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PAULA MARYNELLA ALVES PEREIRA LIMA

POTENCIAL RECOMBINOGÊNICO E CITOTÓXICO DE UM COMPLEXO METÁLICO TERNÁRIO DE COBRE ASSOCIADO A β–DICETONA E 1,10 FENANTROLINA ($CuBTAPhenClO_4$)

PATOS DE MINAS – MG
FEVEREIRO DE 2019

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Dissertação de mestrado apresentada ao Programa de Pós-graduação em Biotecnologia como requisito parcial para obtenção do título de Mestre em Biotecnologia.

Orientador: Prof. Dr. Robson José de Oliveira Junior

Coorientadora: Prof[a.] Dr[a.] Thaise Gonçalves de Araújo

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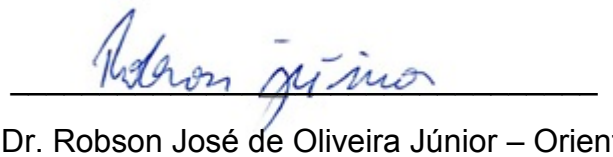
Aprovado em 13 / 02 / 2019

BANCA EXAMINADORA



Prof. Dr. Robson José de Oliveira Júnior – Orientador

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Prof. Dr. Alexandre Azenha Alves de Rezende

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“Há um tempo em que é preciso abandonar as roupas usadas, que já tem a forma do nosso corpo, e esquecer os nossos caminhos, que nos levam sempre aos mesmos lugares. É o tempo da travessia: e, se não ousarmos fazê-la, teremos ficado, para sempre, à margem de nós mesmos”

Fernando Pessoa

Dedicatória

Dedico essa conquista, primeiramente a Deus, minha fortaleza.

À meu marido que sempre me apoiou, incentivou e entendeu minhas ausências, sem voc
teria conseguido: Te amo inf

Aos meus pais, irmãos e cunhada que mesmo de longe sempre me incentivaram: Amo vocês!

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Resumo

O câncer é a segunda maior causa de morte no mundo, sendo o de mama o mais incidente em mulheres brasileiras, após o de pele não-melanoma. A quimioterapia é amplamente utilizada no tratamento dessa doença, contudo, possui efeitos colaterais que debilitam as pacientes. Nesse sentido, torna-se necessária a busca por medicamentos mais seletivos e menos tóxicos, e que, sobretudo, combatam a resistência tumoral. O presente estudo objetivou avaliar o potencial mutagênico/recombinogênico do complexo metálico ternário de cobre associado a β-dicetona e 1,10 fenantrolina (CBP-01) e sua citotoxicidade frente a linhagens mamárias, comparada a fármacos rotineiramente utilizados. As concentrações do composto utilizadas no teste de mutagenicidade/recombinogenicidade *in vivo* foram determinadas pela curva de toxicidade em *D. melanogaster.* Foi realizado o Teste de Mutação e Recombinação Somática – SMART para avaliar o efeito mutagênico e/ou recombinogênico de CBP-01 (0.03mM, 0.06mM, 0.12mM e 0.25mM), Carboplatina (0.5mM) e Cisplatina (0.025mM). Posteriormente, foram conduzidos os ensaios de MTT (brometo-3-(4,5-dimetiltiazol2-il)-2,5-difeniltetrazólio) a fim de se verificar a citotoxicidade de CBP-01, Carboplatina, Cisplatina e Doxorrubicina, em diferentes concentrações (1μM, 5μM, 10μM, 12.5μM, 25μM e 50μM), à linhagens de Câncer de Mama, T-47D (carcinoma ductal), MCF7 (carcinoma luminal) e MDA-MB-231 (triplo-negativa metastática) e à linhagem não tumoral MCF 10A. Foi possível encontrar a dose letal (DL) de CBP-01 em *D. melanogaster* (0,4mM) e por meio do teste SMART foi observado o potencial recombinogênico de CBP-01 apenas na menor concentração (0.03mM) e após sua biotransformação, o que sugere a geração de substâncias reativas capazes de gerar danos ao DNA quando metabolizado. Quanto aos demais fármacos, todos induziram alta frequência de manchas, o que confirma seus potenciais recombinogênico/mutagênico. Os resultados encontrados pelo ensaio de MTT mostraram a seletividade de CBP-01, que apresentou citotoxicidade às linhagens tumorais, especialmente contra as células triplo-negativas, MDA-MB-231 (IC_{50} 2.05 após 72h de tratamento e índice de seletividade de 3.10) quando comparado aos demais quimioterápicos. CBP-01 apresenta-se potencialmente promissor para o tratamento do CM, no entanto, estudos adicionais são necessários para compreender os eventos molecuares mediados por seu tratamento, para que assim sejam estabelecidos novos desenhos terapêuticos para o CM.

Palavras-chave: Câncer de mama. Citotoxidade. CuBTAPhenClO_4. Composto químico. Recombinogenicidade.

Abstract

Cancer is the second leading cause of death woroldwide, and breast is the most common in brazilian women, after non-melanoma skin. Chemotherapy is widely used for treatment of this disease, however, responsible for debilitating side effects. Therefore, it is necessary to search for more selective, less toxic drugs, and effective to combat tumor resistance. The present study aimed to evaluate the mutagenic / recombinogenic potential of the copper ternary metal complex associated with β-diketone and 1,10-phenanthroline (CBP-01), and its cytotoxicity in mammalian lineages, compared to routinely used compounds. The concentrations of the compound for *in vivo* mutagenicity / recombinogenicity test were established by the toxicity curve in *D. melanogaster*. The Somatic Recombination and Mutation Test - SMART was performed to evaluate the mutagenic and / or recombinogenic effect of CBP-01 (0.03mM, 0.06mM, 0,12mM and 0.25mM), Carboplatin (0.5mM) and Cisplatin (0.025mM). Subsequently, the MTT (bromide-3- (4,5-dimethylthiazol-2-yl) - 2,5-diphenyltetrazolium) assays were conducted in order to verify the cytotoxicity of CBP-01, Carboplatin, Cisplatin and Doxorubicin, in diferents concentrations (1μM, 5μM, 10μM, 12.5μM, 25μM e 50μM), to breast cancer lineages T-47D (ductal carcinoma), MCF7 (luminal carcinoma) e MDA-MB-231 (triple-negative metastatic) and non-tumoral lineage MCF 10A. The lethal dose (LD) of CBP-01 in *D. melanogaster* was defined (0.4mM) and through the SMART test the recombinogenic potential of CBP-01 was only observed at the lowest concentration (0.03mM) and after its biotransformation, which suggests the generation of reactive substances capable of damaging the DNA. The other drugs induced high frequency of spots, confirming their recombinogenic / mutagenic potential. The results found by the MTT assay showed the selectivity of CBP-01, which was cytotoxic to tumor cell lines, especially against triple-negative cells, MDA-MB-231 (IC50 2.05 after 72 hours of treatment and selectivity index of 3.10) when compared to the other chemotherapeutic agents. CBP-01 is potentially promising for CM treatment, however, additional studies are needed to understand the molecular events mediated by their treatment, so that new therapeutic designs for CM will may be established.

Keywords: Breast cancer. Cytotoxicity. CuBTAPhenClO_4. Chemical compound. Recombinogenicity.

Lista de abreviaturas e símbolos

$Cu(BTA)(Phen)ClO_4$	Complexo metálico ternário de cobre associado a β–dicetona e 1,10 fenantrolina
CBP-01	Complexo metálico ternário de cobre associado a β–dicetona e 1,10 fenantrolina
CYP19	Enzima citoromo P450 aromatase
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
IFN	Interferon
TGF- β	Fator de transformação do crescimento beta
IDC	Carcinoma ductal invasivo
NOS	Sem outra especificação
NST	Carcinoma invasivo sem nenhum tipo especial
ER	Receptor de estrogênio
PR	Receptor de progesterona
HER2	Receptor 2 do Fator de Crescimento Epidermal Humano
miRNA	MicroRNA
DNA	Ácido Desoxirribonucléico
Cu(II)	Cobre oxidado
Cu(I)	Cobre reduzido
ERRO	Espécies reativas de oxigênio
$O_2^{\bullet -}$	Superóxido
$OH^{\bullet}$	Radical Hidroxila
$RO2^{\bullet}$	Peroxilo
$RO^{\bullet}$	Alcoxilo
HOCl	Ácido hipocloroso
O_3	Ozônio
$ONOO^-$	Peroxinitrito
$^1O_2$	Oxigênio singlete
H_2O_2	Peróxido de hidrogênio
SOD	Superóxido dismutase
GSH	Glutationa peroxidase

GTS	Glutationa S-transferase
DMSO	Dimetil sulfóxido
HBTA	4,4,4-trifluor-1-fenil-1,3-butanodiona
Phen	Fenantrolina
ClO_4^-	Perclorato
CF3	Trifluorometil
SMART	Teste de Mutação e recombinação somática
flr^3	Linhagem flare 3 de *Drosophila melanogaster*
mwh/mwh	Linhagem *multiple wing hairs* de *Drosophila melanogaster*
ORR	Linhagem Oregon R, flr3 de *Drosophila melanogaster*
ST	Cruzamento padrão
Cyp6A2	Enzimas P450
HB	Cruzamento de alta bioativação
MH	Trans-heterozigotos marcados
BH	Heterozigotos balanceados

SUMÁRIO

Introdução

O câncer é um conjunto de doenças de origem multifatorial caracterizada por danos moleculares responsáveis pela transformação e capacidade invasiva das células. Sem sintomas ou sinais definidos, é a segunda maior causa de morte mundialmente, o que o torna um grande problema de saúde pública. Anualmente, mais de 14 milhões de pessoas são diagnosticadas com câncer todo ano e 8,8 milhões morrem pela doença. Segundo estimativas da Organização Mundial da Saúde (OMS), em 2030, serão 21 milhões de novos casos anuais, sendo que 60% ocorrerão em países em desenvolvimento, como o Brasil. Entre as mulheres no mundo, a neoplasia mais frequente é o câncer de mama (CM), menos diagnosticada apenas que o câncer de pele não melanoma (OMS, 2017).

As terapias usadas no tratamento do CM dependem das características histopatológicas e moleculares da doença, bem como do estadiamento e das condições fisícas e psicológicas da paciente. Por se tratar de uma doença heterogênea, cada plano terapêutico deve ser traçado de forma personalizada (ELLSWORTH et al., 2010; YERSAL; BARUTCA, 2014).

Dentre as terapias mais empregadas no CM estão a cirurgia, quimioterapia, radioterapia, hormonioterapia e imunoterapia. Mesmo com tantas alternativas, com o avanço da medicina e dos insumos farmacêuticos, os casos de metástase e recidiva ainda são frequentes. Isso mostra o quão importante é a descoberta e aperfeiçoamento de novos produtos e meios para um diagnóstico mais eficiente e terapia mais eficaz.

Os quimioterápicos são definidos como uma estratégia sistêmica de tratamento, responsáveis por lesionar a molécula de DNA ocasionando a morte das células (ROCHA, 2015). Sua inespecificidade ocasiona efeitos colaterais por vezes debilitantes como náuseas, vômitos, queda de cabelo, erupções cutâneas, fadiga e anemia.

Nesse sentido, torna-se premente a procura por novos medicamentos capazes de associar a ação antineoplásica a uma redução na toxicidade, em um sinergismo entre eficácia e seletividade. Dessa forma, o presente estudo teve como objetivo avaliar o potencial mutagênico/recombinogênico e citotóxico do protótipo $Cu(BTA)(Phen)ClO_4$. Trata-se de um complexo metálico inédito, formado a partir da associação da fenantrolina e a β-dicetona ao cobre (II), chamado genericamente de CBP-01, como resultados promissores em estudos *in vitro* e *in vivo*. O presente

estudo explora sua atividade em diferentes modelos experimentais. Sua síntese e caracterização é descrita por Do Couto Almeida e colaboradores (2015).

Hipotetizou-se que o protótipo CBP-01 não apresentasse efeito mutagênico/recombinogênico em modelos de *Drosophila melanogaster*. Além disso, enquanto quimioterápico, fosse citotóxico a células tumorais mamárias, superando o potencial antineoplásico da Cisplatina, Carboplatina e Doxorrubicina.

Trata-se de uma pesquisa voltada para a bioinorgânica medicinal que visa compreender os efeitos do cobre associado a β-dicetona e 1,10 fenantrolina em meio experimental, que contribuirá com avanços na saúde humana, sobretudo no tratamento de tumores.

CAPÍTULO I

Referencial teórico

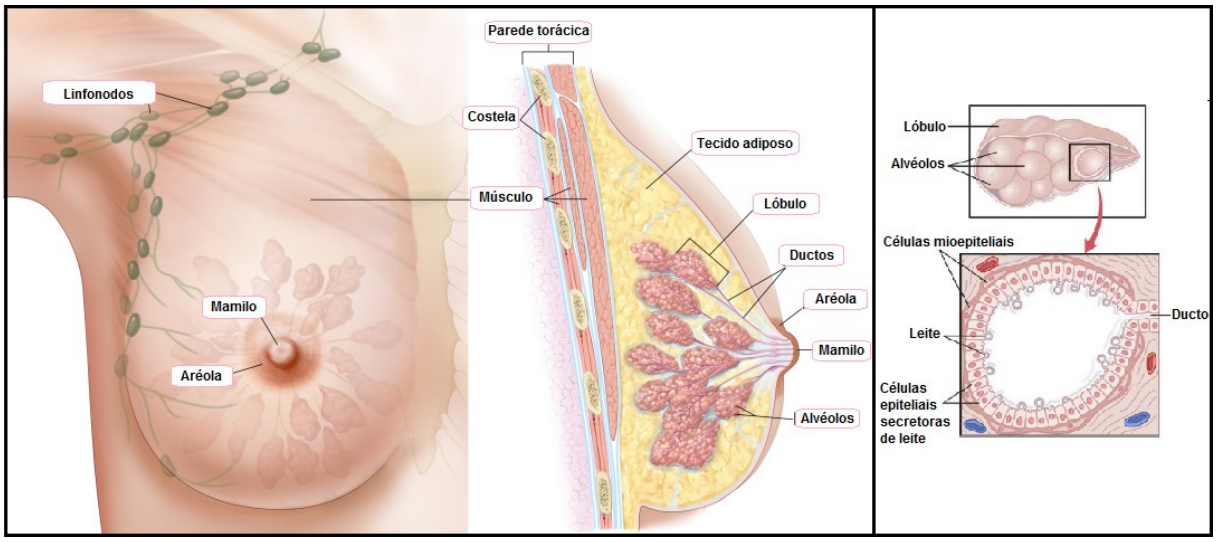
Capítulo formatado de acordo com as normas da Associação Brasileira de Normas Técnicas – ABNT.

2.1 A glândula mamária

A mama é uma projeção hemisférica de dimensão variável, localizada anteriormente aos músculos peitoral maior e serrátil inferior, aderida a estes por uma camada de fáscia constituída de tecido conjuntivo denso não modelado (TORTORA; NIELSEN, 2013). Apresenta, basicamente, três tipos de tecidos: o glandular (parênquima), o adiposo e o fibroso (estroma) (RAMIÃO et al., 2016).

