 1,2,3-triazólicos retinoidais (análogos RT1 e RT3) empregados neste estudo. Os retângulos em vermelho e azul demonstram os locais de modificações moleculares a partir de trocas bioisostéricas que originaram os compostos RT1 e RT3. Adaptado de Aleixo et al. (2017).

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3. Objetivos

3.1. Objetivo geral

Avaliar o potencial citotóxico e os mecanismos celulares envolvidos na resposta ao tratamento com dois análogos retinoidais 1,2,3-triazólicos (RT1 e RT3) em linhagem tumoral de mama MCF-7.

3.2. Objetivos específicos

Avaliar o potencial citotóxico dos retinóides pelo ensaio colorimétrico MTT (brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difeniltetrazólio);

Investigar se os retinóides são capazes de potencializar a citotoxicidade dos agentes antitumorais indutores de danos no DNA 5-fluorouracil, gencitabina, irinotecano, metotrexato, paclitaxel e tamoxifeno pelo ensaio colorimétrico MTT;

Analisar o efeito da combinação terapêutica dos retinóides e agentes antitumorais convencionais pelo índice de combinação (CI), segundo o método de Chou & Talalay;

Avaliar se os retinóides aumentam a morte celular por apoptose e/ou necrose quando combinadas ao tamoxifeno;

Averiguar se os retinóides interferem na proliferação celular, combinados ou não com o tamoxifeno.

Capítulo II – Manuscrito

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Keywords: Retinoid receptors, estrogen receptor, AM580, 1,2,3-triazolic retinoid analogues, MCF-7 cell line

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Abstract: Retinoids are substances that play important roles in cell growth regulation and proliferation, which interact with specific receptors. They have demonstrated increased antitumor effects in breast tumor cells with specific retinoid and estrogen receptors (ER). We evaluated cytotoxic potential of two new retinoid molecules derived from molecular modifications in AM580 and AM80 and its effects on combinations with different antitumor agents in MCF-7 cell line. Cytotoxicity was measured by MTT assay (72h) and combined therapy effect between retinoids and antitumor agents by combination index. The ability to induce cell death and influence on cell proliferation were assessed by morphological cell death assay (6, 24 and 48h) and BrdU incorporation (6h). Our results showed that retinoid derivatives decreased cell viability, caused by cell death and proliferation block. Among combinations, tamoxifen and retinoids potentiated the number of non-viable cells by synergism and additivism, induced cell death and showed anti-proliferative effects. Modifications in AM580 and AM80 produced retinoids with therapeutic potential in ER-positive breast cancer cells, both as single agents and in combinations with tamoxifen. This suggests the possibility of antitumor protocols improvements as well as development of new more efficient target therapies for breast tumors expressing RARα and ER receptors

To
Editor-in-Chief
EXPERIMENTAL CELL RESEARCH

July 04th, 2017
Campo Grande – Brazil

Enclosed please find our manuscript entitled **"Retinoid derivatives for the treatment of breast cancer: studies of cellular and molecular mechanisms"** by Bárbara T. Rós; Natan de David; Diego B. Carvalho, Mariana A. Aleixo, Maria F.C. Matos; Maria T.F.D. Monreal; Rodrigo J. Oliveira; Adriano C.M. Baroni; Renata Matuo, for submisson to *Experimental Cell Research*.

This paper is original work characterizing the mechanism of two new retinoid molecules from molecular modifications in AM580 and AM80 and its effects on combinations with different antitumor agents in MCF-7 cell line. Data showed that retinoids derivatives decreased cell viability caused by cell death and proliferation block. Combination of tamoxifen and retinoids potentiated the number of non-viable cells by synergism and additivism, induced cell death and anti-proliferative effects. This data suggests the possibility of antitumor protocols improvements as well as development of new more efficient target therapies for breast tumors expressing RARα and ER receptors.

We assure that this material has not been published or is under active considerations by another journal. The authors declare that there are no conflicts of interest.

We hope that you and the referees will appreciate our work and look forward hearing from you.

Sincerely yours,

Renata Matuo

Retinoid derivatives for the treatment of breast cancer: studies of cellular and molecular mechanisms

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Abstract

Retinoids are substances that play important roles in cell growth regulation and proliferation, which interact with specific receptors. They have demonstrated increased antitumor effects in breast tumor cells with specific retinoid and estrogen receptors (ER). We evaluated cytotoxic potential of two new retinoid molecules derived from molecular modifications in AM580 and AM80 and its effects on combinations with different antitumor agents MCF-7 cell line. Cytotoxicity was measured by MTT assay (72h) and combined therapy effect between retinoids and antitumor agents by combination index. The ability to induce cell death and influence on cell proliferation were assessed by morphological cell death assay (6, 24 and 48h) and BrdU incorporation (6h). Our results showed that retinoid derivatives decreased cell viability, caused by cell death and proliferation block. Among combinations, tamoxifen and retinoids potentiated the number of non-viable cells by synergism and additivism, induced cell death and showed anti-proliferative effects. Modifications in AM580 and AM80 produced retinoids with therapeutic potential in ER-positive breast cancer cells, both as single agents and in combinations with tamoxifen. This suggests the possibility of improvements in antitumor protocols as well as development of new more efficient target therapies for breast tumors expressing RARα and ER receptors.

Key words: Retinoid receptors, estrogen receptor, AM580, 1,2,3-triazolic retinoid analogues, MCF-7 cell line

Abbreviations

ER, estrogen receptor; PR, progesterone receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; ATRA, All-*trans*-retinoic acid; 9-cis-RA, 9-*cis*-retinoic acid; RT1, 4-(4-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-yl)-1*H*-1,2,3-triazol-1-yl) benzoic acid; RT3, 4-(4-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-yl)-1*H*-1,2,3-triazol-1-yl)aniline; IC_{50}, 50% growth inhibition; 5-FU, 5-fluorouracil; MTX, methotrexate; TAM, tamoxifen; DMSO, dimethylsulfoxide; CI, combination index.

1. Introduction

Breast cancer is the most common cancer type among women [1]. Its incidence was 1.7 million and represented 25.2% of the total number of cancers in the world in 2012 [2]. Its subtypes can be classified according to several characteristics and according to the expression of receptors present in the cell membrane such as estrogen receptor (ER), progesterone receptor (PR) and HER2 receptor tyrosine kinase expression [3].

The natural morphogenesis of mammary glands tissues has cellular processes controlled mainly by steroid hormones such as estrogen type, making ER and PR expression important to cell proliferation. The effects of these hormones are mediated by nuclear receptors ERα and ERβ, through the transcriptional regulation activated by a ligand of target genes, and as membrane components initiating cascades of cytoplasmic cell signaling [4-6]. Studies have shown the correlation between estrogen levels and risk of developing breast cancer [7], with about 80% of breast cancers ERα positive.

In addition to ERs, there are also specific retinoid receptors. Retinoids are substances that play important roles in biological activities such as cell growth regulating, proliferation and differentiation of normal, pre-malignant and malignant cells [8-10], which are linked to interaction with their specific nuclear receptors. The most important source of diversity in signal transduction of retinoids are two types of retinoid receptors: retinoic acid receptor (RAR) and retinoid X receptor (RXR), both contain three subtypes - α, β, and γ. RARs are activated by All-*trans*-retinoic acid (ATRA) and 9-*cis*-retinoic acid (9-cis-RA), and RXRs activated only by 9-cis-RA [11]. In many breast cancer cells, RARα, RARγ, RXRα and RXRβ are expressed in basal conditions [12,13]. In ER-positive cells, which are considered sensitive to retinoids, RARα may be the primary determinant of ATRA sensitivity. According to [14] levels of RARα are higher in ER-positive tumors and their agonists inhibit growth of ER-positive cell lines, while their silencing reduces anti-proliferative effect of ATRA.

