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Ação antineoplásica da C-ficocianina extraída de *Spirulina platensis* em linhagens eritroleucêmicas humanas com e sem o fenótipo de resistência a múltiplas drogas

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Sumário

Abreviações

5-LOX - Araquidonato 5-lipoxigenase

ABC- ATP *binding* cassete

ABCB1- ou MDR1, codifica a Glicoproteína-P

ABCC1- ou MRP1, codifica a glicoproteína MRP1

ABCG2- codifica a Glicoproteína ABCG2 ou BCRP ou MXR

DNA – Ácido desoxirribonucleico

DNR - Daunorrubicina

K562- Linhagem celular não resistente a múltiplas drogas (MDR)

K562-Lucena- Linhagem celular MDR

LMA- Leucemia Mielóide Aguda

LMC- Leucemia Mielóide Crônica

MDR- Resistência a Múltiplas Drogas

MTT - Brometo de (3- (4 5-dimetiltiazol-2-yl)-2 5-difeniltetrazólio

Ph – Cromossomo *Philadelphia*

PTGS2 – Codifica para a proteína Cicloxigenase-2

ROS – Espécies reativas de oxigênio

VCR - Vincristina

Resumo Geral

A C-ficocianina (C-FC), pigmento fotossintético de cianobactérias como a *Spirulina platensis*, possui capacidade antitumoral. Contudo, os mecanismos responsáveis pelo efeito antitumoral permanecem incompletamente compreendidos e o seu papel no fenótipo de resistência a múltiplas drogas (MDR) é bastante restrito. O fenótipo MDR gera insucessos no tratamento quimioterápico, sendo importante investigá-lo. O objetivo desta tese foi avaliar o efeito antitumoral e anti-MDR da C-FC e os mecanismos para geração destes efeitos, em três linhagens eritroleucêmicas humanas: K562 (não MDR); K562-Lucena (MDR/ superexpressão da proteína ABCB1 para efluxo de drogas) e FEPS (MDR/ superexpressão das proteínas ABCB1 e ABCC1 para efluxo de drogas). Realizou-se uma revisão de literatura sobre o tema, além de testes de viabilidade celular (exclusão por azul de Tripan e MTT), verificação de marcação por C-FC por citometria de fluxo, análise de atividade das proteínas de efluxo e avaliação de níveis de espécies reativas de oxigênio (ROS) por fluorimetria, análise de expressão gênica por PCR tempo real (genes para proteínas de efluxo: *ABCB1*; *ABCC1*; genes para proteínas produtoras de mediadores inflamatórios: *PTGS2* e *ALOX5*), e análise de interação da C-FC com: ABCB1, ABCC1 e com os quimioterápicos vincristina (VCR) e daunorrubicina (DNR) por *docking*. Com a revisão constatou-se a ubiquidade de alvos celulares e mecanismos de ação da C-FC, bem como o potencial anti-MDR da molécula. A C-FC inibiu a proliferação nas linhagens K562, K562-Lucena e FEPS (sendo K562 a mais sensível e FEPS a mais resistente), sem gerar citotoxicidade para os macrófagos peritoneais de *Mus musculus*. A C-FC interagiu de modo diferencial com as proteínas de efluxo ABCB1 e ABCC1 afetando a atividade apenas de ABCC1. A mudança de atividade da ABCC1 possivelmente gerou o efluxo da C-FC, pois a FEPS (única linhagem com ABCC1) foi aquela com menor marcação por C-FC e mais resistente. No *docking* proteína-ligante a C-FC interagiu com VCR e DNR de modo semelhante, contudo o aumento de sensibilidade com a combinação C-FC/quimioterápico foi verificado apenas para C-FC + DNR, indicando que a formação de complexo C-FC/DNR deve modificar a interação de C-FC e/ou DNR com a ABCC1. Os mecanismos antitumorais não envolveram níveis significativos de apoptose em relação ao controle, reforçando o papel citostático da C-FC verificado nos ensaios de viabilidade. A C-FC aumentou os níveis de ROS para K562 e K562-Lucena, reduziu a expressão do gene *ALOX5* para K562-Lucena e FEPS, além de aumentar a expressão de *PTGS2* e *ABCB1* para K562-Lucena. Para a linhagem K562-Lucena é possível que a expressão de *PTGS2* e *ABCB1* estejam relacionadas sendo ocasionadas provavelmente via ROS. A C-FC isoladamente gerou inibição de proliferação para K562 e K562-Lucena e combinada com DNR para a FEPS, sendo que o ROS parece estar envolvido na geração dos efeitos para K562 e K562-Lucena. A modulação da expressão gênica de *ALOX5* parece ser um importante alvo celular da C-FC em células MDR, mesmo para linhagens mais resistentes como a FEPS. A C-FC tem capacidade anti-MDR isolada ou em combinação com DNR, sendo provável o envolvimento de vias de sinalização relacionadas a ROS e a modulação da expressão gênica para geração desse efeito.