Cada mama é constituída por uma glândula (Figura 1) composta por 15 a 20 lobos, cada qual com um ducto lactífero excretório que se abre no mamilo. O lobo é formado por compartimentos menores, chamados lóbulos, constituídos de aglomerações alveolares. O epitélio dos alvéolos e dos ductos é simples, exceto em seu pólo basal, o qual é composto por uma camada de células mioepiteliais. Estas são esteladas, semelhantes às do músculo liso, que se contraem e ajudam a impulsionar o leite. Os ductos lactíferos se esvaziam no mamilo, que é uma protusão da mama, ricamente inervada e lubrificada por glândulas sebáceas (BERNE; LEVY, 2009; TORTORA; NIELSEN, 2013).

Figura 1. Tecido mamário normal à esquerda mostrando toda sua estrutura e linfonodos. Á direita, vista ampliada do lóbulo e do alvéolo.



Fonte: Adaptado de GUYTON; HALL (2011) e NCBI (2018).

A glândula mamária desempenha as funções de síntese, secreção e ejeção de leite (TORTORA; NIELSEN, 2013). A partir da puberdade, o estrógeno estimula o crescimento e a ramificação ductal e também contribui para o aumento da deposição

de tecido adiposo, o qual tem grande influência para o tamanho e formato das mamas. O tecido adiposo expressa a enzima citocromo P450 aromatose (CYP19), estimulando a própria síntese de hormônios estrógenos e andrógenos. Durante a gestação há o crescimento, ramificação ductal e o desenvolvimento lóbulo-alveolar (BERNE; LEVY, 2009). Além disso, outros hormônios também contribuem para o desenvolvimento mamário, como o hormônio do crescimento, a prolactina, os glicocorticoides adrenais e a insulina (GUYTON; HALL, 2011).

2.2 Câncer de mama

2.2.1 *Epidemiologia e fatores de risco*

De acordo com dados do INCA são estimados 600 mil novos casos de câncer para o biênio 2018-2019 no Brasil. Entre as mulheres brasileiras, 60 mil serão diagnosticadas com câncer de mama (CM), aproximadamente 56,33 a cada 100 mil mulheres. Destas, 14.206 chegarão à óbito. Portanto, o risco de uma mulher desenvolver o CM no Brasil é de 6,3% e de morrer pela doença é de 1,6% (INCA, 2018). O CM também é o de maior incidência entre as mulheres norte americanas, responsável por 266.120 do total de 878.980 casos de câncer diagnosticados em mulheres (ACS, 2018).

O aumento nos índices epidemiológicos relaciona-se diretamente ao aumento de mamografias realizadas pela população. Esse exame permitiu um diagnóstico precoce da doença, com antecipação de cerca de 1 a 3 anos, detectando casos indolentes. A partir da década de 1990, fatores como a obesidade e uso de hormônios para amenizar os efeitos da menopausa também contribuíram para essa maior incidência (DESANTIS et al., 2013), aumentando o risco de CM em 50% (KEY; REEVES, 2016). A exposição aos hormônios sexuais, que envolve a menarca precoce e/ou menopausa tardia e o uso de terapia hormonal combinada após a menopausa são, portanto, fatores de risco para essa neoplasia (ANDERSON et al., 2014).

Outros aspectos incluem o histórico familiar e mutação nos genes BRCA1 (Breast Cancer 1) e BRCA2 (Breast Cancer 2), exposição a radiação (SIU, 2016), agentes carcinógenos, metais tóxicos, uso de contraceptivos orais, consumo em excesso de alimentos industrializados, sedentarismo, álcool e tabagismo

(ELLSWORT et al., 2010; THAUR et al., 2018). De fato, as alterações genéticas que promovem o câncer podem ser herdadas ou adquiridas, estando ambas inter-relacionadas. As primeiras são de origem parental em que as mudanças estão presentes nas células germinativas. As adquiridas, também chamadas de somáticas, ocorrem ao longo da vida, sendo resultado de erros durante o processo de divisão celular ou decorrentes da exposição a agentes químicos, físicos ou biológicos que culminam em danos no DNA (NCI, 2017).

Raça e etnia também contribuem para a ocorrência do CM. Mulheres da Ásia e llhas do Pacífico apresentam as menores taxas de incidência e mortalidade (DESANTIS et al., 2017). Contudo, sabe-se que a interação de diferentes aspectos, como os reprodutivos, perfil socioeconômico, acesso a cuidados de saúde e mamografia, disponibilidade de tratamento psicológico, fatores culturais, comorbidades (DANFORTH, 2013) e aderência e resposta ao tratamento também influenciam no diagnóstico e sobrevida das pacientes (CURTIS et al., 2008).

A relação entre a dieta e a doença vem sendo estudada há mais de 40 anos. Estudos apontam que o alto consumo de peixes, legumes, vegetais e crucíferos decresce em 25% as chances de CM (KEY; REEVES, 2016). Por outro lado, o consumo exarcebado de alimentos que estimulam a produção de fatores inflamatórios, como refrigerantes açucarados, grãos refinados, carne vermelha e processada, margarina, entre outros, durante a adolescência e o início da vida adulta, aumentam as chances de CM na pré-menopausa (HARRIS et al., 2017).

O sedentarismo também se destaca nesse cenário epidemiológico. O comportamento sedentário é modificável pela prática frequente de exercícios físicos. Exercícios regulares constituem uma ferramenta que controla o balanço energético e previne o acúmulo de reservas de gordura, a qual influencia diretamente na síntese de hormônios (MONNINKHOF et al., 2009).

O consumo de álcool também predispõe ao CM (KEY; REEVES, 2016), uma vez que está associado ao aumento da circulação de estrogênio e seus metabólitos. Sua ingestão ainda está relacionada à maior proliferação tumoral e a regulação da sinalização de citocinas, principalmente interferon (IFN) e fator de transformação do crescimento beta (TGF-β) (WANG et al., 2017). Por fim, padrões reprodutivos também estão ligados à ocorrência desse câncer (DESANTIS et al., 2016). As chances de desenvolver tumores mamários aumenta em mulheres nulíparas (MAKAMA et al., 2017).

Portanto, traçar os fatores de riscos pode favorecer na definição de grupos de maior suscetibilidade (DOSSUS; BENUSIGLIO, 2015). Além disso, o conhecimento a respeito desses aspectos podem conduzir mudanças nos hábitos, o que culminaria em uma menor incidência dessa neoplasia.

2.2.2 *Aspectos patológicos do câncer de mama*

Para a promoção e avanço da doença, células tumorais adquirem diferentes capacidades. Estas incluem: a sustentação dos sinais proliferativos, evasão aos supressores tumorais, resistência a morte celular, imortabilidade replicativa, escape ao sistema imune, instabilidade genômica, desregulação metabólica, indução da angiogênese e metástase (HANAHAN; WEINBERG, 2011).

A grande diversidade molecular e patológica observada em pacientes com CM define sua heterogeneidade (ELLSWORTH et al., 2010). Características como tamanho do tumor, grau histológico, envolvimento de linfonodos e idade da paciente não são suficientes para se estabelecer estratégias terapêuticas efetivas. Além disso, aspectos histológicos não identificam as complexas alterações genéticas e acontecimentos moleculares envolvidos. Por esta razão, tumores com aspectos clínicos e patológicos semelhantes podem apresentar comportamentos diferentes (YERSAL; BARUTCA, 2014).

A 4ª edição da WHO sobre a Classificação de Tumores de Mama publicou, em 2012, a categorização histológica dessas lesões. A terminologia de um tipo comum de CM mudou de carcinoma ductal invasivo (IDC) sem outra especificação (NOS) para carcinoma invasivo sem nenhum tipo especial (NST), omitindo o nome “ductal”. Ester termo transmite suposições histogenéticas não comprovadas (derivação dos tumores do sistema ductal) e o NOS não compreende um grupo uniforme de carcinomas (SINN; KREIPE, 2013).

Os demais subtipos invasivos incluem os carcinomas: lobular invasivo, medular, mucinoso, tubular, cribriforme, metaplásico, apócrino, papilar, adenoide cístico, mucoepidermoide, polimorfo e carcinoma com elementos neuroendócrinos (SINN; KREIPE, 2013). Tumores mamários invasivos correspondem a 80% dos casos (ACS, 2017). Já os não invasivos podem ser classificados como ductal *in situ* (DCIS) e o lobular *in situ* (LCIS) (ACS, 2015).

Em relação aos aspectos moleculares, o CM é dividido em subgrupos (THE CANCER GENOME ATLAS NETWORK, 2012), baseados na expressão de Receptor de Estrogênio (ER), Receptor de Progesterona (PR) e Receptor 2 do Fator de Crescimento Epidermal Humano (HER2), ou pela ausência desses, como em CM triplo negativos (PRAT; PEROU, 2011). Os subtipos incluem:

(i) Luminal A: ER e PR positivos e HER2 negativos (GUIU et al., 2012)

(ii) Luminal B: ER e/ou PR positivos estratificados em HER2 positivo (Luminal-HER) ou HER2 negativo (GOLDHIRSCH et al., 2013).

(iii) Triplo negativos: não expressam ER e PR e não possuem HER2 amplificado (GOLDHIRSCH et al., 2013; BERNARD et al., 2015);

(iv) HER2 positivo: ER e PR negativos e apresentam a amplificação de HER2 (GUIU et al., 2012).

A extratificação do CM em grupos aliada a análises de expressão de genes com base em microarranjos, fornecem informações importantes na definição do tipo de tratamento a ser empregado (SOTIRIOU; PUSZTAI, 2009) e ainda possibilita traçar novas estratégias na busca de agentes quimioterápicos (BERNARD et al., 2015). Ensaios capazes de avaliar outros padrões como o perfil de metililação do DNA, a expressão de microRNAs (miRNA) e a expressão de outras proteínas podem fornecer dados extras para a caracterização mais efetiva da arquitetura molecular do CM (THE CANCER GENOME ATLAS NETWORK, 2012).

2.2.3 *Diagnóstico e estratégias terapêuticas*

Nas últimas duas décadas, apenas países desenvolvidos conseguiram reduzir as taxas de mortalidade por CM. Analistas e pesquisadores atribuem esse fato à eficácia dos programas de controle, destacando-se as ações de detecção precoce e estratégias de tratamento (INCA, 2015).

O diagnóstico do CM no estado inicial da doença, não metastática, conduz ao sucesso terapêutico e a maiores chances de cura (ACS, 2016). O processo de diagnóstico é embasado em três exames: o clínico, o radiológico e a biópsia. No clínico é feita a inspenção e palpação das mamas e dos linfonodos próximos; no radiológico é realizada a mamografia, a ultrassonografia e/ou a ressonância magnética, sendo esta necessária em casos de mamas densas e em mulheres com implantes de silicone. Já a biópsia fornece informações específicas acerca do tumor

(ESMO, 2013; RAJAGURU; PRABHAKAR, 2017). Após diagnóstico, a conduta terapêutica depende das característica da lesão. A cirurgia é amplamente emprega, incluindo a lumpectomia e a mastectomia radical. Na lumpectomia o tumor é removido com margens cirúrgicas,conservando as mamas. Na mastectomia, por sua vez, toda a mama é retirada (NCCN, 2016).

Três aspectos principais devem ser considerados no momento da definição de terapias adjuvantes: i) a responsividade endócrina (após análise de ER e PR); ii) superexpressão de HER2; e iii) risco de recidiva. Tumores que expressam ER/PR são tidos como endócrino sensíveis. A ausência de expressão de ER/PR e superexpressão de HER2 são sugestivos de resposta endócrina incompleta (SBOC, 2011).

As condutas adotadas incluem a quimioterapia, radioterapia, hormonioterapia, terapia alvo e ablação térmica percutânea. A quimioterapia, definida como estratégia sistêmica de tratamento, é administrada intravenosamente para matar ou inibir micrometástases clinicamente indetectáveis após a cirurgia (WHO, 2006). É a principal opção de terapia para pacientes com tumores triplo negativos (LEHMANN et al., 2016).

Já a ablação térmica é uma técnica minimamente invasiva, que consiste na geração de necrose tecidual na região do tumor, seja por aquecimento ou congelamento retrospectivo do tecido. É uma forma de tratamento complementar à terapia sistêmica e efetiva contra o CM (BARRAL et al., 2016).

A radioterapia baseia-se na incidência de raios de alta energia sobre células tumorais presentes na região do tórax (NCI, 2012). Os avanços no tratamento radioterápico vem trazendo inúmeros benefícios, como a diminuição de quadros de radiodermatites e o não compromentimento de órgãos próximos como o coração e pulmões. Os riscos de recidiva tumoral também são relativamente menores em pacientes submetidas à radioterapia após mastectomia (MCGALE et al., 2014).

A hormonioterapia inclui duas abordagens. A primeira é voltada para a inibição da produção de estrogênio com a utilização de inibidores da enzima aromatase, adotada principalmente em mulheres menopausadas. Na outra são utilizados moduladores seletivos do ER, como o tamoxifeno ou o fulvestrant (SPRING et al., 2016).

Já os anticorpos monoclonais compõem a estratégia de terapia alvo neutralizando proteínas tumorais específicas. Estão diretamente relacionados à

melhora do prognóstico das pacientes que expressam o HER2 por meio da administração do anticorpo trastuzumabe. No entanto, o custo ainda é um fator limitante (FILPULA, 2007; KALIKS, 2016; PUSZTAI et al., 2016).

2.3 Terapias oncológicas baseadas em quimioterápicos

A quimioterapia é uma forma de tratamento baseada em compostos químicos (BRASIL, 2014). O primeiro quimioterápico antineoplásico foi desenvolvido a partir do gás mostarda para o tratamento de linfomas. No ano de 1946 surgiram as publicações de estudos clínicos sobre este gás e seus os efeitos (INCA, 2017).

Os quimioterápicos atuam sobre diversas moléculas (FONTES et al., 2005) como ácidos nucléicos, lipídios e proteínas. Em geral, causam lesões no DNA, que quando não reparadas, conduzem à morte celular (O'CONNOR, 2015; PUIGVERT et al., 2016). Assim, são classificados de acordo com seu mecanismo de ação, sua estrutura química e sua ação fisiológica (REDDY; COUVREUR, 2010). Destacam-se:

- os agentes alquilantes, como a Cyclophosphamida, responsáveis pela inativação química da base nitrogenada guanina (EMADI et al., 2009);
- os inibidores de topoisomerase I, como o Topotecan e inibidores de topoisomerase II, como a Doxorrubicina e Etoposide, os quais conduzem à quebra do DNA por tensão (WIJDEVEN et al., 2016).;
- compostos de coordenação com platina, como a Cisplatina e seus análogos que se ligam avidamente ao DNA, mais especificamente à guaninas, gerando ligações cruzadas (CHABNER; CALABRESI, 1995; WIJDEVEN et al., 2016);
- agentes antimitóticos, no qual estão inclusos o Taxol e Docetaxel, responsáveis por estabilizar as fibras do fuso (OLSON et al., 2017; MOHR et al., 2017);
- os antimebólitos, dentre os quais estão o Gemcitabine, Methotrexate e o Fluorouracil que inibem a síntese de nucleotídeos purínicos e pirimídicos, bloqueando a replicação e proliferação celular (CHABNER; CALABRESI, 1995; WIJDEVEN et al., 2016).