Studies have shown the relationship between ER expression and RARα retinoid receptor subtype expression in MCF-7 breast tumor cell line [14-16]. In this cell line, specific RARα ligands demonstrated better results on cell proliferation control compared with non-specific RAR ligands [17-19].

Retinoid treatments and simultaneous ER markers may increase antitumor effects, which make them promising agents for breast cancer therapy [20]. In

addition, they may induce differentiation and/or apoptosis in tumor cells, have anti-proliferative and anti-oxidant activity, and may influence on carcinogenesis [21]. Because they present varied characteristics in response to cancer cells, retinoid treatments have great potential as chemotherapeutic or chemopreventive agents. Thus, the present study evaluated the activity of two new retinoid molecules RT1 and RT3 obtained from molecular modifications of the compounds AM580 and AM80, selective RARα ligands, and the effect of combinations with different antitumor agents, in human breast adenocarcinoma MCF-7cell line.

2. Materials and Methods

2.1. Synthesis of retinoids 1,2,3-triazolic derivatives retinoids

Molecules used in this study were identified as RT1 and RT3 (Figure 1). Compounds were synthesized and kindly provided by Laboratory of Pharmaceutical Chemistry of Faculty of Pharmaceutical Sciences, Food and Nutrition of Federal University of Mato Grosso do Sul. Route of retinoids synthesis has been described by [22].).

2.2. Cell line and culture conditions

Human breast adenocarcinoma MCF-7 cell line was cultured in Dulbecco's Modified Eagle Medium-DMEM (Gibco®) supplemented with 10% fetal bovine serum (Gibco®) (v/v), 0.1% penicillin (100U/ml)/streptomycin (100µg/ml) (v/v) (LGC biotechnology®) and incubated at 37°C with 5% CO_2 atmosphere.

2.3. Antitumor agents

Concentrations of antitumor agents used in this study were previously defined by pilot experiment. For all agents, 50% growth inhibition (IC_{50}) value was used for combination with different concentrations of arotinoids retinoids. 1.25µM 5-fluorouracil (5-FU); 10µM gemcitabine; 5µM irinotecan; 325nM methotrexate (MTX); 1.25nM paclitaxel and 19µM tamoxifen (TAM) were used.

2.4. MTT colorimetric assay

Cell viability was determined by MTT colorimetric test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide - Invitrogen®), based on [23]. 3 x 10^3 cells/well were seeded in 96-well plates and maintained for 24h in incubator.

Treatments were performed for 72h with different doses of isolated agents RT1 (10, 25, 100 and 150μM) and RT3 (5, 10, 25, 50 and 100μM), combined or not with different antitumor agents in simultaneous treatment. At the end of treatments, plates were incubated with 0.3 mg/mL MTT for 4h. Culture medium was removed and 100μL of dimethylsulfoxide (DMSO) was added to read at 540nm. Two independent experiments were performed in quintuplicates.

2.5. Combination index

From cell viability results, combination index (CI) of combined treatments was calculated using the drug combination method based on the principle of median effect of the law of mass action which constitutes the theoretical basis for CI - isobologram equation which allows the quantitative determination of drug interactions described by [24] and [25], through CompuSyn software (http://www.combosyn.com/) and algorithms. The index was calculated based on the affected fraction of cells (non-viable cells) obtained in the MTT assay against the different treatments, taking into consideration the doses, allowing to estimate if treatments presented additive, synergic or antagonistic effect, depending on the combined concentrations.

2.6. Cell death due to apoptosis or necrosis

Cell death was evaluated by morphological assay with ethidium bromide and acridine orange, according to [26], with modifications. 3 x 10^5 cells/well were seeded in 12-well plates and kept in incubator for 24h. Treatments were carried out for 6, 24 and 48h, using 35μM RT1 and 22μM RT3, combined or not with tamoxifen (19μM). The concentrations used were chosen based on the results of the MTT colorimetric assay and combination index.

Cells were collected by trypsinization, centrifuged at 1200 rpm for 5min and the supernatant discarded. Slides were prepared with 20μl of cell suspension and 2μl of dye containing ethidium bromide (100μg/ml) and acridine orange (100μg/ml) (1:1). Two independent experiments were performed with two replicates each. For each replicate 100 cells were analyzed on a 400x magnification fluorescence microscope immediately after preparation. Cells were classified in: i) viable cells: green intact nucleus; ii) cells in initial apoptosis: dense areas of chromatin condensation in the green nucleus; iii) cells in late apoptosis: dense areas of chromatin condensation in orange; iv) necrotic cells: intact orange nucleus [27,28] (Figure 2).

2.7. Cell proliferation assay

Cell proliferation assay was performed by BrdU incorporation according to [29] and [30], with modifications. 7.5 x 10^5 cells/well were seeded in 6-well plates containing sterilized round coverslips and kept in incubator for 24h. Treatments were performed for 6h, using 35μM RT1 and 22μM RT3, combined or not with tamoxifen (19μM). After 5h of treatment 50μM BrdU was added to the culture medium and incubated at 37°C for 1h. Culture medium was removed, cells were washed with PBS and fixed with 4% PFA (paraformaldehyde pH=7.4 in phosphate buffer) for 30-60min. Cells were washed 5x with PBS for approximately 20min, treated with 2N HCl for 30min for denaturation, washed again 3x with PBS for approximately 10min and incubated in phosphate buffer containing 5% FBS and 0.1% Triton X-100 for 1-2h.

Labeling was performed with the primary anti-BrdU antibody (IIB5) (monoclonal mouse IgG - Sc 32323 – Santa Cruz Biotechnology (RRID: AB_626766), diluted at the concentration 1:200 in PBS containing 5% FBS and 0.1% Triton X-100) overnight. Cells were washed 5x with PBS for approximately 20 min, incubated with secondary Goat Anti-Mouse IgG antibody (Alexa Fluor® 568) diluted at the concentration 1:1000 in PBS with 5% PBS and 0.1% Triton X-100 for 1h in the dark, and washed 5x with PBS. Coverslips were removed and transferred to slides containing one drop of antifade containing DAPI (Invitrogen®). Two independent experiments were performed with two replicates each, and 100 cells per replicate were analyzed in a fluorescence microscope at a magnification of 400x.

2.8. Statistical analyzes

In order to compare quantitative data of different treatments, ANOVA/Tukey test was employed according to data distribution. Treatments with retinoids were compared to respective controls (bars in the same color). Analyzes were performed with Graph-PadPrism software (version 5.01; Graph-Pad Software Inc., San Diego, CA, USA), considering statistically significant differences when $p<0.05$.

3. Results

3.1. Retinoids decrease cell viability

Cell viability was assessed by MTT assay for different concentrations of RT1 and RT3, associated or not with 5-FU, gemcitabine, paclitaxel, MTX, irinotecan and TAM. Results demonstrated that both retinoids reduced cell viability after 72h. RT1

associated with 5-FU (Figure 3A), gemcitabine (Figure 3C), paclitaxel (Figure 3E), MTX (Figure 4A) and irinotecan (Figure 4C) showed a slight increase in sensitivity of RT1, however, when combined with TAM (Figure 4E), this increase was more pronounced. RT3 combined with 5-FU (Figure 5A) and paclitaxel (Figure 5E) presented no RT3 sensitivity effect at some concentrations, and combinations with gemcitabine (Figure 5C), MTX (Figure 6A) and irinotecan (Figure 6C) showed a slight increase in RT3 sensitivity at some concentrations, but when combined with TAM (Figure 6E), this increased sensitivity was more pronounced.