Palavras-chave: Inibição de proliferação; ABCB1; ABCC1; *Docking*.

Abstract

The C-phycocyanin (C-PC), a photosynthetic pigment of cyanobacteria such as *Spirulina platensis*, has antitumor capacity. However, the mechanisms responsible for the antitumor effect remain incompletely understood and their role in the multidrug resistance (MDR) phenotype is quite restricted. The MDR phenotype generates failures in the chemotherapeutic treatment, being important to investigate it. The aim of this thesis was to evaluate the antitumor and anti-MDR effect of C-PC and the mechanisms to generate these effects, in three human erythroleukemic cell lines: K562 (non-MDR); K562-Lucena (MDR/ overexpression of protein ABCB1 for drugs efflux) and FEPS (MDR/ overexpression of proteins ABCB1 and ABCC1 for drugs efflux). We conducted a literature review, besides cell viability tests (Trypan blue exclusion and MTT), verification of C-PC labeling by flow cytometry, analysis of efflux proteins activity and evaluation of reactive oxygen species (ROS) levels by fluorimetry, gene expression analysis by real-time PCR (genes for efflux proteins: *ABCB1*; *ABCC1*; genes for inflammatory mediators: *PTGS2* e *ALOX5*), and interaction analysis of C-PC with: ABCB1, ABCC1 and with the chemotherapeutic vincristine (VCR) and daunorubicin (DNR) by docking. With the review we verified the ubiquity of cellular targets and mechanisms of action of C-PC, as well as the anti-MDR potential of this molecule. The C-PC inhibited proliferation in the K562, K562-Lucena and FEPS cell lines (K562 being the most sensitive and FEPS the most resistant), without generating cytotoxicity for the peritoneal macrophages of *Mus musculus*. The C-PC interacted differentially with the ABCB1 and ABCC1 efflux proteins affecting activity only of ABCC1. The change in ABCC1 activity possibly generated C-PC efflux, since FEPS (single cell line with ABCC1) was the one with the lowest C-PC and the most resistant. In the protein-ligand docking the C-PC interacted with VCR and DNR in a similar way, however the increase of sensitivity with the combination C-PC/chemotherapeutic was verified only for C-PC+DNR, indicating that the formation of complex C-PC/DNR should modify the interaction of C-PC and/or DNR with ABCC1. Antitumor mechanisms did not involve significant levels of apoptosis in relation to the control, reinforcing the cytostatic role of C-PC verified in the viability tests. The C-PC increased levels of ROS for K562 and K562-Lucena, reduced expression of the *ALOX5* gene for K562-Lucena and FEPS, and increased expression of *PTGS2* and *ABCB1* for K562-Lucena. For the K562-Lucena cell line it is possible that the expression of *PTGS2* and *ABCB1* are related being probably caused by ROS. The C-PC alone generated proliferation inhibition for K562 and K562-Lucena and combined with DNR for FEPS, and ROS appeared to be involved in the generation of effects for K562 and K562-Lucena. Modulation of *ALOX5* gene expression appears to be an important cellular target of C-PC in MDR cells, even for more resistant cell lines such as FEPS. The C-PC has anti-MDR capability alone or in combination with DNR, probably with the involvement of ROS-related signaling pathways and the gene expression modulation for this effect.