Contudo, as células tumorais evadem ao tratamento, adquirindo resistência a esses fármacos. A regulação positiva da expressão de proteínas de transporte de

drogas, a inibição da via apoptótica e a ativação das vias de reparo de danos ao DNA como forma de lidar com o estresse genotóxico induzido por vários quimioterápicos (WIJDEVEN et al., 2016) são exemplos de mecanismos adotados para a manutenção do crescimento da lesão.

Com a finalidade de melhorar os resultados terapêuticos, diminuindo a toxicidade e a resistência aos fármacos, há um crescente interesse no desenvolvimento e descoberta de novas drogas (TWOMEY et al.,2017). No entanto, torna-se imprescindível o controle de danos provocados por esses compostos (SILVA et al., 2003). Realizar ensaios de mutagenicidade/recombinogenicidade e carcinogenicidade de drogas com potencial quimioterápico são necessários e possibilitam identificar o real dano que algumas drogas podem provocar às células.

2.3.1 *Complexos metálicos como agentes quimioterápicos*

Evidências empíricas sobre o uso terapêutico de metais datam do século XVI (THOMPSON; ORVIG, 2003). O progresso na área de química inorgânica medicinal tornou positiva a utilização de complexos metálicos como drogas. Estes exercem papel essencial na busca de ferramentas tecnológicas para o tratamento clínico de tumores. Nesse contexto, vários compostos com toxicidade reduzida e alta especificidade vêm sendo desenvolvidos (GOWDA et al., 2014).

O composto inorgânico cis-diamminedichloroplatinum (II) cis-[Pt $(NH_3)_2(Cl)_2$], conhecido como cisplatina ou sal de Peyrone (em referência a Michel Peyrone, 1845) é pioneiro como medicamento anticancerígeno contendo platina. No século XIX, foi usada como droga antiproliferativa. A cisplatina e seus similares são complexos de metais pesados, tendo no centro da molécula um átomo de platina rodeado por dois átomos de cloreto e duas moléculas de amônia na posição cis (THOMPSON; ORVIG, 2003; GOWDA et al., 2014a).

A cisplatina é o complexo metálico mais conhecido e usado no tratamento do câncer. Vários outros similares já foram desenvolvidos como a carboplatina, oxaliplatina, nedaplatina, heptaplatina e lobaplatina (WANI et al., 2016). Nestes, quando a platina se liga ao DNA causa a torção da molécula, inibe a transcrição e provoca a morte das células tumorais (GOWDA et al., 2014).

Uma característica relevante dos metais é a perda de elétrons para formar íons carregados positivamente, que tendem a ser solúveis em fluidos biológicos,

desempenhando suas funções. Enquanto os íons metálicos são deficientes em elétrons, o DNA e proteínas são ricos dessas partículas, o que tendencia a sua interação com o quimioterápico (ORVIG; ABRAMS, 1999). Portanto, torna-se interessante investigar e explorar essa interação (GOWDA et al., 2014), uma vez que o sucesso clínico da cisplatina forneceu a “prova de conceito” para investigar metais essenciais e não essenciais unidos a agentes anticancerígenos (FREZZA et al., 2010).

Nesse contexto, complexos contendo cobre, ouro e zinco têm apresentado resultados promissores no tratamento neoplásico (FREZZA et al., 2010). O interesse em complexos de cobre baseia-se no seu potencial antimicrobiano, antiviral, anti-inflamatório, antitumoral e inibidor enzimático (IAKOVIDIS et al., 2011). O cobre é absorvido no estômago e na primeira porção do intestino delgado sendo carreado complexado à albumina, histidina e proteínas transcupreína de alto peso molecular através da veia porta até o fígado. A entrada de cobre nas células deve ser rigorosamente contraloda, pois quando em excesso, é altamente tóxico. Além disso, os íons cobre podem assumir os estados oxidado Cu(II) ou reduzido Cu(I), agindo como um cofator catalítico na atividade redox de enzimas como a citocromo oxidase e a superóxido dismutase. Participa, portanto, da respiração mitocondrial, absorção de ferro, remoção de radicais livres, bem como da produção destes ao interagir com o oxigênio molecular e peróxido de hidrogênio (LOWNDES; HARRIS, 2004; RIVÉRO-MULLER et al., 2007), aumentando, dessa forma, a produção de espécies reativas de oxigênio (ERO) (SISSI et al., 2005).

ERO é um termo usado para denominar coletivamente radicais de oxigênio, como superóxido ($O_2^{\bullet -}$), radical hidroxila ($OH^{\bullet}$), peroxilo ($RO2^{\bullet}$) e alcoxilo ($RO^{\bullet}$), bem como agentes oxidantes e/ou moléculas que são convertidas facilmente em radicais, como o ácido hipocloroso (HOCl), ozônio (O_3), peroxinitrito ($ONOO^-$), oxigênio singlete ($^1O_2$) e peróxido de hidrogênio (H_2O_2) (WISEMAN; HALLIWELL, 1996).

Compostos à base de cobre têm a capacidade de aumentar a quantidade de ERO nas células, comprovada pela detecção de maior atividade das enzimas superóxido dismutase (SOD) e da catalase. As ERO também podem ocasionar a depleção da atividade da enzima glutationa peroxidade (GSH), e provocar a mudança conformacional no sítio ativo da enzima glutationa S-transferase (GTS). Uma vez que essas enzimas são ponto chave no equilíbrio redox celular, o decréscimo de suas atividades desencadeia um maior acúmulo de ERO dentro das

células, com consequente estresse oxidativo e danos às biomoléculas (ZAFAR et al., 2017), como lipídios, proteínas e ácidos nucléicos (SISSI et al., 2005). Esses danos oxidativos causam alterações estruturais na mólecula de DNA, como mutações, recombinações, rearranjamentos, deleções, inserções, que, quando não reparados, contribuem para a oncogênese (WISEMAN; HALLIWELL, 1996).

Sabe-se que as células de CM apresentam níveis de espécies reativas de oxigênio (ERO) mais elevados do que as células normais, contudo, essas células tumorais são mais suscetíveis a ERO exógenos, tornando um mecanismo terapêutico promissor. Portanto, a combinação de moléculas redox-ativas com a terapia convencional pode ser útil para potencializar os tratamentos e superar os mecanismos de resistência. Sendo o CM uma doença heterogênea, é importante caracterizar melhor o estado redox dos diferentes subtipos e definir as características de expressão gênica associadas à homeostase redox (HECHT et al., 2016).

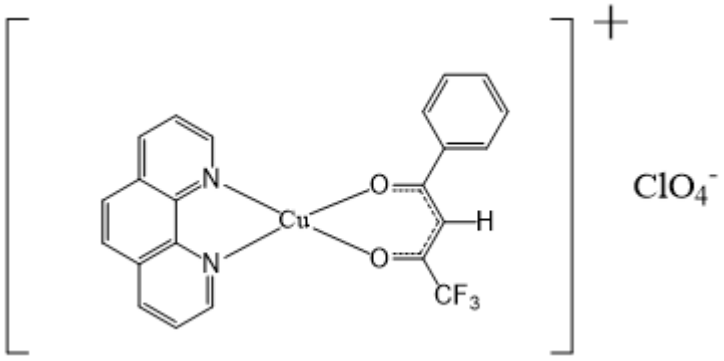
Quando combinado a ligantes tridentados assimétricos, o cobre inibe a atividade do proteassoma e induz a apoptose em células tumorais de próstata e sanguíneas (FREZZA et al., 2010; HINDO et al., 2009). Portanto, íons metálicos como o cobre não só possuem reatividade interessante, como também permitem diferentes combinações e geometrias com outros ligantes. Estes fatores o coloca como forte candidato na síntese de moléculas terapêuticas de amplo espectro (RUIZ-AZUARA; BRAVO-GÓMEZ, 2010).

2.3.2 *Complexo metálico de cobre (II) associado a β–dicetona e 1,10-fenantrolina (CBP-01)*

Almeida e colaboradores, em 2015, sintetizaram e descreveram o complexo ternário [Cu(O-O)(N-N) X], onde O-O refere-se 4,4,4-trifluor-1-fenil-1,3-butanodiona (HBTA); N-N refere-se 1,10-fenantrolina (Phen) e X é ocupado pelo íon ClO_4^- (Figura 2), chamado de Cu(BTA)(Phen)ClO_4 (CBP-01). É um complexo que apresenta coloração esverdeada, não higroscópico, estável ao ar e à luz e solúvel em solventes orgânicos, como dimetil sulfóxido (DMSO) e acetonitrilo. O íon de cobre tem a geometria quadrada-piramidal distorcida e se liga a β–dicetona via átomos de oxigênio e o N-doador heterocíclico (1,10 fenantrolina) liga-se por dois átomos de

nitrogênio heterocíclicos. O íon perclorato ocupa a posição apical, fracamente ligado, completando a esfera de coordenação (do COUTO ALMEIDA et al., 2015).

Figura 2: Representação da estrutura química do complexo metálico Cu(BTA)(Phen)ClO_4.



Fonte: do Couto Almeida et al., (2015).

A presença do anel aromático de fenantrolina fundido ao complexo é necessária para preservar a atividade antiproliferativa, uma vez que sua estrutura possibilita sua interação com o DNA (RUIZ-AZUARA; BRAVO-GOMEZ, 2010). Kljun e Turel (2017) demonstraram que complexos de cobre associados a 1,10 fenantrolina são excelentes candidatos para testes de mecanismo de ação e citotoxicidade em diferentes linhagens celulares. Já a β–dicetona é utilizada como ligante funcional na produção de complexos metálicos. A molécula CF3, presente na estrutura da β–dicetona, aumenta a lipofilicidade e melhora a absorção celular do composto, conferindo maior citoxicidade em linhagens tumorais como HeLa, de câncer de pulmão, osteosarcoma e câncer de mama (KLJUN; TUREL, 2017). Ambos ligantes ainda apresentam efeito sobre a seletividade do complexo e grau de atividade biológica em células tumorais e não tumorais, propriedades conferidas devido ao seu poder de modular as propriedades redox do cobre (RUIZ-AZUARA; BRAVO-GOMEZ, 2010).

O desenho dos complexos metálicos são feitos com base em três pontos principais: i) o composto deve conter um metal essencial para diminuir a toxicidade; ii) os ligantes (quelantes) devem favorecer a configuração cis em torno do íon metálico; e iii) e o conjunto deve apresentar diferente grau de hidrofobicidade para favorecer a absorção e distribuição da droga pelas células. Essa configuração é

garantida pela associação do cobre com a fenantrolina e a β–dicetona (RUIZ-AZUARA; BRAVO-GOMEZ, 2010).

Encontra-se em fase de estudos clínicos (BRAVO-GÓMEZ et al., 2015) uma classe de complexo metálico patenteada e registrada com o nome de Casiopeínas® - fórmula geral [Cu(N-N) (O-O)] NO3 ou [Cu(N-N) (O-N)] NO3 (RUIZ-AZUARA, 1992; RUIZ-AZUARA, 1993). As Casiopeínas clivam as ligações fosfodiéster do DNA através da formação de um oxo-complexo metálico que gera radicais $OH^{\bullet}$ e modifica quimicamente as bases nitrogenadas, induzindo o processo de apoptose. Além disso, apresenta baixa toxicidade. Esse complexo metálico ainda causa atraso no ciclo celular devido a danos na maquinaria de controle do ciclo (RIVERO-MULLER et al., 2007; CHAVEZ-GOZALEZ et al., 2017).

2.4 Ensaios *in vivo* em *Drosophila melanogaster*

Popularmente conhecida como a mosca da fruta, a *D. melanogaster* (Fig. 3) foi um dos organismos pioneiros para estudos genéticos. Logo após a redescoberta de trabalhos de Mendel, no laboratório do Dr. T. H. Morgan, a *D. melanogaster* passou a ser um organismo experimental para estudos genéticos. Trata-se de um eucarioto que possui sistema enzimático semelhante ao dos mamíferos, apresentando 80% de homologia genética com mamíferos (GRAF; VAN SCHAIK, 1992; MIKLOS; RUBIN, 1996), de pequeno tamanho (3 a 4 mm), de fácil diferenciação do macho da fêmea, baixo custo de manutenção em laboratório, ciclo de vida curto, número elevado de progênie, reduzido número de cromossomos, favorecendo a obtenção de resultados rápidos e confiáveis (GRAF; VAN SCHAIK, 1992).

Figura 3: Casal de *Drosophila melanogaster*. Fêmea à esquerda e macho à direita, o qual apresenta pente sexual no primeiro par de pernas (indicado pelas setas).

Fonte: http://www. sc.didaxis.pt/hereditariedade/drosophila.htm.

O disco imaginal em larvas de *D. melanogaster* é formado por apenas uma camada celular, que durante a metamorfose, prolifera-se mitoticamente para formar as estruturas epidérmicas da mosca adulta. As células deste disco têm um ciclo celular muito semelhante ao das células somáticas de mamíferos (EEKEN et al., 2002). Alterações genéticas em algumas dessas células leva à formação de células mutantes que são detectadas como manchas nas asas de moscas adultas (GUZMÁN-RINCON; GRAF, 1995), bem como a deleção de supressores tumorais também pode levar a formação de tumores nos indivíduos adultos. A mutação é definida como sendo alterações no DNA, seja em sua sequencia nucleotídica, definda como gênica, ou na reorganização da estrutura da dupla hélice (translocação, inversão ou mesmo ganho ou perda de parte do cromossomo), sendo conhecida como cromossômica (SILVA et al., 2003). Sabe-se que a mutação é um evento importante na carcinogênese (MARTINCORENA; CAMPBELL, 2015).

Portanto, estágios de desenvolvimento distintos, a disponibilidade de várias ferramentas e reagentes, sequência genômica conhecida, capacidade de ativar enzimaticamente promutágenos e procarcinógenos, semelhenças fisiológicas e elevada homologia entre a *D. melanogaster* e o homem, fazem desse um excelente modelo *in vivo* de estudos em toxicologia. Fornece, nesse contexto, resultados concernentes à mutagenicidade/recombinogenicidade aguda e/ou crônica e carcinogenicidade do compostos avaliados, bem como permite estudar o estresse oxidativo (ONG et al., 2015).

2.4.1 *Teste para detecção de mutação e recombinação somática (SMART)*

O teste SMART (*Somatic Mutation and Recombination Test*) é usado para detecção de mutações e recombinaçöes somáticas através da perda de heterozigose em genes que estabelecem a expressão de fenótipos detectáveis nas asas de *Drosophila melanogaster* (GRAF et al., 1984; GRAF; WURGLER, 1996). Permite avaliar uma série de acontecimentos tais como: mutações pontuais, recombinação mitótica, deleções ou padrões específicos de translocação (GRAF et al., 1984). As três linhagens mutantes de *D. melanogaster* utilizadas para realização do teste SMART são: linhagem *flare 3* (*flr*^{3}), linhagem *multiple wing hairs* (*mwh/mwh*) e linhagem *ORR* (*Oregon R, flr*^{3}) (GRAF et al., 1984; GRAF; VAN SCHAIK, 1992).