Based on cellular viability results, combination index (CI) of combined treatments was calculated using the method described by Chou & Talalay [25] based on physical, chemical and mathematical principles of mass action law, employing The CompuSyn program. The main characteristics of CI method are: i) derived from theory and equation; ii) has established algorithms; iii) has automated computer simulation; iv) is flexible in its use; and v) achieves quantitative conclusions and pharmacodynamic indexes [25,31-34]. CI results complement data observed in cell viability curves, once fractions of affected cells obtained in MTT assay as well as doses and IC_{50} are taken into account. They demonstrated that combination of RT1 and 5-FU (Figure 3B) presented antagonism, additive and synergistic effects, depending on the doses used. Combinations of RT1 with gemcitabine (Figure 3D), paclitaxel (Figure 3F), irinotecan (Figure 4D) and TAM (Figure 4F) showed additive and synergistic effects, increasing the rate of cell death. While the combination of RT1 and MTX showed synergistic activity (Figure 4B).

RT3 CI values showed that RT3 combination with 5-FU (Figure 5B), paclitaxel (Figure 5F), MTX (Figure 5B), and irinotecan (Figure 6D) presented antagonism and additive effects. Combination of RT3 and gemcitabine (Figure 5D) was antagonistic and synergistic, whereas combination with TAM (Figure 6F) was additive and synergistic.

3.2. Retinoids cause cell death by apoptosis or necrosis

Cell viability results demonstrated that TAM increased RT1 and RT3 sensitivity. This increased sensitivity can occur both by increased cell death and/or replication blockage. Thus, cell death was investigated by apoptosis or necrosis employing morphological assay with ethidium bromide and acridine orange. Both retinoids induced cell death by apoptosis from 6h treatments (Figure 7A - gray bars),

being that after 48h (Figure 7A - striped bars) values were statistically similar or higher than TAM.

In RT1+TAM, apoptotic profile obtained was similar to RT1 alone at 6h and 24h (Figure 7A), but different at 48h. RT1 and RT3 presented slight apopotosis increase after 6h (Figure 7A - gray bars), a higher apoptosis increase after 24h (Figure 7A - black bars), and a pronounced increase after 48h (Figure 7A - striped bars) in both treatments, however, RT1 remained smaller than RT3, but statistically equal to TAM treatment. RT3+TAM combination showed a similar behavior to TAM at 6h and 24h, but different at 48h, presenting a higher and statistically significant apoptotic cells. When comparing RT3 and RT3+TAM, there are statistically differences between all times, and RT3+TAM showed profile similar to TAM.

Assessing cell death by necrosis, RT1 induced necrosis since 24h (Figure 7B - black bars), whereas this effect was more pronounced with RT3 after 6h (Figure 7B - gray bars). RT1+TAM presented a similar profile to RT1 alone, mainly in the first 24h (Figure 7B -black bars) and both presented statistically similar results to TAM. RT3+TAM was similar to RT3 in the first 24h and showed increased necrosis when compared to TAM, especially after 48h (Figure 7B - striped bars).

3.3. Retinoids interfere in cell proliferation

BrdU is a synthetic nucleoside analogous to thymine that is incorporated into cell DNA strands during replication. BrdU incorporation can be used to evaluate cell proliferation activity. Cell viability results showed that TAM increased RT1 and RT3 sensitivity and this increase can occur by cell death and/or replication blockage. Thus, cell proliferation was investigated from the labeling of cells with BrdU by immunofluorescence for RT1 and RT3 associated or not with TAM (Figure 8). Results showed that both retinoids reduced cell proliferation, as well as TAM. Combined treatments RT1+TAM and RT3+TAM presented similar results to TAM. Results showed that there was anti-proliferative activity both in retinoid treatment alone and in its combinations with TAM.

4. Discussion

New therapeutic targets such as RARα receptor in responsive estrogenous breast tumors are considered primary determinants of breast tumors sensitivity to retinoids [16,35,36]. Studies have shown that depending on cell type, RARα induction

may be associated with differentiation, anti-proliferative effect and/or apoptosis [10,37-40]. In our study, ER-positive MCF-7 cell line presents RARα receptor [41] to investigate antitumor potential of these retinoids.

Structural design of retinoid triazolic agents RT1 and RT3 evaluated in this study, according to [22], was inspired by positive results of several arotinoid antitumor agent analogues. AM580 and AM80 are selective RARα linkers and they were chosen as the leaders molecules for RT1 and RT3 synthesis via ClickChemistry. Molecular modifications using bioisosterism approach were performed in central linker, amide group, and carboxylic acid part of AM580 and AM80, in order to develop analogues with lower toxicity, higher potency in resistant tumor cells [42], selectivity to RARα receptor, and improvements in physico-chemical, pharmacokinetic and pharmacodynamic characteristics of the molecule.

According to [31], therapeutic combination is advantageous in cancer treatment, once it allows to affect multiple molecular targets with therapeutic efficacy increase, dosages reduction and consequently toxicity decrease, as well as reduction or delay in the acquisition of resistant phenotypes. In our study, cellular viability of retinoids was evaluated in combination or not with antitumor agents that have different mechanisms of action. 5-fluorouracil when converted to its active metabolites can be incorporated into DNA and/or RNA and inhibits the thymidylate synthase enzyme responsible for nucleotide synthesis [43]. Gemcitabine, when converted to its metabolites, can be incorporated into DNA leading to the termination of DNA strand that has being synthesized by DNA polymerase, or may inhibit ribonucleotide reductase enzyme, decreasing amounts of dCTP for replication [44]. Irinotecan is a drug that converts to SN-38, inhibits topoisomerase I activity by stabilizing the cleavage complex between topoisomerase and DNA, resulting in DNA breaks that result in replication inhibition and apoptosis [45]. Methotrexate is an antifolate with antineoplasic and immunosuppressive activities that binds and inhibits dihydrofolate reductase, leading to thymidylate synthesis inhibition, which results in DNA and RNA synthesis inhibition [46]. Paclitaxel is a microtubules inhibitor, which stabilizes microtubules when polymerized, leading to cell death [47]. Tamoxifenis a non-steroidal antineoplasic agent selective for estrogen receptor and binds to ER preventing that estrogen binds to it. In this way, reduction in DNA synthesis occurs [48].

Our results demonstrate that retinoids decrease cell viability of MCF-7 breast cancer line. According to [49], combinations of various retinoids with tamoxifen show strong synergistic inhibition of proliferation in ER-positive and ER-negative human breast cancer cells. This data corroborates the results obtained in our cell viability and CI tests for tamoxifen, in which it showed synergistic and additive activity in combination with both retinoid triazolics analogs. The most promising results were observed in combination of retinoids with TAM, which can be justified by the greater selectivity to RARα receptor of RT1 and RT3, due to the fact that tamoxifen is an estrogen antagonist (binds to ER) and MCF-7 cell line presents these ligands in their conformation.

The ideal combination of drugs is one that has a synergistic effect without increased systemic toxicity, and that combination with additive effect and favorable toxicity profile may also have clinical benefits [50]. According to [34], none of approximately 20 different methods of synergism/antagonism evaluation in literature has all characteristics that CI method presents, for this reason it is the most cited for drug combination analyzes. In our study it was observed from this method that the best combination was that employed retinoids with TAM, once they presented synergistic and additive activity in both associations.

Cell viability reduction may be related to two processes: cell death and/or cell cycle arrest. Vitamin A derivatives and retinoic acid have been identified as apoptosis inducers and they reduce cell proliferation in human breast adenocarcinoma cells, once they regulate antitumor activity through RARα receptor activation, encoded by the RARA receptor gene [36, 51]. Our results showed greater apoptosis and/or necrosis induction, mainly in RT3+TAM, at 24h for necrosis and 48h for apoptosis. While RT1+TAM presented apoptosis increase after 48h.

Replication inhibition evaluation was performed with the purpose of complementing the results of cell death by apoptosis and/or necrosis, once if retinoids influence in cell viability, possibly they would also be responsible for blocking cell proliferation. It is known that ERα receptors, important in breast cancer, are therapeutic targets and predict the response to drugs such as tamoxifen, because it is a competitor of estrogen in ERα binding [6,52,53]. According to [54], ER-positive MCF-7 human cell line when treated with different concentrations of retinoic acid or synthetic derivatives, associated or not with tamoxifen has an additive effect on cell proliferation inhibition. Our results corroborate with literature data,

whereas both retinoids reduced proliferating cells. At the same time, RT1+TAM and RT3+TAM combinations reduced further the number of cells.