Keywords: Proliferation inhibition; ABCB1; ABCC1; Docking.

Introdução

1.1. Câncer

Segundo o Instituto Nacional do Câncer (INCA) "Câncer é o nome dado a um conjunto de mais de 100 doenças que têm em comum o crescimento desordenado (maligno) de células que invadem os tecidos e órgãos, podendo espalhar-se (metástase) para outras regiões do corpo. Dividindo-se rapidamente, estas células tendem a ser muito agressivas e incontroláveis, determinando a formação de tumores (acúmulo de células cancerosas) ou neoplasias malignas (INCA, 2018)".

O câncer trata-se de uma doença essencialmente genética, uma vez que ocorre devido a mutações em genes relacionados ao processo de divisão celular (mitose). Assim, quando protooncogenes adquirem mutações tornam-se oncogenes e passam a determinar uma multiplicação celular excessiva. Não somente os protoconcogenes podem adquirir mutações, mas também os genes supressores de tumores, que uma vez mutados não são capazes de exercer o controle para a prevenção de crescimento celular inapropriado. As mutações em protooncogenes e genes supressores de tumores podem ocorrer devido a fatores externos (tabaco, organismos infecciosos, nutrição inadequada, agentes químicos e radiação) e fatores internos (mutações hereditárias) (Rivoire *et al.*, 2001).

Essa patologia é uma das principais causas de morbidade e mortalidade em países desenvolvidos e em desenvolvimento (Fidler *et al.*, 2017). Há sete anos de acordo com a Organização Mundial da Saúde (WHO) o câncer causava mais mortes que doenças coronárias e acidente vascular cerebral (WHO, 2011), sendo esperados para o ano de 2025 cerca de 20 milhões de novos casos da doença (Bray, 2014).

1.2. Leucemias

A leucemia é um tipo de câncer dos mais comuns em todo o mundo, apresentando 250 mil casos por ano (Ferlay *et al.*, 2012). A leucemias é uma patologia hematológica causada por glóbulos brancos anormais produzidos na medula óssea, sendo esta a definição clássica da doença. Desse modo, ocorre uma produção exagerada e descontrolada de células sanguíneas anormais, diminuindo assim a produção de células sanguíneas saudáveis, podendo provocar aumento de sangramentos, várias infecções e anemia grave (Inamdar e Bueso-Ramos, 2007). Além disso, as células leucêmicas podem se espalhar para outros órgãos como o baço,

cérebro, linfonodos e outros tecidos (Inamdar e Bueso-Ramos, 2007). As leucemias são subclassificadas de acordo com sua evolução clínica em aguda ou crônica (Arber *et al.*, 2016), existindo assim quatro tipos principais, aquelas de precursor linfoide: Leucemia linfoide aguda (LLA) e Leucemia linfoide crônica (LLC) e aquelas de precursor mieloide: Leucemia mieloide aguda (LMA) e Leucemia mieloide crônica (LMC).

As linhagens eritroleucêmicas pesquisadas na presente tese são do tipo LMC, que é uma neoplasia mieloproliferativa caracterizada hematologicamente por um excesso de granulócitos (neutrófilos, basófilos, eosinófilos). Existem três fases para a LMC, sendo estas: a fase crônica, a acelerada e a blástica ou aguda. Aproximadamente 85% dos diagnósticos ocorrem na fase crônica e 15% nas fases mais agressivas (acelerada e blástica). As características da fase blástica são: ≥ 20% de blastos no sangue periférico ou na medula óssea, infiltrado extramedular de células blásticas e grandes focos de blastos em uma biópsia de medula óssea (Hanlon e Copland, 2017).