No SMART, a recombinação genética pode gerar células homozigóticas para os mutantes *flr*^{3} e *mwh*, resultando em fenótipos específicos. Um pêlo *mwh*, por exemplo, se caracteriza pelo crescimento de pelos múltiplos, ao passo que o *flr*^{3} é um único pelo encurtado e amorfo. Durante o desenvolvimento da mosca, devido à divisão celular, células mutantes usualmente formam um cluster de clones que apresentam fenótipos similares, chamado de ponto mutante. A frequência de pontos mutantes formados, por meio da exposição das larvas a um composto, permite identificar se ele é genotóxico (LOMBARTOD et al., 2015).

O teste possibilita a avaliação da ação direta e indireta de agentes mutagênicos e antimutagênicos. A ação direta é avaliada através do cruzamento ST (*standard*) devido ao nível basal de enzimas P450 (*Cyp6A2*), ao passo que a ação indireta é investigada por meio do do cruzamento HB (*high bioactivation*) em razão da linhagem utilizada possuir altos níveis de P450, o que possibilita a ação do composto somente após sua metabolização (FROLICH; WURGLER, 1989; GRAF; VAN SCHAIK, 1992; SANER et al., 1996; SAPANÓ; GRAF, 1998; ORSOLIN et al., 2016).

As análises de dois diferentes tipos de descendentes, trans-heterozigotos marcados (*mwh* +/+ *flr*^{3}) (MH) e heterozigotos balanceados (*mwh* +/ *TM3, Bd*^{s}) (BH), que apresentam o fenótipo borda lisa da asa e borda serrilhada da asa, respectivamente, determinam a atividade recombinogênica de genotoxicinas. A progênie MH pode manifestar o fenótipo *mwh* ou *flare* (manchas simples), fenótipo *mwh* e *flare* (manchas gêmeas), que podem ser ocasionados tanto por eventos mutagênicos quanto recombinogênicos. Já na progênie BH todos os eventos recombinogênicos são inviabilizados devido ao cromossomo balanceador, sendo assim, apenas o fenótipo *mwh* será visualizado (manchas simples) (GRAF et al., 1984; SPANÓ; GRAF., 1998; GRAF et al., 1992; FREI et al., 1992).

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CAPÍTULO II

A new copper ternary complex associated with β-diketone and 1,10 phenanthroline ($CuBTAPhenClO_4$) is a promised compound for the treatment of triple-negative breast cancer

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Abstract

Triple-Negative Breast Cancer (TNBC) is a biologically aggressive neoplasm with poor prognosis due to the lack of effective treatment which options include surgery, radiation, and mainly chemotherapy. However, chemorresistence and severe side effects remain a challenge in treating this breast cancer subtype. Metallodrugs of copper have been emerged as novel therapeutic agents exploring ion-binding ligands to overcome the limitations of toxicity. This work was focused on the interface between molecular biology and bioinorganic chemistry when assessing the cytotoxic and effect of new copper ternary metal complex associated with β-diketone and 1,10 phenanthroline (CBP-01) against breast cancer tumor cells. The recombinogenic / mutagenic potential was also evaluated though Somatic and Recombination test – SMART in *Drosophila melanogaster*. For MTT assay CBP-01 was tested in different concentrations (1μM; 5μM; 10μM; 12,5μM; 25μM; 50μM) for 24, 48 and 72h, and the cellular responses were compared to Carboplatin (CARB), Cisplatin (CIS) and Doxorrubicin (DOX). For SMART test, CBP-01 was evaluated at 0.03mM, 0.06mM, 0.12mM and 0.25mM. In the cellular models, the dose-dependent profile was identified for the four drugs and the cell viability differed between the times of treatment. Considering the triple-negative cell line (MDA-MB231), CBP-01 was clearly more effective with lower IC_{50} (2.05) and higher SI (3.10) than the other compounds. CBP-01 presented mutagenic / recombinogenic potential only in the lowest concentration (0.03mM) and after biometabolization. In the highest concentrations the frequency of mutant spots was lower, suggesting its ability to trigger apoptosis. Triple-Negative Breast Cancer (TNBC) is a biologically aggressive neoplasm with poor prognosis due to the lack of effective treatment, therefore, our

data provide vital new prospects for TNBC treatment, and may yield new directions for drug discovery.

Keywords: Triple-Negative Breast Cancer. Cytotoxic. Chemotherapy. Copper complex. Recombinogenic.

1. Introduction

Breast cancer (BC) is the most common neoplasm among women worldwide. In 2018, over 2 million of new cases were diagnosed and 600.000 deaths were recorded (Bray et al., 2018). Breast tumors are molecularly classified in four main subtypes, with distinct outcomes. The less aggressive express at least one of the hormonal receptors (estrogen receptor-ER, and/or progesterone receptor-PR), and the human epidermal growth factor receptor 2 (HER2) (Haque et al., 2012; Yersal et al., 2019) . Triple negative breast cancer (TNBC) does not express any of these markers and has challenged clinical practice.

In fact, TNBC is a more aggressive subtype (Alluri and Newman, 2014) associated with a poorer prognosis (Alluri and Newman, 2014; Dubuc et al., 2019). It represents a highly relevance group once patients do not benefit from endocrine or anti-HER2 therapies (Denkert et al., 2017). Chemoterapy is a standard therapeutic approach for TNBC at all stages and usually fails due to toxicity and chemoresistance (Bianchini et al., 2016). Therefore, is urgent the identification of new molecular targets and effective compounds for antineoplasic fields (Dubuc et al., 2019).

Doxorrubicin (DOX) and Platinum-based drugs are commonly used for TNBC treatment (Bianchini et al., 2016; Shi et al., 2018). The primary action of DOX includes the inhibition of topoisomerase I and II, intercalating into DNA (Tacar et al., 2013) and generating reactive oxygen species (ROS) (Tacar et al., 2013; Orsolin et al., 2015). However, resistance to DOX has been lead to increased doses, resulting in adverse side effects including cardiotoxicity and suppression of bone marrow hematopoietic function (Shi et al., 2018).

Platinum compounds, as cisplatin (CIS) and carboplatin (CARB), have important role in TNBC, especially in patients with BRCA1/2 mutations (Bianchini et al., 2016). However, BRCA wild-type patients have been shown a limited response to these agents (P Basourakos et al., 2017). Moreover, as DOX, CIS therapeutic potential is also limited due to severe side effects (including nephrotoxicity, gastrointestinal dysfunction, myelosuppresion) (Tuncer et al., 2010), and high incidence of chemoresistance (Galluzzi et al., 2012). These limitations are also verified for Platin analogues, such as carboplatin (Gore et al., 1989).

In this adverse context, complex of redox active metals, like copper, represent an important group of metallodrugs for tumor treatment. Copper active compounds present mechanisms of action and biodistribution different than the platinum drugs already used, and may be effective against tumors that are resistant to conventional chemotherapy (Bravo-Gómez et al., 2009; De-Vizcaya-Ruiz et al., 2000; Gracia-Mora et al., 2001; Mejia and Ruiz-Azuara, 2008). The mechanisms of cooper compounds action include the generation of reactive oxygen species (ROS) (Tisato et al., 2010), intercalation with DNA, and induction of apoptosis (Rodríguez-Mercado et al., 2017; Ma et al., 2017; Poloni, 2017). In TNBC (MDA-MB-231) cells, copper complexes have shown to be quite promising, with selective citotoxicity, increasing expression of p53 and Bax (Bcl-2 associated protein X), inducing cell cycle arrest and apoptosis (Foo et al., 2018). However, evaluation of genotoxic potential of cooper compounds is relevant while it is suggested as an antineoplastic drug.

Drosophila melanogaster fly is an eukaryotic organism used for decades to monitor genetic damage caused by chemical agents (Graf and van Schaik, 1992; Miklos and Rubin, 1996; Nepomuceno, 2015). It has the ability to activate

enzymatically pro-mutagens and pro-carcinogens *in vivo*, considered an optimized model for the detection of genotoxic activity (Graf et al., 1984; Nepomuceno, 2015; Machado et al., 2016; Orsolin et al., 2016; Silva-Oliveira et al., 2016; Oliveira et al., 2017; Naves et al., 2018.). According to Adams et al. (2000), genetic and metabolic similarity between fly and humans reinforces the importance of *D. melanogaster* as an adequate experimental platform for the study of human diseases related to replication, repair pathways, translation and drug metabolism.

Somatic mutation and recombination test (SMART) that uses *D. melanogaster* as an experimental model was developed by Graf and collaborators in 1984, and improved by Graf and van Schaik (1992). This bioassay is well described and widely used in toxicology, for mutagenic and recombinogenic evaluation of different compounds (Graf and Singer, 1992; Graf et al., 1998; Orsolin et al., 2016), such as antineoplastic drugs (Danesi et al., 2010; De Campos et al., 2017). It is based on the premise that during the embryonic development of *D. melanogaster*, the cells of the imaginal disk multiply themselves mitotically to form the body of the adult fly (Guzman-Rícon and Graf, 1995). Over treatment, substances that damage the DNA (Graf and Singer, 1992; Graf et al., 1998) lead to loss of heterozygosity and expression of recessive genes, giving rise to a clone of mutant cells and that can be detected by means of mutant trichomes on the wing of the adult individual (Guzman-Rícon and Graf, 1995; Spanó et al., 2001).

The present study aimed to assess the cytotoxic and selective potential of the ternary complex of copper associated the β–dicetone and 1,10 phenanthroline ($CuBTAPhenClO_4$) (CBP-01), through 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay in three breast cancer cell lines, T-47D, MCF7 and MDA-MB-231, and a non-neoplastic cell line, MCF 10A. The results were

compared to the treatment with CARB, CIS and DOX in order to provide insights into the better behavior of our new cooper-based chemical compound for TNBC treatment. In addition, the mutagenic / recombinogenic potential of a new copper ternary metal complex associated with β-diketone and 1,10-phenanthroline ($CuBTAPhenCLO_4$) (CBP-01) was evaluated through SMART test in *D. melanogaster.*

2. Materials and methods

2.1 Chemical agents

High purity chemicals and reagents were purchased from commercial sources. DOX (Adriblastina®, CAS 25316-40-9, batch 5PL5111, registred, imported and distributed by the laboratory Pfizer, São Paulo – Brazil) was used as positive control at 0.4 mM in SMART assay. This concentration was based on previous studies that demonstrated the induction of homologous recombination in *D. melanogaster* (Orsolin et al., 2012; Machado et al., 2013; Orsolin et al., 2015; Vasconcelos et al., 2017; Lima et al., 2018; Braga et al., 2018).

Cisplatin (CIS) – 0.5mM, CAS 15663-27-1, was purchased from the company Sigma-Aldrich® and used at 0.025mM (Danesi et al., 2010; de Campos et al., 2017). Concentration of Carboplatin (CARB) or B-Platin® CAS 41575-94-4, batch 18010333, produced by Blau Farmacêutica S.A, São Paulo – Brazil, was defined according to De Campos et al. (2017).

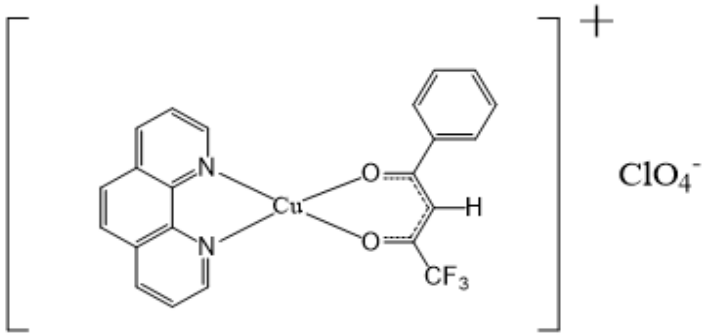
The effects of platinum complexes (CIS and CARB), and DOX were compared to CBP-01. The 3-[4,5-dimethylthiazole-2—yl]2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich® and concentrations of 1μM; 5μM;

10μM; 12,5μM; 25μM; 50μM for CBP-01, DOX, CIS and CARB were used in cellular experiments. For *D. melanogaster*, ethanol 5% was used as negative control and for dilution of the compounds. All dilutions were prepared immediately before use.

2.2 Copper complex $CuBTAPhenClO_4$ (CBP-01)

The copper ternary complex CBP-01, assigned from this study, was previously synthesized and characterized by do Couto Almeida et al. (2015). It is a compound of greenish coloration, non-hygroscopic, stable to air and light, soluble in organic solvents such as dimethyl sulfoxide (DMSO) and acetonitrile, with molecular weight of 558,46g/mol. The chemical formula is shown in Figure 1.

Fig.1. Representation of the chemical structure of copper ternary metal complex associated with β-diketone and 1,10-phenanthroline (CuBTAPhenCLO4) (CBP-01).



Source: do Couto Almeida et al., (2015)

2.3 Cell culture

For the *in vitro* assays, four human breast cell lines were obtained from the American Type Culture Collection. All cell lines were cultured in appropriate medium supplemented with 10% of Fetal Bovine Serum (FBS) (Gbico®), and 50 μg/L of gentamycin (Cultilab). The lineage MCF 10A (non-neoplastic) was used as control

and grown in DMEM F12 medium (Gbico®), enriched with 10ng/mL of Epidermal Growth Factor (EGF) (Gbico®), 1mg/mL of Hydrocortisone (Sigma-Aldrich®), and 10mg/mL of insulin (Gbico. The cell lines MCF7 (luminal carcinoma, ER^+), and T-47D (ductal carcinoma, PR^+) were maintained in RPMI-1640 medium (Sigma-Aldrich®), and the last supplemented with 10mg/mL of insulin. MDA-MB-231 lineage (metastatic triple-negative) was cultured in Leibovitz medium (Gbico®).

Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37° C. The medium was exchanged every two days until 80% cell confluence for further experiments.

2.4 MTT assay

Cell viability was evaluated by 3-[4,5-dimethylthiazole-2—yl]2,5-diphenyltetrazolium bromide - MTT methodology as described by Mosmann (1983), with some modifications.

Briefly, cells were seeded in 200 µL growth medium at a density of 10^4 cells per well onto 96-well plates and treated with 1µM; 5µM; 10µM; 12.5µM; 25µM, and 50µM of CBP-01, CARB, CIS and DOX. Controls included untreated cells (viability control), and wells with only medium. Cells treated with only DMSO (diluent) were also included. Subsequently, the plates were incubated at 37 °C. The experiments were carried out in triplicate for 24, 48 and 72h.

MTT reagent (10% p/v) was then added to the cells for 4 h incubation at 37°C. Then, the supernatant was discarded and 200µL of DMSO was placed in all wells. The optical density (OD) values at an absorbance frequency of 570 nm were measured by an ELISA Reader (IndiaMART, DD Bioinfotech / Nathupura, New Delhi).

The mean OD of the treated cells was compared to the mean OD of the control wells (Mosmann, 1983). The percentage of cell viability was given by the following formula (F1):

$$Cell\ Viability\ (\%) = \frac{(At - Ab)}{(Ac - Ab)}\ x\ 100 \quad (F1)$$

At = absorbance of treated cells

Ab = absorbance of the negative control (without cells)

Ac = absorbance of viability control (untreated cells)

2.5 Selectivity index (SI)

Selectivity index (SI) of all drugs was determined based on IC_{50} value calculated from each treatment described above. The calculation was given by the following formula (F2):

$$Selectivity\ index\ (IS) = \frac{IC50\ of\ non - tumoral\ cell\ line\ (MCF\ 10A)}{IC50\ of\ tumoral\ cell\ lines} \quad (F2)$$

According to Badisa et al (2009) SI ≥ 2 was considered significant.