Evaluating the general context, results indicate that although cellular proliferation data indicates reduction of proliferating cells, the greater interference in cell viability was due to cell death by apoptosis. We believe that the molecular modifications of AM580 and AM80 compounds, evaluated in this work, produced retinoid derivatives (RT1 and RT3) with therapeutic potential for breast cancer, both as single agents and in combinations, since they are capable of enhancing tamoxifen activity. These observations suggest the possibility of improvements in antitumor protocols, as well as the development of more efficient treatments.

Conflict of interest statement

None declared.

Acknowledgments

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Figure Legends

Figure 1. Chemical structure of the retinoid derivatives RT1 and RT3 used in this study.

Figure 2: Morphological assay with ethidium bromide and acridine orange. Cellular classification: (A) viable cells; (B) cells in initial apoptosis; (C) cells in late apoptosis; and (D) necrotic cells.

Figure 3: Cell viability evaluation of RT1 combination with different antitumor agents by MTT assay. Different RT1 concentrations were combined with IC50 agents (A) 5-FU, (C) gemcitabine, (E) paclitaxel. Curves represent results of at least two independent replicates and bars the standard deviation. Combination indexes (CI) were calculated according to Chou & Talalay, for RT1 combinations with different antitumor agents: (B) 5-FU, (D) gemcitabine and (F) paclitaxel, by CompuSyn program from cell viability results. CI>1.2 antagonistic effect; 0.8<CI<1.2 additive effect and CI<0.8 synergism.

Figure 4: Cell viability evaluation of RT1 combination with different antitumor agents by MTT assay. Different RT1 concentrations were combined with IC50 agents (A) MTX, (C) irinotecan and (E) tamoxifen. Curves represent results of at least two independent replicates and bars standard deviation. Combination indexes (CI) were calculated according to Chou & Talalay, for RT1 combinations with different antitumor agents: (B) MTX, (D) irinotecan e (F) tamoxifeno, by CompuSyn program from cell viability results. CI>1.2 antagonistic effect; 0.8<CI<1.2 additive effect and CI<0.8 synergism.

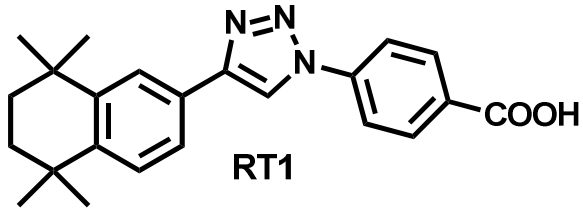
Figure 5: Cell viability evaluation of RT3 combination with different antitumor agents by MTT assay. Different RT3 concentrations were combined with IC50 agents (A) 5-FU, (C) gemcitabine (E) paclitaxel. Curves represent results of at least two independent replicates and bars standard deviation. Combination indexes (CI) were calculated according to Chou & Talalay, for RT3 combinations with different antitumor agents: (B) 5-FU, (D) gemcitabine e (F) paclitaxel, by CompuSyn program

from the cell viability results. CI>1.2 antagonistic effect; 0.8<CI<1.2 additive effect and CI<0.8 synergism.

Figure 6: Cell viability evaluation of RT3 combination with different antitumor agents by MTT assay. Different RT3 concentrations were combined with IC50 agents (A) MTX, (C) irinotecan and (E) tamoxifen. Curves represent results of at least two independent replicates and bars standard deviation. Combination indexes (CI) were calculated according to Chou & Talalay, for combinations of RT3 with different antitumor agents: (B) MTX, (D) irinotecan e (F) tamoxifen, by CompuSyn program from the cell viability results. CI>1.2 antagonistic effect; 0.8<CI<1.2 additive effect and CI<0.8 synergism.

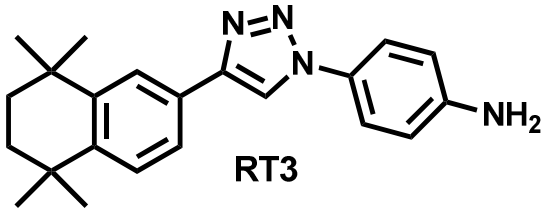
Figure 7. Cell death by (A) apoptosis or (B) necrosis after treatments with RT1 and RT3. Retinoids IC50 were used in combination or not with tamoxifen (TAM) for 6h (gray bars), 24h (black bars) and 48h (striped bars). 100 cells per replicate were analyzed, classified by morphological aspect and differential staining by ethidium bromide and orange acridine. Graphs represent mean and standard error of the average of two independent experiments with two replicates. The statistical differences between treatments of the same experimental time (bars of the same color) were analyzed by ANOVA/Tukey, being that different letters indicating a statistical difference ($p<0.05$).

Figure 8: Cell proliferation employing BrdU labeling by immunofluorescence after treatments with RT1 and RT3. 100 cells per replicate were analyzed. Retinoids IC50 were used in combination or not with tamoxifen (TAM). (A) Proliferating cells (red); cells nuclei (blue); merge of red and blue; (a) control; (b) TAM; (c) RT1; (d) RT1+TAM; (e) RT3; and (f) RT3+TAM. (B) Graphs represent mean and standard error of the average of two independent experiments with two replicates. Statistical differences between treatments were analyzed by ANOVA/Tukey, different letters indicating a statistical difference ($p<0.05$).



RT1

4-(4-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-yl)-1*H*-1,2,3-triazol-1-yl)benzoic acid



RT3

4-(4-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-yl)-1*H*-1,2,3-triazol-1-yl)aniline

Figure 1.

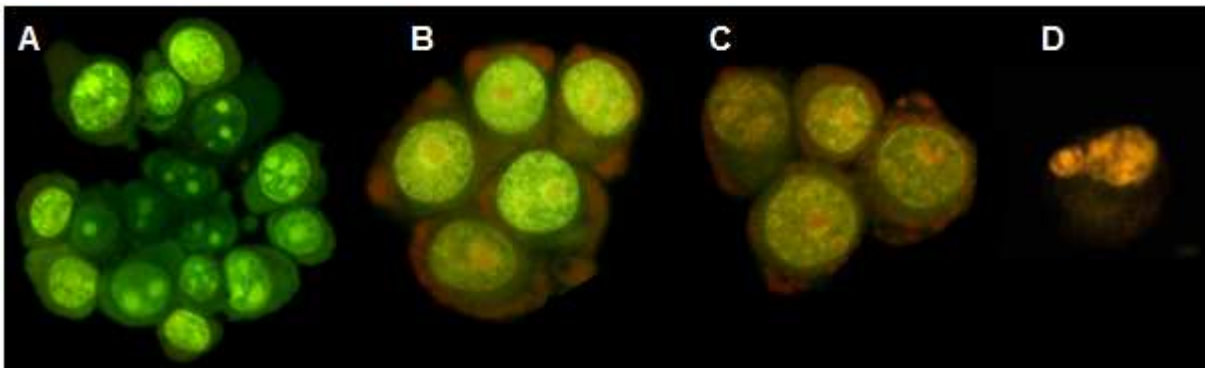


Figure 2.

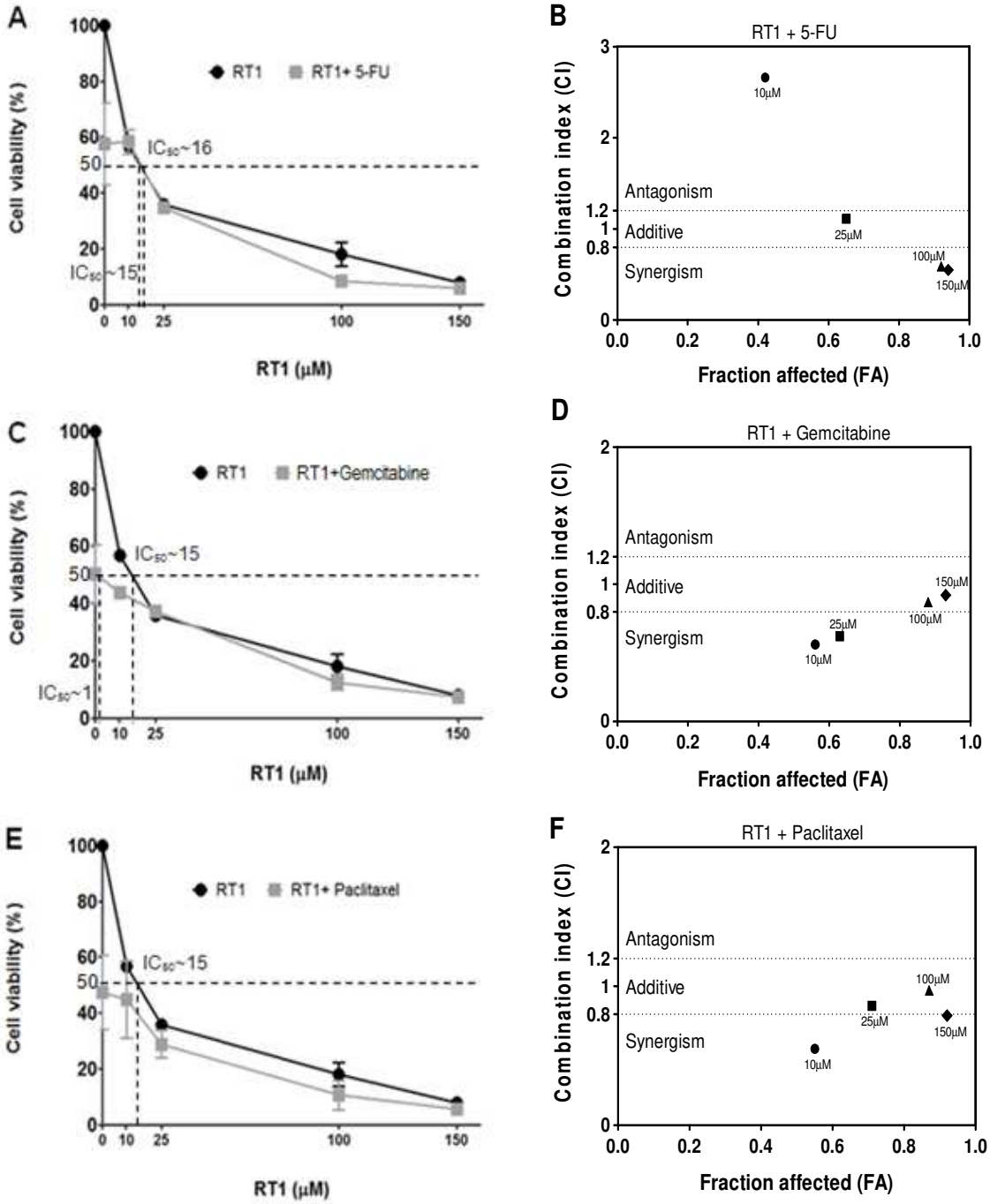

A
Cell viability (%)
RT1
RT1+ 5-FU
IC50~16
IC50~15
RT1 (μM)
B
RT1 + 5-FU
Combination index (CI)
Antagonism
Additive
Synergism
10μM
25μM
100μM
150μM
Fraction affected (FA)
C
Cell viability (%)
RT1
RT1+Gemcitabine
IC50~15
IC50~1
RT1 (μM)
D
RT1 + Gemcitabine
Combination index (CI)
Antagonism
Additive
Synergism
10μM
25μM
100μM
150μM
Fraction affected (FA)
E
Cell viability (%)
RT1
RT1+ Paclitaxel
IC50~15
RT1 (μM)
F
RT1 + Paclitaxel
Combination index (CI)
Antagonism
Additive
Synergism
10μM
25μM
100μM
150μM
Fraction affected (FA)

Figure 3.

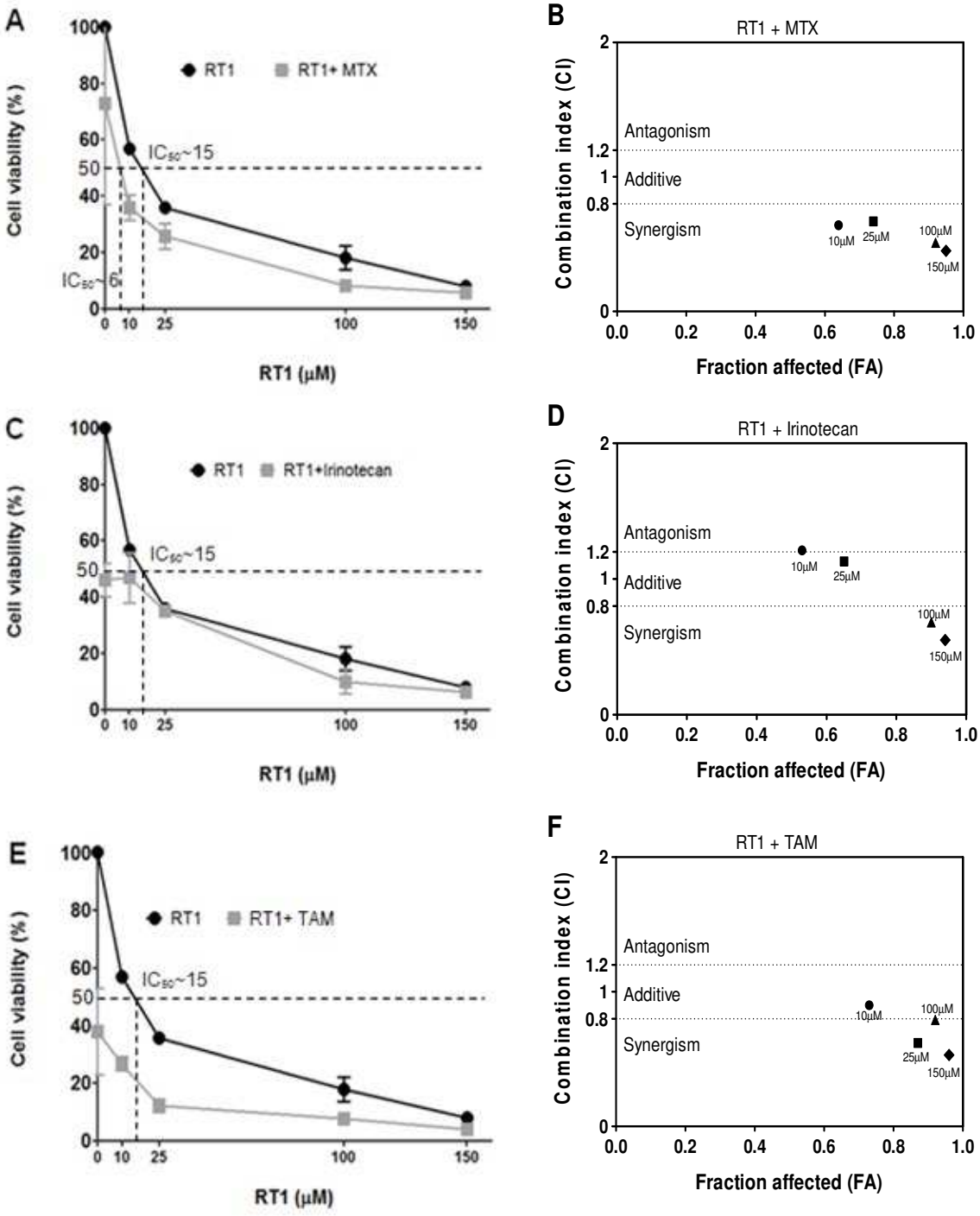

A
Cell viability (%)
RT1
RT1+ MTX
IC50~15
IC50~6
RT1 (μM)
B
RT1 + MTX
Combination index (CI)
Antagonism
Additive
Synergism
10μM
25μM
100μM
150μM
Fraction affected (FA)
C
Cell viability (%)
RT1
RT1+Irinotecan
IC50~15
RT1 (μM)
D
RT1 + Irinotecan
Combination index (CI)
Antagonism
Additive
Synergism
10μM
25μM
100μM
150μM
Fraction affected (FA)
E
Cell viability (%)
RT1
RT1+ TAM
IC50~15
RT1 (μM)
F
RT1 + TAM
Combination index (CI)
Antagonism
Additive
Synergism
10μM
100μM
25μM
150μM
Fraction affected (FA)

Figure 4.