2.6 Strains and crosses of *Drosophila melanogaster*

Three different strains of *D. melanogaster* were used in the SMART assay: (ii) females *flr-3 (flr^3/In(3LR)TM3, ri pp sep l(3)89Aabx^{34e}* and *Bd^s*; (ii) females *ORR;flr3 (ORR; flr3/In(3LR)TM3, ri pp sep l(3) 89Aabx^{34e}* and *Bd^s*; (iii) and males *mwh*(*mwh/mwh*).

The SMART was carried out in two crosses. In the standard (ST) cross virgin females *flr*^{3} were crossed with males *mwh*. The descendants have basal levels of the cytochrome P450 enzymes and enable the direct evaluation of mutagenic agents. The second was a High bioactivation (HB) cross, in which virgin females *ORR* were crossed with males *mwh*. This crossing enables greater biotransformation due to high level of P450 (Graf et al., 1989; Graf and van Schaik, 1992; De Rezende et al., 2011).

Both crosses produced two types of progeny: the marked trans-heterozygous (MH, *mwh*+/+*flr*^{3}), with smooth wing edge phenotype, and individuals balancer heterozygous (BH, *mwh*+/+*TM3*) with a serrated wing (Guzmán-Rincón and Graf, 1995) MH and BH individuals were analyzed.

2.7 Toxicity test in *Drosophila melanogaster*

The toxicity assay was performed in order to establish the concentration of CBP-01 to SMART test. Fifty larvae obtained from the ST cross and 50 from HB cross were counted and placed in separate tubes containing 1.5g of culture medium (mashed potatoes) for *D. melanogaster* (Spanó et al., 2001) and 5.0mL of different concentrations of CBP-01 (0.03mM, 0.06mM, 0.12mM, 0.25mM, 0.50mM, 1.00mM, 2.00mM and 4.00mM), alone or in association with DOX (0.4mM). The concentration of CBP-01 was based on previous studies conducted with Casiopeina II-gly and Casiopeina III-Ea (Jiménez et al., 2016; M. Vidal et al., 2017). The emerged flies were counted. The number of surviving flys per treatment provided an indicator of the toxicity of the compounds (Orsolin et al., 2016).

2.8 Somatic mutation and recombination test (SMART) in *Drosophila melanogaster*

The SMART test was performed according to the methodology proposed by Graf et al., (1984) and improved by Graf and van Schaik (1992), with modifications. The crosses were described in section 2.2.

Briefly, after the crosses, flies were transferred to a flask containing hatching medium, a layer of yeast (*Saccharomyces cerevisiae*) supplemented with sugar under a solid base of agar (4% w/v). Oviposition occurred for a period of 8h. After 72h, the third instar larvae were washed and placed in each glass vials containing 1.4g of mashed potato flakes (HIKARI®) (Spanó et al., 2001). CBP-01 (0.03mM, 0.06mM, 0.12mM, 0.25mM, 0.50mM, 1.00mM, 2.00mM and 4.00mM) was added in each tube diluted in 5% ethanol, alone or in association with DOX. CARB (0.025Mm), CIS (0.5mM), the positive control (DOX 0.4mM) and the negative control (ethanol 5%) were included. All compounds were tested in two independent experiments, under optimal laboratory conditions (25 ±4°C and 65% RH) in BOD-type chamber (Model: SL224, SOLAB – Equipamentos para Laboratórios,São Paulo, SP, Brazil). Third stage larvae were subjected to a chronic treatment, during about 48h, until development of the pupal stage.

2.9 Analysis the SMART test

After underdoing metamorphosis, the adult flies were transferred to vessels containing 70% (v/v) ethanol for subsequent mounting. The wings were removed with entomological forceps and mounted on coded slides containing Faure solution (30g of gum arabic, 50mL of distilled water, 200g of chloral hydrate and 16mL of glycerol). The wings from both the dorsal and ventral surfaces were analyzed under a light

microscope, at a magnification of 400x (Graf et al., 1984). Frequency and size of single and twin spots were recorded.

2.10 Statistic analysis

Statistical analysis for the cytotoxicity MTT assay was carried out in GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, EUA) program, and group differences were determined by one-way analysis of variance (ANOVA) and the Tukey HSD post hoc. The IC50 (concentration that inhibits 50% of cell growth) was determined from a non-linear regression, which relates the percentage of cell viability as a function of the logarithm of the concentrations tested. The graphs were plotted with mean ± standard deviation data. A p-value less than 0.05 was considered as statistically significant.

For the Toxicity test (TX), statistical comparisons of survival rates were performed with the Chi-squared (X^2) test for ratios of independent samples, using the program GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, EUA), with significance level of $p<0.05$.

The statistical analysis of SMART test was carried out in accordance with the multiple decision procedure proposed by Frei and Würgler (1988), at a significance level of 5%, resulting in different diagnoses: positive, weakly positive, negative and inconclusive. For each treatment, the frequency of the type of spot (small or large single spot and twin spot), and the total frequency of spots per fly were recorded (Kastenbaum and Bowman, 1970). The comparison was made in pairs (CBP-01 *vs* negative control/ CARB *vs* negative control/ CIS *vs* negative control; DOX *vs* negative control; and CBP-01+DOX *vs* positive control).

According to Abraham (1994) the calculation of recombinogenic activity was based on the frequency of induction of mutant spots per 10^5 cells/division. Comparisons of induction of mutant spots in descendants MH and BH were performed as follows: (i) Frequency of mutation (FM)= frequency of clones in BH individuals/ frequency of clones in MH individuals/ (ii) Frequency of recombination (FR) = 1 - frequency of mutation (FM).

3. Results and Discussion

3.1 Drug Cytotoxicity Screening

The effects of CBP-01 on breast cancer cells viability were evaluated through an MTT assay. Considering our aimed to characterize new antineoplastic compounds, comparisons with routinely drugs are particularly interesting. So, we also performed a concomitant MTT assay with CARB, CIS and DOX in the same concentrations of CBP (1μM; 5μM; 10μM; 12,5μM; 25μM; 50μM). The dose-dependent profile was identified for the four drugs (Figure S1) with highest toxicity at the highest dosages for all cultivated lineages.

In addition, the cellular effects differed between treatment times, showing the role of metabolism in response to chemotherapeutic agents (Herling et al., 2011). In fact, metabolites from anti-tumor agents may alter drug therapeutic efficacy (W Edwardson et al., 2015).

Ganeshpandian et al (2014), performed *in vitro* cytotoxicity assay, and treated MCF7 cells with a copper (II) complexes of type $[Cu(L)(2,9\text{-dmp})](ClO_4)_2$ and CIS. They also observed that both drugs were cytotoxic and this effect was potentialized after metabolization. Metallodrugs are characterized by the high

capacity to react with biomolecules and to generate active metabolites (Butcher et al., 2004; Wang et al., 2015). Copper complexes bind to molecular oxygen and hydrogen peroxide generating ROS and leading to oxidative damage of DNA, lipids and proteins (Slator et al., 2018). We inferred that the longer the exposure time of the cells to these compounds, more severe is the oxidative damage, leading to apoptosis (Dixon and Stockwell, 2014; Redza-Dutordoir and Averill-Bates, 2016).

CBP-01 was effective against the tumor cell lines, especially against MDA-MB-231 (Figure S1A-S1C). This chemical compound also reduced the viability of the non-tumorigenic cell line MCF-10A with IC_{50} of 6.37uM after 72 hours of treatment (Table 1). However, this behavior was also detected for CIS and DOX.

Table 1. IC_{50} values of the copper ternary metal complex associated with β-diketone and 1,10 phenanthroline (CBP-01), Carboplatin (CARB), Cisplatin (CIS) and Doxorrubicin (DOX) based on cytotoxicity assay – MTT with Breast Cancer cell lines (T-47D, MCF7 and MDA-MB-231) and non-tumorigenic breast line (MCF 10A). The values were calculated for each treatment (24, 48 and 72h).

	IC_{50} (μM)											
Compound	**24 hours**				**48 hours**				**72 hours**			
	MCF 10A	T-47D	MCF7	MDA-MB 231	MCF 10A	T-47D	MCF7	MDA-MB 231	MCF 10[a]	T-47D	MCF7	MDA-MB 231
CBP	15.11	1.07	8.03	3.03	9.33	0.04	0.42	3.18	6.37	ND1	1.37	2.05
CARB	ND2	ND2	ND2	ND2	ND2	23.33	ND2	ND2	ND2	9.83	ND2	ND2
CIS	27.32	9.13	30.41	ND2	34.31	7.07	17.10	36.63	16.24	1.98	2.82	33.67
DOX	ND2	19.77	41.65	ND2	11.38	4.72	15.86	ND2	6.75	ND1	1.41	42.35

Not determined– IC_{50}<1μM
Not determined – IC_{50}>50μM

Copper is an essential trace metal that plays an important role in many biological functions. It is cofactor of several enzymes (Kumar et al., 2015) with oxy-reduction properties (Tisato et al., 2010). Copper transporters are overexpressed in tumors, increasing its uptake by malignant cells (Tacar et al., 2013; Ullah et al., 2011; Peng et al., 2006). This property has been explored for oncology fields (Gouda et al., 2018; Denoyer et al., 2018). Moreover, this metal is also required in the genesis of new blood vessels as an angiogenic mediator cofactor (Tisato et al., 2010).

In fact, metals are tightly regulated under normal conditions and aberrant metal ion concentrations are associated with pathological disorders (Freeza et al., 2010). To overcome this limitation, in the present study we used a coordinated complex that presented potential anticancer property, even better than CARB and CIS.

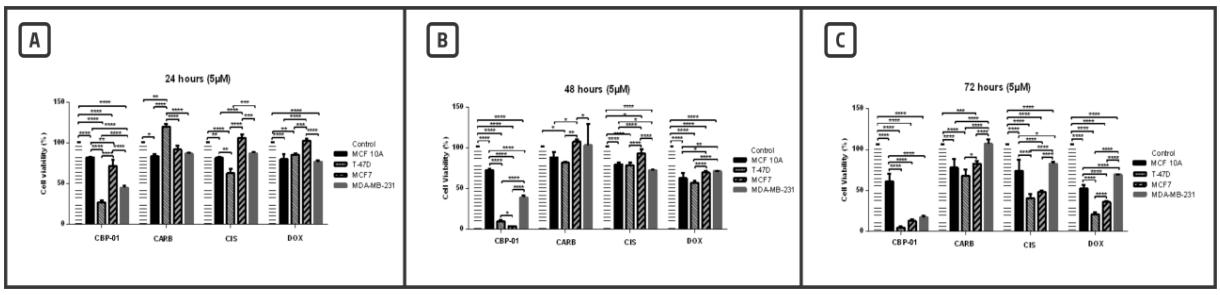
Comparing with other compounds, CARB was less toxic to MCF 10A cell line. However, less effective in inhibiting the proliferation of MCF7 and MDA-MB-231. It seems to be more cytotoxic to T-47D cells (Figure S1D-S1F). CIS and DOX also showed significant inhibitory activity against hormone-dependent lineages. Considering CIS (Figure S1G-S1I), when effective against MDA-MB-231 after 72 hours (IC_{50} 33.63uM - Table 1), it was even more cytotoxic to the non-tumorigenic cell (IC_{50} 16.24uM - Table 1). A similar behavior was observed for DOX (Figure S1J-S1L), with inhibition of triple-negative cell viability only after 72 hours, however, with impairment of MCF 10A cells.

Previous *in vitro* cytoxicity assays with CARB, CIS and DOX, showed growth inhibition effects in estrogen-dependent MCF7 and triple-negative MDA-MB-231 cell lines, in a dose and a time-dependent manner, enhancing apoptotic index (Tyagi et al., 2004). The same was observed by Thomadaki and Scorilas (2007), in the breast

cancer cell line MCF7, demonstrating that these drugs modulate the expression of apoptosis-related genes, such as Bcl-2. These data corroborate our results, once CARB, CIS and DOX where toxic against hormone-dependent cells.

Considering the chemotherapeutic compounds, CBP-01 seems to be more effective against the triple-negative cells MDA-MB-231 with IC_{50} lower than 3.2uM in all treatment times (Table 1). This is a tumor subtype with restricted strategies for treatment (Bianchini et al., 2016) and, in this context, our results are promising. We chose the concentration of 5uM (based on IC_{50} value of CBP-01 for MCF 10A) to compare the cytotoxicity of all tested drugs (Figure 2).

Fig. 2. Cell viability (%) of mammary cell lines treated with the copper ternary metal complex associated with β-diketone and 1,10 phenanthroline (CBP-01), Carboplatin (CARB), Cisplatin (CIS) and Doxorrubicin (DOX), performed by MTT assay. Cytotoxicity of the chemical compounds was evaluated in MCF 10A (non-neoplastic); T-47D (ductal carcinoma, PR+); MCF7 (luminal carcinoma, ER+), and MDA-MB-231 (triple-negative). Concentration of 5uM was chosen based on IC50 value of CBP-01 for MCF 10A cells. In (A) treatment for 24h, in (B) for 48h and (C) for 72h.



Although platinum-based agents are used in the standard treatment of TNBC in neoadjuvant and adjuvant chemotherapy, its remains controversial mainly because of toxicity (Bianchini et al., 2016; Sihori et al., 2008) and chemoresistence (Leung et

al., 2016). Corroborating our results, in a study with a salicylate phenantroline copper (II) complex, it was observed that the compound inhibited the growth of four breast cancer cell lines (MCF7, T-47D, MDA-MB-231 and BT-20) and induced apoptosis in a concentration-dependent manner. Moreover, the effects were higher in TNBC cell lines, MDA-MB-231, and BT-20, with reduced levels of the anti-apoptotic proteins expression Bcl-2 and Bcl-xL and attenuated tumor grow of MDA-MB-231 xenografts, after treatment (Fan et al., 2017).

Copper complexes have been shown promising results as antineoplastic agents (do Couto Almeida et al., 2015; Jiménez et al., 2016) with proven efficacy in *in vitro* treatment of tumor cells resistant to platinum compounds (Wehbe et al., 2017). The CBP-01 presented higher selectivity index (SI) for TNBC cell lines, in all treatment times, compared to CARB, CIS, and DOX (Table 2). According Mahavorasirikul et al (2010) SI of greater than 3 are considered highly selective. Thus, these data demonstrated that cooper-based compounds are promising as chemotherapeutic compounds and CBP-01 is particularly interesting for TNBC treatment.

Table 2. Selectivity index (SI) values of the copper ternary metal complex associated with β-diketone and 1,10 phenanthroline (CBP-01), Carboplatin (CARB), Cisplatin (CIS) and Doxorrubicin (DOX) for Breast Cancer cell lineages T-47D, MCF7 and MDA-MB-231 compared to the non-neoplastic MCF 10A. SI was calculated according to According to Badisha et al (2009).