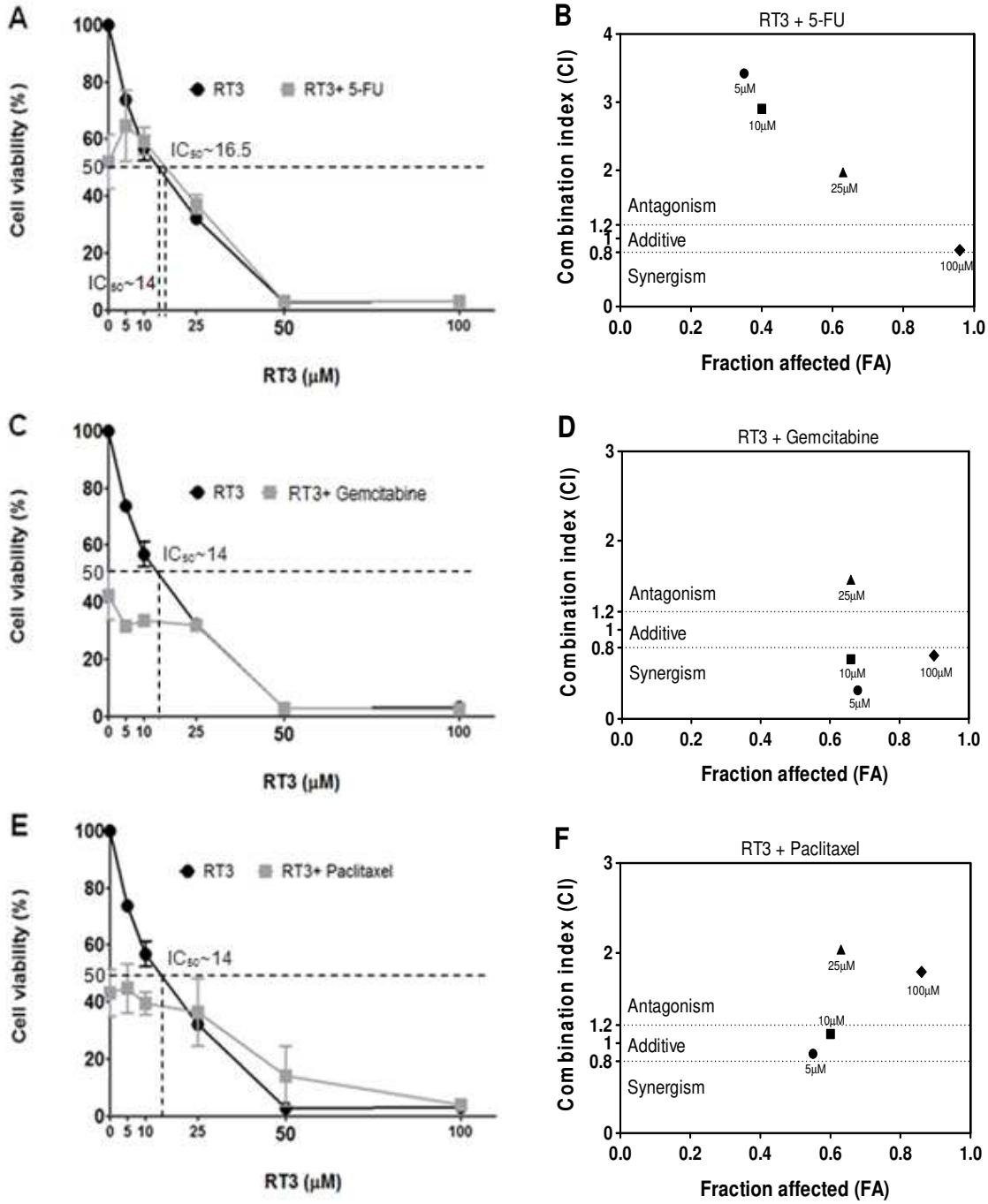

A
Cell viability (%)
RT3
RT3+ 5-FU
IC50~16.5
IC50~14
RT3 (µM)
B
RT3 + 5-FU
Combination index (CI)
5µM
10µM
25µM
100µM
Antagonism
Additive
Synergism
Fraction affected (FA)
C
Cell viability (%)
RT3
RT3+ Gemcitabine
IC50~14
RT3 (µM)
D
RT3 + Gemcitabine
Combination index (CI)
Antagonism
Additive
Synergism
25µM
10µM
100µM
5µM
Fraction affected (FA)
E
Cell viability (%)
RT3
RT3+ Paclitaxel
IC50~14
RT3 (µM)
F
RT3 + Paclitaxel
Combination index (CI)
25µM
100µM
10µM
5µM
Antagonism
Additive
Synergism
Fraction affected (FA)

Figure 5.

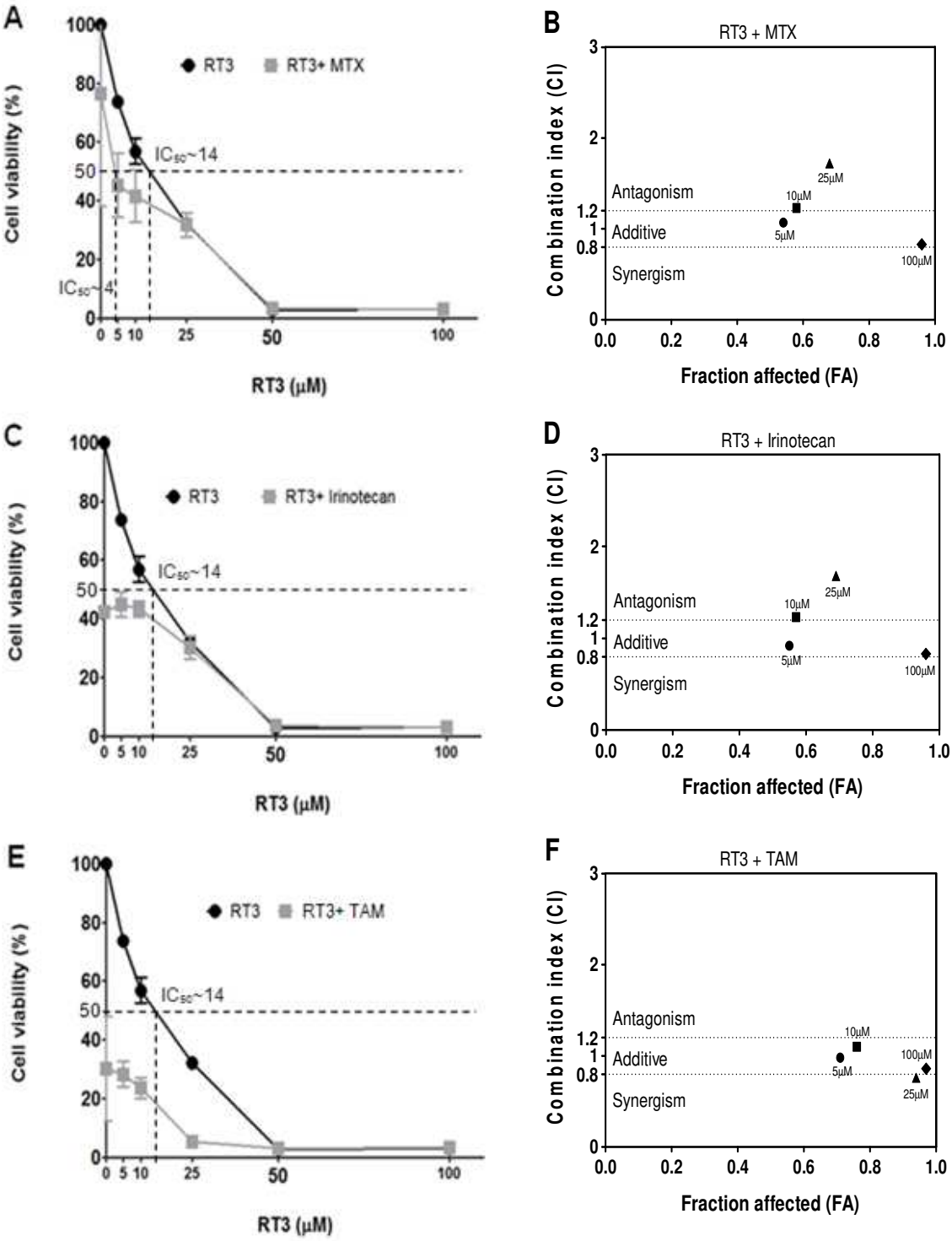

A
Cell viability (%)
RT3
RT3+ MTX
IC50~14
IC50~4
RT3 (μM)
B
RT3 + MTX
Combination index (CI)
Antagonism
Additive
Synergism
25μM
10μM
5μM
100μM
Fraction affected (FA)
C
Cell viability (%)
RT3
RT3+ Irinotecan
IC50~14
RT3 (μM)
D
RT3 + Irinotecan
Combination index (CI)
Antagonism
Additive
Synergism
25μM
10μM
5μM
100μM
Fraction affected (FA)
E
Cell viability (%)
RT3
RT3+ TAM
IC50~14
RT3 (μM)
F
RT3 + TAM
Combination index (CI)
Antagonism
Additive
Synergism
10μM
5μM
100μM
25μM
Fraction affected (FA)

Figure 6.

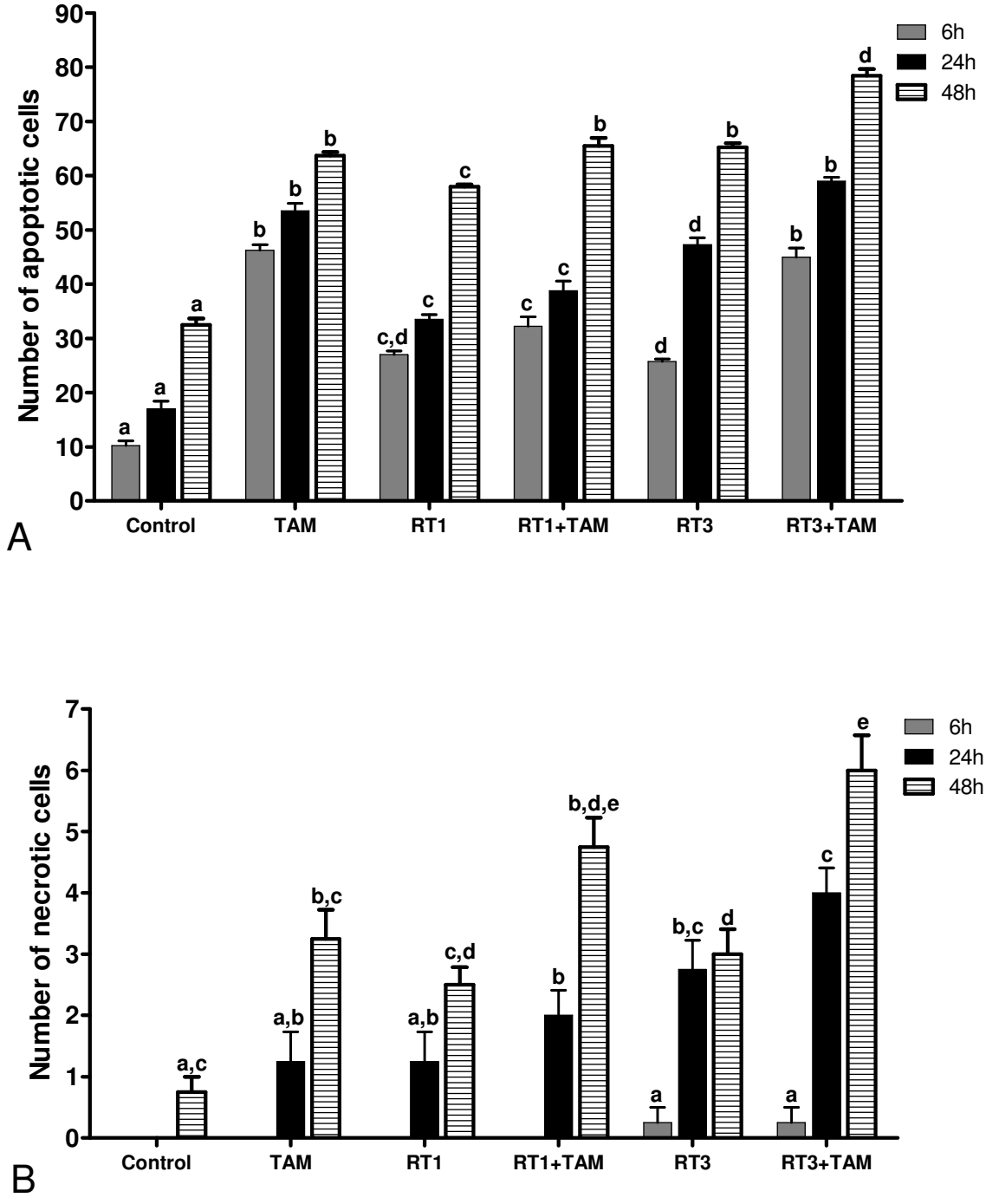
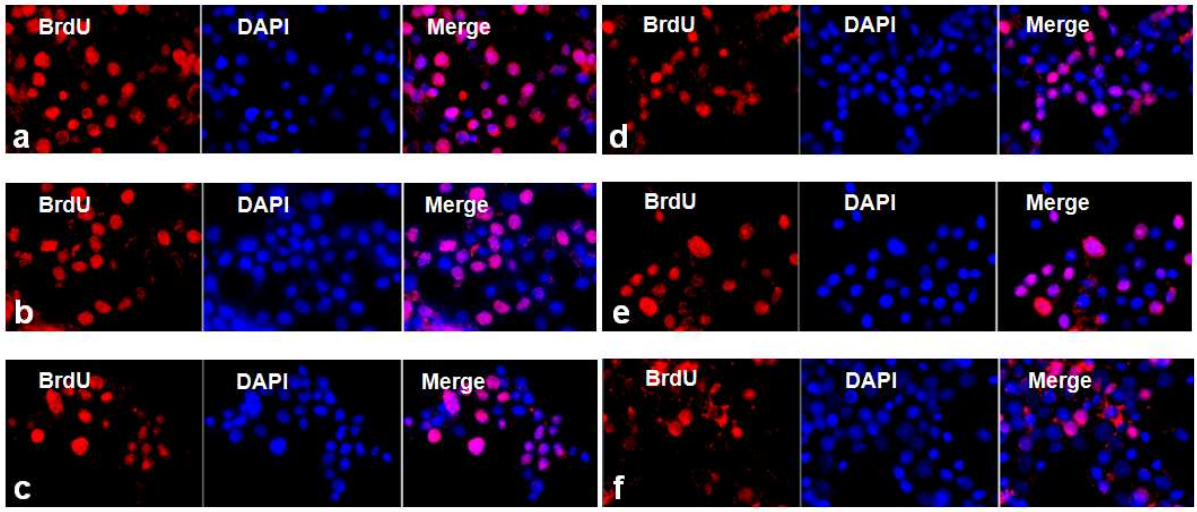
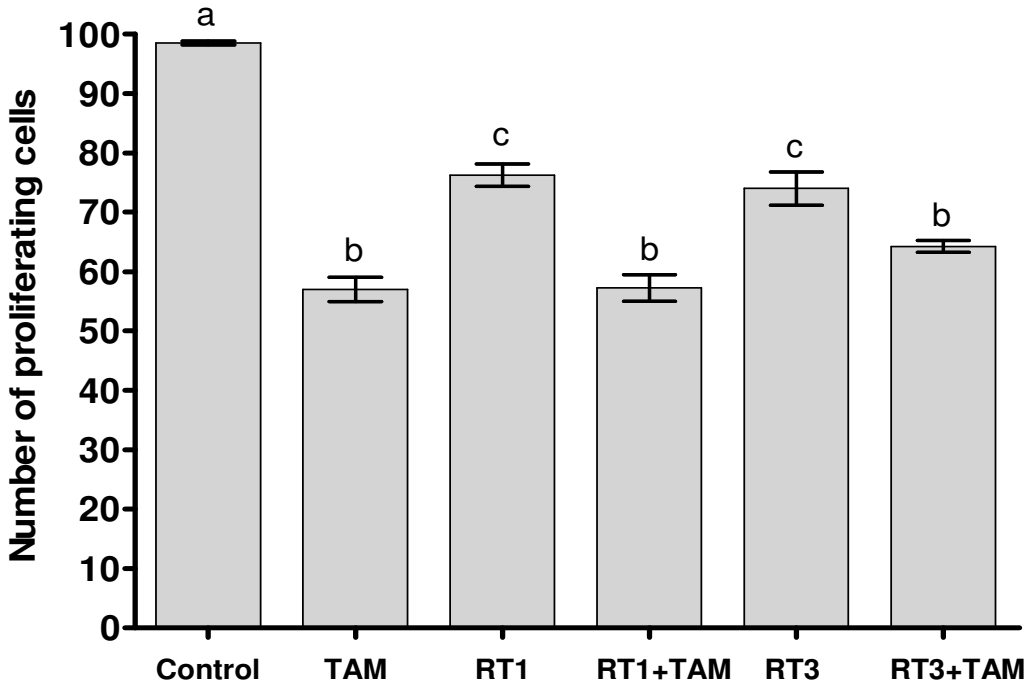