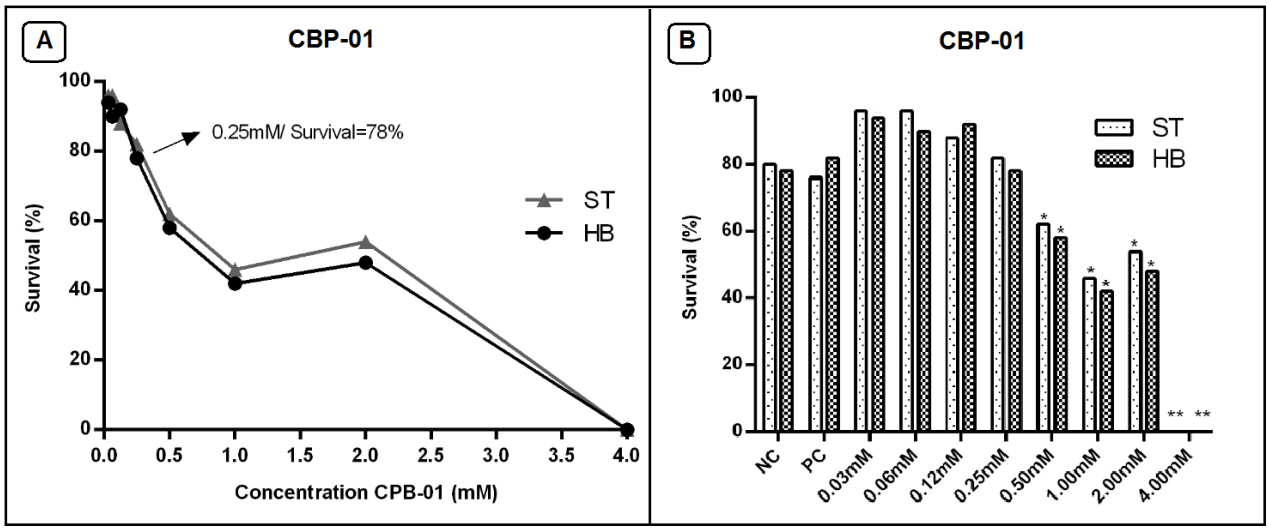
	SI								
	24 hours			48 hours			72 hours		
Compound	MCF 10A ×T-47D	MCF 10A ×MCF7	MCF 10A ×MDA-MB 231	MCF 10A ×T-47D	MCF 10A ×MCF7	MCF 10A ×MDA-MB 231	MCF 10A ×T-47D	MCF 10A ×MCF7	MCF 10A ×MDA-MB 231
CBP-01	14.12	1.88	4.98	233.25	22.21	2.93	ND	4.64	3.10
CARB	ND	ND	ND	ND	ND	ND	ND	ND	ND
CIS	2.99	0.89	ND	4.85	2.00	0.93	8.20	5.75	0.48
DOX	ND	ND	ND	2.41	0.71	ND	ND	4.78	0.15

ND – Not determined.

3.2 Recombinogenic potential of CBP-01 in somatic cells of *Drosophila melanogaster*

Copper toxicity was investigated through TX test. The lethal dose was established and was used for the design of SMART test. The survival rates of flies are shown in Figure 2. The highest concentration of 4.00mM CBP-01, alone or with DOX, was lethal to all individuals. The survival frequency of 60% was observed for CBP-01 at 0.5mM (Figure 3A and 3B) with statistical difference when compared to negative control. On the other hand, CBP-01 at 0.03 mM, 0.06 mM, 0.12 mM and 0.25 mM had a survival of over 70% (Figure 3A and 3B), with no statistical difference when compared to negative control.

Fig. 3. Survival rate of *Drosophila melanogaster* obtained from standard (ST) crosses and high bioactivation (HB) crosses for Somatic Mutation and Recombination test – SMART. Different concentrations of the new copper ternary metal complex associated with β-diketone and 1,10 phenanthroline (CBP-01) were used.



NC: Negative control (ethanol 5%)
PC: Positive control (Doxorrubicin 0.4mM)
*Statistical difference ($P<0.05$) comparing to negative control according to the X^2 test for ratios for independent samples
**(LD) Lethal dose

Copper, at high concentrations, can cause lipid peroxidation, damage to proteins and DNA, which would make it potentially toxic to non-tumor cells (Gaetke; Chow, 2003; Tisato et al., 2010; Saghiri et al., 2015), limiting its use as a chemotherapeutic agent. We observed that CBP-01 showed no significant toxicity at the lowest concentrations (until 0.25mM). The survival rate of the flies was up to 70% until 0.25mM concentration, showing that CBP-01 had similar toxicity to other cooper-based compounds such as Casiopeina II-gly (Jimenéz et al., 2016) and Casiopeina III-Ea (M. Vidal et al., 2017), who are in phase 1 of clinical studies in Mexico. Based on the TX result the four lowest concentrations of CBP-01 (0.03mM, 0.06mM, 0.12mM, 0.25mM) were used for the SMART test in order to evaluate the damage

caused by the compound to the DNA of somatic cells of *D. melanogaster*, as well as its modulating effect on DOX.

Table 3 shows the test of somatic mutation and recombination – SMART in *D. melanogaster*, MH and BH descendants of the ST and HB crosses treated with CBP-01 alone. In the MH progenies, in ST cross, CBP-01 did not show significant difference in the total number of spots when compared to the negative control ($p<0.05$). However, in HB cross, at the lowest concentration of CBP-01 (0.03 mM) we identified a significant increase of spots when compared to negative control. The difference between the crosses is related to P450 levels, since ST crossed individuals present a basal level of this enzyme allowing evaluating damages caused by direct action of genotoxins (Graf et al., 1984). HB crossed individuals have a high level of P450, which makes it possible to identify genotoxic damages of metabolites generated through the biotransformation of xenobiotics (Frölich and Würgler, 1989; Graf and Van Schaik, 1992; Saner et al., 1996; Silva et al., 2003).

Table 3: Summary of results obtained in the marked trans-heterozygous descendants (MH) and balancer-heterozygous (BH) of *Drosophila melanogaster* derived from the standard cross (ST) and high bioactivation cross (HB) treated with different concentrations of the new copper ternary metal complex associated with β-diketone and 1,10 phenanthroline (CBP-01). Positive control (Doxorrubicin 0.4mM) and negative control (5% ethanol in distilled water).

Treatments		N^0. of flies (*N*)	Spots per fly (n^0. of spots) statiscal diagnosis[a]											Spots with *mwh* clone[c] (*n*)	Mean clone size class[c,d] (*î*)	Frequency of formation / 10^5 cells per cells division[d]		Recombination (%)
DOX (mM)	CBP-01 (mM)		Small single (1-2 cels)[b] *m* = 2			Large single (>2 cels)[b] *m* = 5			Twin *m* = 5			Total spots *m* = 2					Observed	Control corrected	
mwh/flr^3 (MH)																		
Cross ST																		
0	0	60	0.37	(22)		0.05	(3)		0.00	(0)		0.42	(25)		25	1.40	0.56		
0.4	0	60	0.48	(29)	i	0.92	(55)	+	0.80	(48)	+	2.20	(132)	+	110	3.42	18.06	18.86	95.12
0	0,03	60	0.38	(23)	.	0.03	(2)	i	0.05	(3)	i	0.47	(28)	-	26	1.85	0.80	0.00	
0	0,06	60	0.32	(19)	-	0.07	(4)	i	0.02	(1)	i	0.40	(24)	-	24	2.13	0.89	0.00	
0	0,12	60	0.23	(14)	-	0.07	(4)	i	0.02	(1)	i	0.32	(19)	-	19	1.79	0.56	0.10	
0	0,25	60	0.12	(7)	-	0.07	(4)	i	0.02	(1)	i	0.20	(12)	-	12	2.75	0.69	0.16	
Cross HB																		
0	0	60	0.78	(47)		0.17	(10)		0.00	(0)		0.95	(57)		57	2.00	1.95		
0.4	0	60	1.52	(91)	+	1.98	(119)		0.25	(15)	+	3.75	(225)	+	222	3.25	18.06	18.12	88.98
0	0,03	60	1.35	(81)	+	0.05	(3)	-	0.03	(2)	i	1.43	(86)	+	86	1.51	2.09	0.36	52.15
0	0,06	60	0.83	(50)	-	0.18	(11)	i	0.00	(0)	i	1.02	(61)	-	60	1.77	1.77	0.00	
0	0,12	60	0.82	(49)	-	0.10	(6)	-	0.03	(2)	i	0.95	(57)	-	57	1.70	1.58	1.58	
0	0,25	60	0.60	(36)	-	0.07	(4)	-	0.02	(1)	i	0.68	(41)	-	41	1.85	1.27	0.71	
mwh/TM3 (BH)																		
Cross HB																		
0	0	30	0.33	(10)		0.03	(1)		[f]			0.37	(11)		11	1.36	0.48		
0.4	0	30	0.93	(28)	+	0.17	(5)	i				1.10	(33)	+	33	1.82	1.99	1.55	
0	0.03	30	0.73	(22)	+	0.07	(2)	i				0.80	(24)	+	24	1.29	1.00	0.52	

Marker-trans-heterozygous flies *(mwh/flr^3)* and balancer-heterozygous flies *(mwh/TM3)* were evaluated.
[a] Statistical diagnoses according to Frei and Würgler (1988, 1995): +, positive; -, negative; i, inconclusive. *m* = multiplication factor for significantly negative results. Level of significance $P \leq 0.05$.
[b] Including rare *flr^3* single spots.
[c] Considering *mwh* clones from *mwh* single and twin spots.
[d] Frequency of clone formation: clones/flies/48,800 cells (without size correction) Frei et al. (1992).
[f] Only *mwh* single spots can be observed in heterozygous individuals *mwh/TM3*, since the balancer chromosome TM3 does not contain the mutant gene *flr^3*.

We inferred that CBP-01, after metabolization, produced reactive substances, which interacted with DNA and led to greater expression of mutant phenotypes. It is known that the metabolism of certain drugs leads to the production of ROS (Silva et al., 2003). Previous studies have indicated that the main mechanism of action of copper complexes involves the generation of ROS (Blackman et al., 2012; Graf and Lippard, 2012; Santini et al., 2014; Agbale et al., 2016; Tabti et al., 2017; de Souza et al., 2019).

Comparisons of the clone frequencies observed in the MH and BH descendants treated with CBP-01 alone at 0.03mM (Table 3) were performed to quantify the percentage of mutagenic and recombinogenic potential of the test samples (Santos et al., 1999; Sinigaglia et al., 2006). In the MH progeny, mitotic recombination and several other types of mutagenic events may occur, but in the BH individuals, all recombinogenic events are eliminated owing to multiple inversions present on the *TM3* balancer chromosome (Graf et al., 1994; Spanó and Graf., 1998; Graf et al., 1992; Frei et al., 1992). We found that the spots induced by CBP-01 at 0.03mM in MH progenies were mainly due to recombination (52.15%), but the complex also induced mutagenic events (47,85%) at the lowest concentration, as presented in Table 3.

Our group has already studied a similar ternary complex of Copper (II) with Doxycycline and 1,10-Phenanthroline on somatic cells of *D. melanogaster* and we found that this compound significantly increased the frequencies of mutant cells in both ST and HB crosses, mostly by a recombinogenic effect (Lopes et al., 2018). Additionally, Serment-Guerrero et al., (2017) performed a DNA breakage test in bacterial cultures with Cassiopeins, a copper complex in clinical phase I in Mexico, and found that this drug caused different double-strand breaks (DSBs), probably due

to oxidative damage. In humans cells, DSBs are repaired for homologous recombination repair pathways (Poplawsi et al., 2010), suggesting the recombinogenic damages caused by CBP-01 in our study.

Interestingly, the data were dose-dependent with a decrease in the number of spots with increasing compound concentration at both crosses. Results obtained in the TX support the findings in the SMART test. Although the concentration of 0.25 mM was not sufficiently toxic, we cannot point that cellular damage did not occur after the treatment of the flies.

Thus, in the SMART test, as the concentration of CBP-01 increased (from 0.03 to 0.25 mM), damage may have progressively increased leading to cellular apoptosis, rendering the expression of the mutant phenotype in the fly's wing unfeasible and resulting in lower frequency of spots without causing the lethality of the individual. In an earlier study, Jiménez et al. (2016) tested the synergism between the genotoxic and oxidative potential of Casiopeina II-gly, demonstrating that increased drug concentration led to increased oxidative stress. However, the authors identified an increased activity of the enzymes superoxide dismutase (SOD) and catalase (CAT) protecting the DNA against damage and, therefore, the expression of mutant spots was not affected by the treatment. We suggest that the same mechanism may have occurred in the individuals analyzed, however, tests aimed at oxidative stress need to be performed to confirm this hypothesis.

Regarding the mutagenic agent used as positive control, we observed that DOX presented a significant amount of spots, mainly induced by recombinogenic events. Several studies with *D. melanogaster* in SMART test used this drug as positive control and reported its genotoxic effect associated with recombination (De Rezende et al., 2011; Machado et al., 2012; Orsolin et al., 2015; Silva-Oliveira, 2016;

Oliveira et al., 2017). DOX is a lipophilic antineoplastic agent that has high penetrability. It intercalates to the DNA molecule and causes single and double-stranded DNA breaks, targeting enzymes topoisomerases, preventing DNA replication (Tacar et al., 2012).

In Table 4, the results of treatments with CARB (0.5mM) and CIS (0.025mM), in ST and HB crosses, and in MH progeny can be visualized. When compared to the negative control, both had a high frequency of spots, showing their mutagenic / recombinogenic effects. The BH (mwh/TM3) descendants from de ST and HB crosses were analyzed to calculate the percentage of recombinogenic and mutagenic events. We found that CARB and CIS induced spots were mainly due to recombination (66.66% and 86.71% in ST cross; 67.16% and 86.98% in HB cross, respectively).

Table 4: Summary of results obtained in the marked trans-heterozygous descendants (MH) and balancer-heterozygous (BH) of *Drosophila melanogaster* derived from the standard cross (ST) and high bioactivation cross (HB) treated with Carboplatin (CARB) (0.5mM), and Cisplatin (CIS) (0.025mM) and negative control (5% ethanol in distilled water).