Number of apoptotic cells
90
80
70
60
50
40
30
20
10
0
6h
24h
48h
a
a
a
b
b
b
c,d
c
c
c
b
c
d
d
b
b
b
d
Control
TAM
RT1
RT1+TAM
RT3
RT3+TAM
A
Number of necrotic cells
7
6
5
4
3
2
1
0
6h
24h
48h
a,c
a,b
b,c
a,b
c,d
b
b,d,e
a
b,c
d
a
c
e
Control
TAM
RT1
RT1+TAM
RT3
RT3+TAM
B

Figure 7.


BrdU
DAPI
Merge
a
d
b
e
c
f

A


Number of proliferating cells
100
90
80
70
60
50
40
30
20
10
0
a
b
c
b
c
b
Control
TAM
RT1
RT1+TAM
RT3
RT3+TAM

B

Figure 8.

ANEXOS

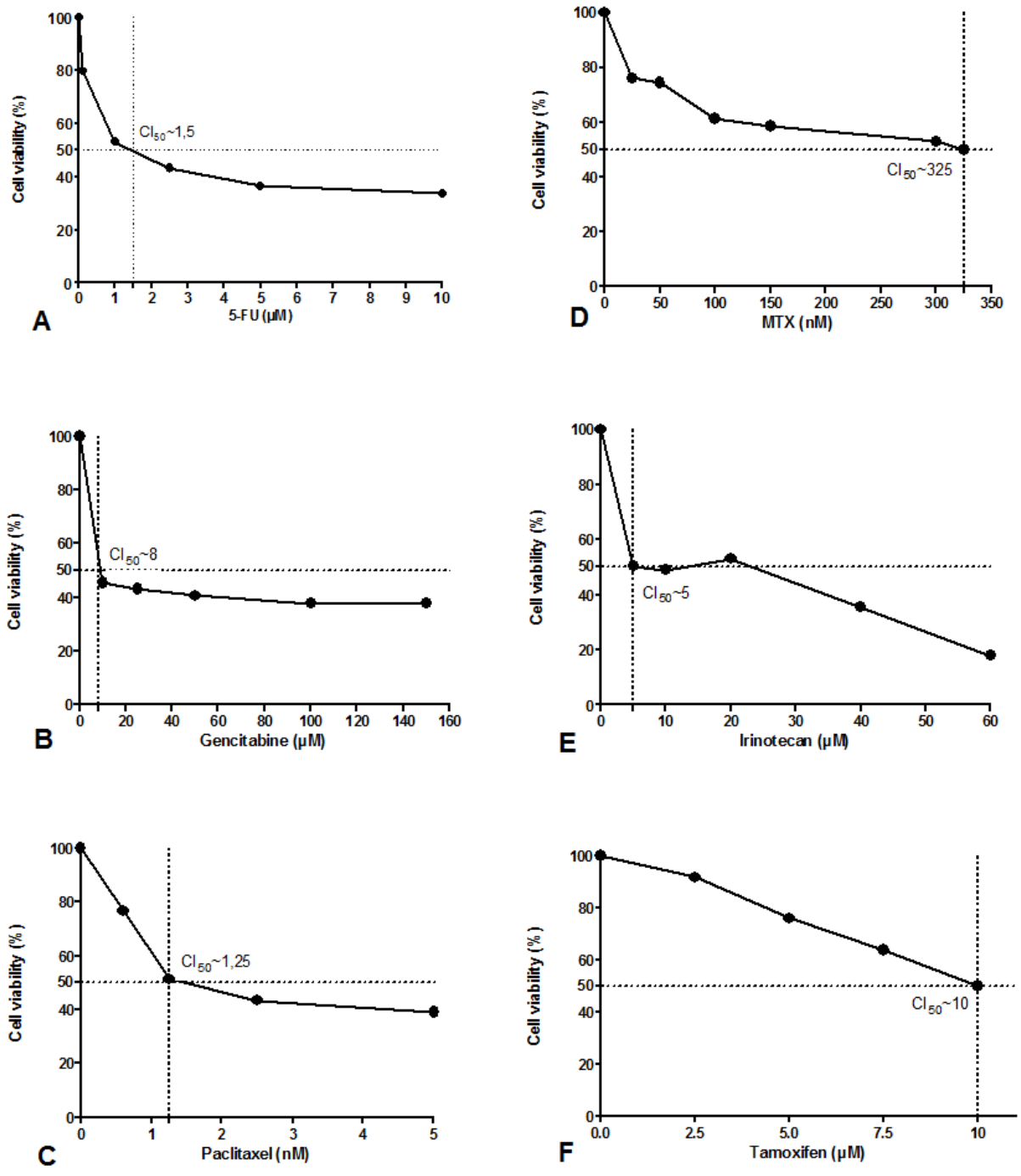


Figura 1. Avaliação da viabilidade celular dos diferentes agentes antitumorais pelo ensaio MTT. Diferentes concentrações dos agentes foram testadas e suas respectivas CI_{50}: (A) 5-FU, (B) gencitabina, (C) paclitaxel, (D) MTX, (E) irinotecano e (F) tamoxifeno. As curvas representam resultados de duas repetições independentes.