Treatments		N⁰. of flies (*N*)	Spots per fly (n⁰. of spots) statiscal diagnosis[a]											Spots with *mwh* clone[c] (*n*)	Mean clone size class[c,d] (*î*)	Frequency of formation / 10^5 cells per cells division[d]		Recombination (%)
CARB (mM)	CIS (mM)		Small single (1-2 cels)[b] *m* = 2			Large single (>2 cels)[b] *m* = 5			Twin *m* = 5			Total spots *m* = 2					Observed	Control corrected	
mwh/flr³ (MH)																		
Cross ST																		
0	0	60	0.37	(22)		0.05	(3)		0.00	(0)		0.42	(25)		25	1.40	0.85		
0.5	0	60	24.72	(1489)	+	1.17	(70)	+	0.28	(17)	+	26.17	(1576)	+	1563	1.28	32.33	31.77	66.66
0.0	0,025	60	7.25	(435)	+	4.15	(249)	+	1.32	(79)	+	12.72	(763)	+	728	2.34	31.52	31.15	86.71
Cross HB																		
0	0	60	0.78	(47)		0.17	(10)		0.00	(0)		0.95	(57)		57	2.00	1.95		
0.5	0	60	25.27	(1516)	+	0.85	(51)	+	0.12	(7)	+	26.23	(1574)	+	1622	1.24	32.64	30.89	67.16
0.0	0.025	60	6.82	(409)	+	2.58	(155)	+	0.73	(44)	+	10.13	(608)	+	595	2.13	22.31	20.37	89.96
mwh/TM3 (BH)																		
Cross ST																		
0	0	30	0.10	(3)		0.03	(1)		[f]			0.13	(4)		4	2.00	0.27		
0.5	0	30	9.03	(271)	I	0.23	(7)	I				9.27	(278)	-	278	1.18	10.78	10.54	
0.0	0.025	30	1.03	(31)	I	0.57	(17)	I				1.60	(48)	-	48	2.35	4.19	3.93	
Cross HB																		
0	0	30	0.33	(10)		0.03	(1)					0.37	(11)		11	1.36	0.48		
0.5	0	30	8.97	(269)	+	0.27	(8)	I				9.23	(277)	+	277	1.18	10.72	10.24	
0.0	0.025	30	1.37	(41)	+	0.37	(11)	I				1.73	(52)	+	52	1.71	2.91	2.45	

Marker-trans-heterozygous flies *(mwh/flr³)* and balancer-heterozygous flies *(mwh/TM3)* were evaluated.
[a] Statistical diagnoses according to Frei and Würgler (1988, 1995): +, positive; -, negative; i, inconclusive. *m* = multiplication factor for significantly negative results. Level of significance $P \leq 0.05$.
[b] Including rare *flr³* single spots.
[c] Considering *mwh* clones from *mwh* single and twin spots.
[d] Frequency of clone formation: clones/flies/48,800 cells (without size correction) Frei et al. (1992).
[f] Only *mwh* single spots can be observed in heterozygous individuals *mwh/TM3*, since the balancer chromosome TM3 does not contain the mutant gene *flr³*.

Our data corroborate previous results with CARB, in which this platinum-based compound was shown to be mutagenic / recombinogenic in *D. melanogaster* using the SMART test (de Campos et al., 2017; Danesi et al., 2010). According to King and colleagues (2014) CARB is responsible for decreasing the fruit fly fecundity rate. In Chinese hamster ovary cells this same compound inhibited cell growth and induced the formation of micronuclei (de Souza, 2016). Danesi et al., (2010) also evaluated CIS by the SMART test and identified recombinogenic effects on progenies of both crosses, ST and HB.

Cisplatin interacts with nucleophilic sites of DNA purines causing distortions in the double-helix (Eastman, 1987; Pinto and Lippard, 1985). The drug inhibits replication, suppresses transcription, causes cell cycle damage, and inactivates antioxidant system enzymes such as glutathione (Timerbaev et al., 2006; Koberle et al., 2010). Its cytostatic and genotoxic effects have been previously evaluated in zebrafish and human cells (Gajski et al., 2015).

Zaidi et al., (2014) also performed genotoxicity and oxidative stress tests in vivo comparing trinuclear copper (II) - tin (IV) (CuSn2 (Trp)) to cisplatin demonstrating that low doses of cisplatin are already sufficient to cause DNA damage, unlike CuSn2 (Trp), which required higher doses to cause genotoxic effect. These data highlight the potential of copper-based compounds and their promising properties when compared to drugs already incorporated in clinical practice.

4. Conclusion

CBP-01 was selectively cytotoxic to BC cell lines, especially to MDA-MB-231 (triple-negative phenotype), when compared to other drugs. Further analyses are

necessary to explore intrinsic mechanisms of the antineoplastic potential of CBP-01 in human breast tumorigenic cells. On the other hand, CBP-01 caused lower damages to somatic cells of *D. melanogaster* when compared to CARB and CIS. In addition, we suggest the involvement of mechanisms associated with oxidative stress, which need to be validated. In summary, different bioassays in other biological models are interesting to confirm the potential of CBP-01 as antineoplastic drug.

Conflict of interest statement

The authors declare that there are no conflict of interest.

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Highlights

- A novel copper complex has antineoplastic potential.
- CPB-01 is cytotoxic to Breast Cancer cell lines in a dose and time-dependent manner and more effective against the triple-negative cells MDA-MB-231 than Carboplatin, Cisplatin and Doxorrubicin.

- CBP-01 presented recombinogenic effect only at the lowest concentration tested and after biometabolization in *D. melanogaster*.
- Carboplatin and Cisplatin presented recombinogenic effect.
- CBP-01 is a promising agent for cancer therapy

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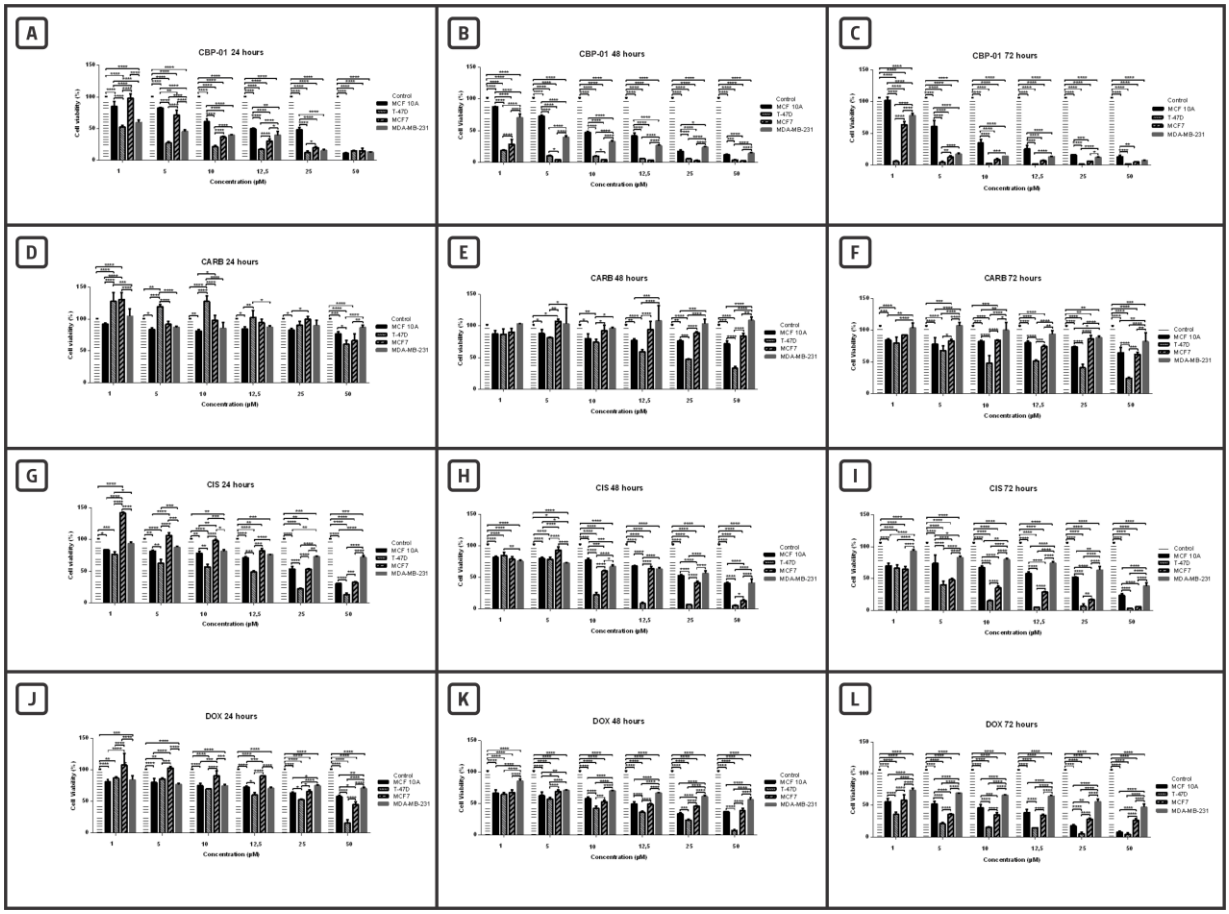
Supplementary Figure

Fig. S1. Cell viability (%) of mammary cell lines treated with copper ternary metal complex associated with β-diketone and 1,10 phenanthroline (CBP-01), Carboplatin (CARB), Cisplatin (CIS) and Doxorrubicin (DOX), performed by MTT assay. Cytotoxicity of the chemical compounds was evaluated in MCF 10A (non-neoplastic); T-47D (ductal carcinoma, PR+); MCF7 (luminal carcinoma, ER+), and MDA-MB-231 (triple-negative). In (A), (B) and (C) treatment with CBP-01 for 24, 48 and 72h, respectively. In (D), (E) and (F) treatment with CARB for 24, 48 and 72h, respectively. In (G), (H) and (I) treatment with CIS for 24, 48 and 72h, respectively. In (J), (K) and (L) treatment with DOX for 24, 48 and 72h, respectively.



CAPÍTULO III

Recombinogenic potential of a new copper ternary complex with β-diketone and 1,10-phenanthroline ($CuBTAPhenClO_4$) associated with doxorubicin in *Drosophila melanogaster*

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Abstract

One of the most widely used therapeutic modalities for the treatment of cancer is chemotherapy. Although crucial for the overall survival of patients, major challenges remain to be overcome, such as multidrug resistance and severe side effects. Drugs routinely used such as doxorubicin (DOX) are known for their genotoxic potential and toxicity. On the other hand, copper active compounds have shown promising results in cancer treatment. The present study aimed to evaluate the polypharmacological recombinogenic / mutagenic potential of a new copper ternary metal complex with β-diketone and 1,10 phenanthroline (CBP-01) at concentrations of 0.03mM, 0.06mM, 0.12mM and 0.25mM when associated with DOX (0.4mM), through the Somatic and Recombination test – SMART in *Drosophila melanogaster*. Polypharmacological strategies are routinely used and particularly interesting in drug discovery. When associated with DOX, CBP-01 potentialized recombinogenic events of DOX, probably involving oxidative stress mechanisms. The results were verified for ST and HB crosses. After metabolization, the products were even more reactive, capable in generating greater damages with consequent increase in the expression of mutant spots in *D. melanogaster*. Therefore, additional assays in different animals are necessary to understand the pharmacological interaction of CBP-01 with DOX and the intrinsic mechanisms of this polypharmacology

Keywords: Chemoterapy. Copper complex. Doxorrubicin. Polypharmacology. Recombinogenic.

1. Introduction

Cancer is a worldwide health problem. Although evolution in treatment strategies have produced a steady decline in cancer rates in developed countries, metastasis remains a critical problem [1]. Chemotherapy is the most important strategy that systematically attack tumor circulating cells [2].

In fact, the deregulation of molecular mechanisms in transformed cells offers opportunities to concentrate the drugs action [3]. However, over the years, much evidence has shown mechanisms of multidrug resistance (MDR). Drug resistance limits therapeutic efficacy and exposes healthy tissues to toxicity [4]. The severe side effects [5] limit the dose and compromise their efficacy [6].

Doxorubicin, one of the most widely used chemotherapeutics, causes the death of tumor cells through the inhibition of topoisomerase activity and high production of free radicals. However, doxorubicin does not target only tumor cells, leading to adverse effects such as liver, brain, kidney and heart toxicity, and also drug resistance [7].

Complexes containing essential metals, such as copper, have been shown promising results in cancer treatment [8,9]. Copper complexes features like its redox potential and geometry optimize DNA binding. Under aerobic conditions or in the presence of intracellular oxidants, these drugs can generate ROS through Fenton chemistry. If the complex is bound to DNA, ROS generation will induce the oxidation of the molecule and leads to strand breaks [10].

In this context, the concept of polypharmacology arouses great interest in the treatment of cancer, especially when it comes to the triad of death, which consists of tumor growth, metastasis and drug resistance. The combination of

chemotherapeutics, in addition to making it possible to target a greater diversity of molecules, also decreases the adaptability and evasion of tumor cells [11].

However, multi drug regimen can leads to adverse clinical reactions and toxicity [12]. Thus, the evaluation of genotoxic potential of drugs combinations in *Dosophila melanogaster* is relevant. According to Dar et al [13], *D. melanogaster* provides a powerful system for evaluating drug combinations as well as their toxic effect.

Different genes involved in cell cycle regulation are highly conserved between *D. melanogaster* and human [14]. In addition the metabolic similarity between fly and humans reinforces the importance of *D. melanogaster* as an adequate experimental platform for the study of human diseases and drug metabolism [15]. This is a sofisticate and low cost model that has been used for decades to monitor genetic damages caused by chemical agents [16-19], including mutagenic / recombinogenic activity [20,18].

Somatic mutation and recombination test (SMART) that uses *D. melanogaster* as an experimental model is well described and widely used for mutagenic and recombinogenic evaluation of chemical, natural and synthetic agents [21-22,18,23], including those with antineoplastic action [24,19,25]. It also allows for greater knowledge about the risks and benefits of combining antineoplastic agents [26]. The smart test detects the loss of heterozygosity and the expression of recessive genes [27-28] caused by DNA damage [21-22] gives rise to a clone of mutant cells that can be detected by means of mutant trichomes on the wing of the adult [27-28].

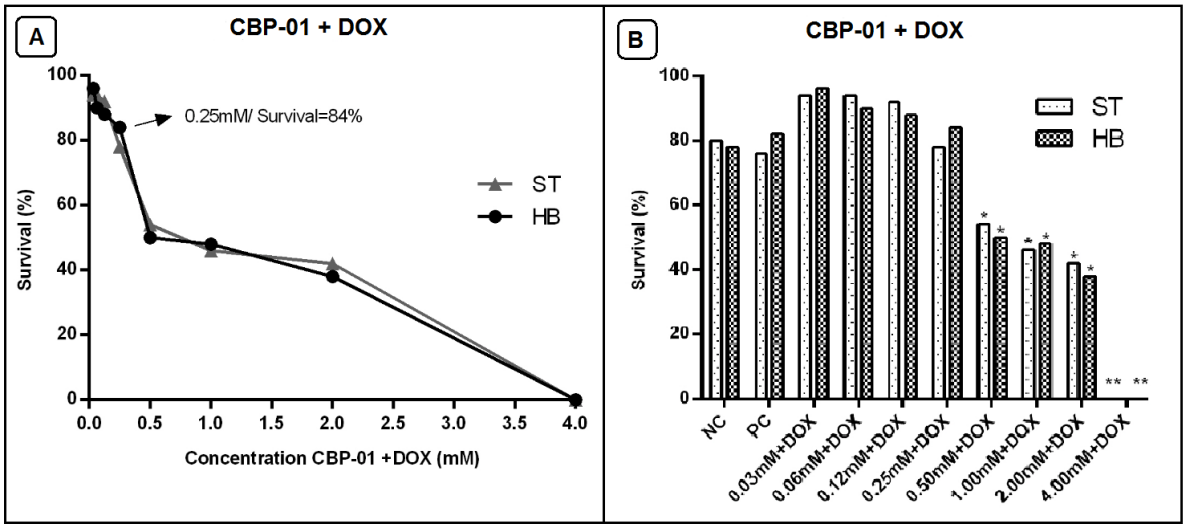
In this context, the present study aimed to evaluate the mutagenic / recombinogenic potential of a new copper ternary metal complex with β-diketone and

1,10-phenanthroline ($CuBTAPhenCLO_4$) (CBP-01) associated with doxorubicin (DOX), through SMART test in *D. melanogaster.*

2. Results and Discussion

The survival rates observed in the toxicity test are presented in Figure 1. CBP-01 at 4.00mM associated with doxorubicin was letal to all lineages of *D. melanogaster*. The survival frequency of CBP-01 at 0.5mM was less than 60% when compared to negative control ($p<0.05$) (Figure 1A and 1B). On the other hand, all concentrations of CBP-01 lower than 0.5mM (0.03mM, 0.06mM, 0.12mM and 0.25mM) presented a survival up to 70% (Figure 1A and 1B), with no statistical difference when compared to negative control.

Fig. 1. Percentage of survival of *Drosophila melanogaster* treated with different concentrations of the new copper ternary metal complex associated with β-diketone and 1,10 phenanthroline (CBP-01) associated with doxorubicin (0.4mM).



NC: Negative control (ethanol 5%)
PC: Positive control (Doxorrubicin 0.4mM)
*Statistical difference ($P<0.05$) comparing to negative control according to the X^2 test for ratios for independent samples

**(LD) Lethal dose

Treatments of CBP-01 with DOX (0.4mM) are presented in Table 1. When compared to the negative control, DOX induced significative spots frequency. The comparison between the frequencies of spots of MH and BH individuals revealed that recombination is the main response to the treatment of DOX alone (88.98%).

Table 1: Summary of results obtained in the marked trans-heterozygous descendants (MH) and balancer-heterozygous (BH) of *Drosophila melanogaster* derived from the standard cross (ST) and high bioactivation cross (HB) treated with different concentrations of the new copper ternary metal complex associated with β-diketone and 1,10 phenanthroline (CBP-01) , positive control (Doxorrubicin 0.4mM) and negative control (5% ethanol in distilled water).

Treatments		N⁰. of flies (*N*)	Spots per fly (n⁰. of spots) statiscal diagnosis[a]											Spots with *mwh* clone[c] (*n*)	Mean clone size class[c,d] (*i*)	Frequency of formation / 10^5 cells per cells division[d]		Recombination (%)	Indução[e] (%)
DOX (mM)	CBP-01 (mM)		Small single (1-2 cels)[b] *m* = 2			Large single (>2 cels)[b] *m* = 5			Twin *m* = 5			Total spots *m* = 2					Observed	Control corrected		
mwh/flr³ (MH)																			
Cross ST																			
0	0	60	0.37	(22)		0.05	(3)		0.00	(0)		0.42	(25)		25	1.40	0.56			
0.4	0	60	0.48	(29)	i	0.92	(55)	+	0.80	(48)	+	2.20	(132)		110	3.42	18.06	18.86	95.12	
0.4	0,03	60	1.17	(70)	+	1.30	(78)	+	0.95	(57)	-	3.42	(205)	f+	191	3.10	13.98	4.39	94.49	76.72
0.4	0,06	60	0.88	(53)	+	1.78	(107)	+	1.47	(88)	+	4.13	(248)	+	228	3.77	26.59	17.30	97.29	8.27
0.4	0,12	60	1.47	(88)	+	1.57	(94)	+	1.22	(73)	+	4.25	(225)	+	242	3.05	17.16	7.58	93.12	59.81
0.4	0,25	60	1.38	(83)	+	1.72	(103)	+	1.83	(110)	+	4.93	(296)	+	272	3.34	23.55	13.53	93.94	28.26
Cross HB																			
0	0	60	0.78	(47)		0.17	(10)		0.00	(0)		0.95	(57)		57	2.00	1.95			
0.4	0	60	1.52	(91)	+	1.98	(119)	+	0.25	(15)	+	3.75	(225)	+	222	3.25	18.06	18.12	88.98	
0.4	0,03	60	3.22	(193)	+	5.68	(341)	+	0.70	(42)	+	9.60	(576)	+	565	3.57	57.43	40.27	97.67	122,24
0.4	0,06	60	3.22	(193)	+	3.10	(186)	f+	0.45	(27)	+	6.77	(406)	+	401	2.97	26.74	9.32	91.32	48,57
0.4	0,12	60	1.77	(106)	-	4.02	(241)	+	0.73	(44)	+	6.52	(391)	+	382	3.63	40.48	24.46	95.73	34,99
0.4	0,25	60	1.88	(113)	f+	2.53	(152)	f+	0.57	(34)	+	4.98	(299)	+	337	3.18	25.99	8.00	97.38	55,85
mwh/TM3 (BH)																			
Cross ST																			
0	0	30	0.10	(3)		0.03	(1)		[f]			0.13	(4)		4	2.00	0.27			
0.4	0	30	0.40	(12)	+	0.00	(0)	i				0.40	(12)	+	12	1.25	0.49	0.25		
0.4	0.03	30	0.50	(15)	i	0.07	(2)	i				0.57	(17)	i	17	1.41	0.77	0.30		
0.4	0.06	30	0.40	(12)	i	0.03	(1)	i				0.43	(13)	i	13	1.69	0.72	2.19		
0.4	0.12	30	0.50	(15)	i	0.10	(3)	i				0.60	(18)	i	18	1.94	1.18	1.03		
0.4	0.25	30	0.37	(11)	i	0.10	(3)	i				0.47	(14)	i	14	1.79	0.82	1.09		
Cross HB																			
0	0	30	0.33	(10)		0.03	(1)					0.37	(11)		11	1.36	0.48			
0.4	0	30	0.93	(28)	+	0.17	(5)	i				1.10	(33)	+	33	1.82	1.99	1.55		
0.4	0.03	30	0.63	(19)	i	0.17	(5)	i				0.80	(24)	i	24	1.71	1.34	0.66		
0.4	0.06	30	0.83	(25)	-	0.30	(9)	i				1.13	(34)	-	34	2.00	2.32	4.37		
0.4	0.12	30	0.90	(27)	-	0.13	(4)	i				1.03	(31)	-	31	1.71	1.73	0.39		
0.4	0.25	30	0.57	(17)	i	0.00	(0)	+				0.57	(17)	+	17	1.24	0.68	1.48		

Marker-trans-heterozygous flies *(mwh/flr³)* and balancer-heterozygous flies *(mwh/TM3)* were evaluated.

[a] Statistical diagnoses according to Frei and Würgler (1988, 1995): f+, weak positive; +, positive; -, negative; i, inconclusive. *m* = multiplication factor for significantly negative results. Level of significance $P \leq 0.05$.

[b] Including rare *flr³* single spots.

[c] Considering *mwh* clones from *mwh* single and twin spots.

[d] Frequency of clone formation: clones/flies/48,800 cells (without size correction) Frei et al. (1992).

[e] Calculated as(DOX alone – CBP-01 + DOX / DOX alone) x 100, according to Abraham (1994)

[f] Only *mwh* single spots can be observed in heterozygous individuals *mwh/TM3*, since the balancer chromosome TM3 does not contain the mutant gene *flr³*.

The association of CBP with DOX in the ST significantly increased the number of spots, when compared to the positive control. The interaction between CBP-01 and DOX potentiated the recombinogenic effect of DOX (76.72%, 8.27%, 59.81% and 28.26%) in all tested concentrations (0.03mM, 0.06mM, 0.12mM, 0.25mM, respectively). These results revealed the increase in the frequency of mutant spots in the individuals of both crosses. However, potentialization was more evident in the HB cross descendants. The differences ST and HB are related to the levels of cytochrome P450.

The damages caused in *D. melanogaster* somatic cells by DOX are associated with free radicals and recombinogenic mechanisms [7,29]. Copper also has the ability to generate ROS [30] leading us to suggest that the excessive production of these molecules or even the reduction of antioxidant defenses can lead to redox imbalance, causing damage to the DNA with consequent significant increase of expression of the mutant phenotype in the wings of the flies. Our hypothesis is supported by data from Kankala et al [31] that demonstrated that the interaction of copper with H_2O_2 generated by DOX produced ROS via Fenton's reaction. However, such validation need to be conducted experimentally.

3. Conclusion

We emphasize the importance of polypharmacological studies, since the combination of drugs provides a greater range of targets and helps to overcome the inherent challenges of tumor resistance. Althought we proposed that cotreatment with CBP-01 potenzialized the effect of doxorubicin, different bioassays in other

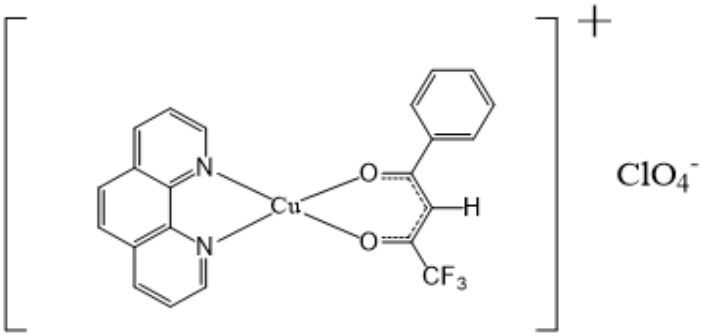
biological models are interesting to evaluate the pharmacological interaction of CBP-01 with doxorubicin and its contribution in the generation of DNA damage.

4. Materials and methods

4.1 Chemical agents

The copper ternary complex CBP-01, assigned from this study, was previously synthesized and characterized by do Couto Almeida et al. [32]. It is a compound of greenish coloration, non-hygroscopic, stable to air and light, soluble in organic solvents such as DMSO and acetonitrile, with molecular weight of 558,46g/mol. The chemical formula is shown in Figure 1.

Fig.2. Representation of the chemical structure of copper ternary metal complex associated with β-diketone and 1,10-phenanthroline (CuBTAPhenCLO4) (CBP-01).



Source: do Couto Almeida et al., [32]

DOX (Adriblastina®, CAS 25316-40-9, batch 5PL5111, registred, imported and distributed by the laboratory Pfizer, São Paulo – Brazil) was used as a positive control and associated with CBP-01. The concentration of DOX was 0.4mM based on previous studies that demonstrated the induction of homologous recombination in *D.*

melanogaster under this condition [33-34,29,35-36]. Ethanol 5% was used in dilution all of the compounds, which were prepared immediately before use.

4.2. Strains and crosses of *Drosophila melanogaster*

The SMART was carried out in two crosses. In the standard (ST) cross virgin females *flr*^{3} (*flr-3 (flr*^{3}*/In(3LR)TM3, ri pp sep l(3)89Aabx*^{34e} and *Bd*^{s}) were crossed with males *mwh (mwh/mwh*). The descendants have basal levels of the cytochrome P450 enzyme enabling the direct evaluation of mutagenic agents. The second was a High bioactivation (HB) cross, in which virgian females (*ORR ORR;flr3 (ORR; flr3/In(3LR)TM3, ri pp sep l(3) 89Aabx*^{34e} and *Bd*^{s}) were crossed with males *mwh (mwh/mwh*). In this cross is possible to evaluate the indirect damages caused by mutagenic agents, due to biotransformation through high levels of P450 [37,16,38].

Both crosses resulted in two types of progeny: the marked trans-heterozygous (MH, *mwh*+/+*flr*^{3}), with smooth wing edge phenotype, and individuals balancer heterozygous (BH, *mwh*+/+*TM3*) with the serrated appearance wing [27]. MH and BH individuals were analyzed in this study.

4.3 Somatic mutation and recombination test (SMART) in *Drosophila melanogaster*

For establish the concentration of CBP-01 associated with doxorubicin, the toxicity assay was firstly performed. Fifty larvae obtained from each crossing, ST and HB, were counted and placed in separate tubes containing 1.5g of culture medium (mashed potatoes) for *D. melanogaster* [28] and 5.0mL of different concentrations of CBP-01 (0.03mM, 0.06mM, 0.12mM, 0.25mM, 0.50mM, 1.00mM, 2.00mM and 4.00mM) in association with DOX (0.4mM). The emerged flies were counted.

According Orsolin et al [23] the number of surviving *D. melanogaster* in toxicity test per treatment provided an indicator of the toxicity of the compounds.

The methodological procedure employed for SMART was according to Graf et al [20] and improved by Graf and Van Schaik [16], with modifications. After the crosses (described in 2.2 section), eggs were collected over a period of 8h in flasks containing hatching medium, and a layer of yeast (*S. cerevisiae*) supplemented with sugar under a solid base of agar (4% w/v). The third instar larvae (72 ± 4h after) were washed and placed in each glass vials containing 1.5g of mashed potato flakes (HIKARI®) [28]. In each tube, the mashed potato flakes was rehydrated with 5 mL of CBP-01 (0.03mM, 0.06mM, 0.12mM and 0.25mM) in association with DOX, diluted in 5% ethanol. For the positive control and negative control, doxorubicin and ethanol 5% was used, respectively. The larvae were subjected to a chronic treatment, during approximately 48h, until pupal stage. All compounds were tested in duplicate in B.O.D incubator, under optimal laboratory conditions, temperature of 25 ±4°C and with relative humidity of 65%.

After hatching, the adult flies were fixed in 70% ethanol (v / v). The wings were removed and placed on microscope slides with Faure's solution (30g of gum arabic, 50mL of distilled water, 200g of chloral hydrate and 16mL of glycerol). The wings were analyzed under a light microscope, at a magnification of 400x (Graf et al., 1984). Frequency and size of mwh, flr and twin spots were recorded in a standard diagram.

4.4 Statistic analysis

In toxicity test, comparisons concerning survival rates were accomplished through the Chi-square test . For each treatment, a total of 60 flies (30 fameles and

30 males) were analyzed. At a significance level of 5%, the statistical analysis was carried out in accordance with the multiple decision procedure proposed by Frei and Würgler [39], resulting in four different diagnoses: positive, weakly positive, negative and inconclusive. The frequency of each type of spot (small or large single spot and twin spot), and the total frequency of spots per fly from each treatment were compared between CBP-01+DOX and positive control.

Mutant spots in descendants MH and BH were compared according to the frequency of mutation per 10^5 cells/division, leading to the calculation of recombinogenic activity as follows: (i) Frequency of mutation (FM)= frequency of clones in BH individuals/ frequency of clones in MH individuals; (ii) Frequency of recombination (FR) = 1 - frequency of mutation (FM) [40]. In addition, the percentage of induction of recombination was calculated, according to the formula: (DOX alone – CBP-01+DOX/DOX alone)×100.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Highlights

- CBP-01 associated with Doxorrubicin presents mutagenic/recombinogenic potential *in vivo.*
- CBP-01 potentialized the recombinogenic effect of Doxorrubicin in ST and HB crosses
- CBP-01 associated with Doxorrubicin increased the frequency of mutant spots, especially in the descendants of the HB cross

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5 Conclusão

O composto CBP-01 apresentou potencial recombinogênico apenas na menor concentração testada em *D. melanogaster* e após sua biotransformação por enzimas P450, o que sugere que os metabolitos gerados podem causar danos a biomoléculas, como o DNA. Quanto aos outros fármacos testados, Carboplatina, Cisplatina e Doxorrubicina, todas induziram expressivamente a formação de manchas mutantes. Além disso, o CBP-01 apresentou atividade antitumoral *in vitro* e seletivo às células tumorais mamárias. Quando comparado às outras drogas, Carboplatina, Cisplatina e Doxorrubicina, foi mais efetivo contra a linhagem triplo negativa, com fenótipo conhecidamente mais agressivo. Portanto, composto avaliado apresenta-se potencialmente promissor para o tratamento do CM. No entanto, estudos adicionais são necessários para compreender os eventos molecuares mediados por seu tratamento, para que assim sejam estabelecidos novos desenhos terapêuticos para o CM.

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