A LMC é caracterizada geneticamente pelo cromossomo Filadélfia (Ph) (Figura 1), cromossomo encontrado em mais de 90% dos pacientes com LMC (Rowley, 1973; Hanlon e Copland, 2017). O cromossomo Ph trata-se do cromossomo 22 reduzido (Avelino *et al.*, 2017) que surge da fusão da porção 3' do gene Abelson (ABL) do braço longo do cromossomo 9 com a porção 5' da região do cluster de ponto de interrupção (BCR) no braço longo do cromossomo 22. O produto da fusão destes cromossomos é uma onco-proteína (BCR-ABL) de 210 kDa, localizada no citoplasma (Ben-Neriah *et al.*, 1986) com atividade de tirosina cinase que é essencial para a proliferação desregulada das células mieloides (Hanlon e Copland, 2017). Um tipo de LMC que apresenta o cromossomo Ph são as eritroleucemias, caracterizadas pela transformação maligna de eritroblastos.

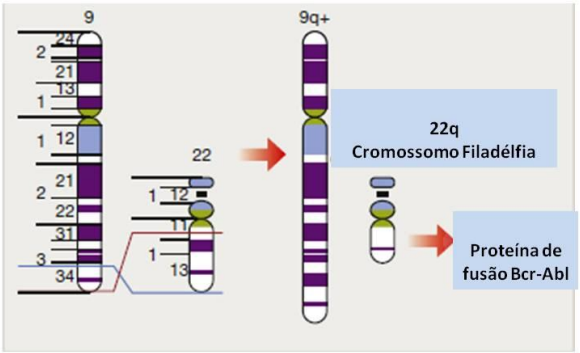

Figura 1 – O cromossomo Filadélfia ou 22 reduzido: Oriundo da fusão do braço longo do cromossomo 9 com o braço longo do cromossomo 22 (Adaptado de Hanlon e Copland 2017).

1.3. Resistência a múltiplas drogas

Um dos principais desafios para o sucesso da quimioterapia continua sendo o fenótipo de resistência a múltiplas drogas (MDR). O fenótipo MDR consiste na capacidade das células tumorais resistirem ao tratamento com um vasto espectro de drogas não relacionadas estruturalmente (Gottesman *et al.*, 2002; Singh *et al.*, 2017). Esse fenótipo pode desenvolver-se em função da exposição à quimioterapia por tempo prolongado (extrínseco) ou através de alterações genéticas das células tumorais (intrínseco) dificultando o sucesso quimioterapêutico (Gottesman *et al.*, 2002), mesmo quando são administradas doses adequadas dos medicamentos. Segundo Gottesman *et al.*, (2002) e Wu *et al.*, (2014) alguns dos fatores celulares que levam à resistência a múltiplas drogas são:

a) Superexpressão de proteínas transmembrana para o efluxo de drogas: As proteínas da família ABC (do inglês ATP-binding cassete) compartilham a sequência e a homologia estrutural, sendo identificados até o momento, 48 genes ABC para humanos (Dean *et al.*, 2001). Essas proteínas contribuem para a resistência uma vez que realizam, com gasto de ATP, o efluxo de drogas, diminuindo assim as concentrações intracelulares de fármacos (Ambudkar *et al.*, 1999; Dean *et al.*, 2001). A atuação dessas proteínas é um dos fatores melhor compreendidos para a resistência (Gottesman e Pastan, 1993).

A estrutura básica da maioria dos transportadores da família ABC conta com dois domínios transmembrana (do inglês TMD1 e TDM2) associados a dois domínios de ligação a nucleotídeos (NBD1 e NBD2) de modo a formar um canal onde existe um sítio de ligação para o substrato a ser transportado (Figura 2). A maioria das 48 proteínas ABC humanas descritas são transportadores de membrana que realizam o transporte de pequenas moléculas, possivelmente contra um gradiente de concentração (Szöllősi et al., 2018).

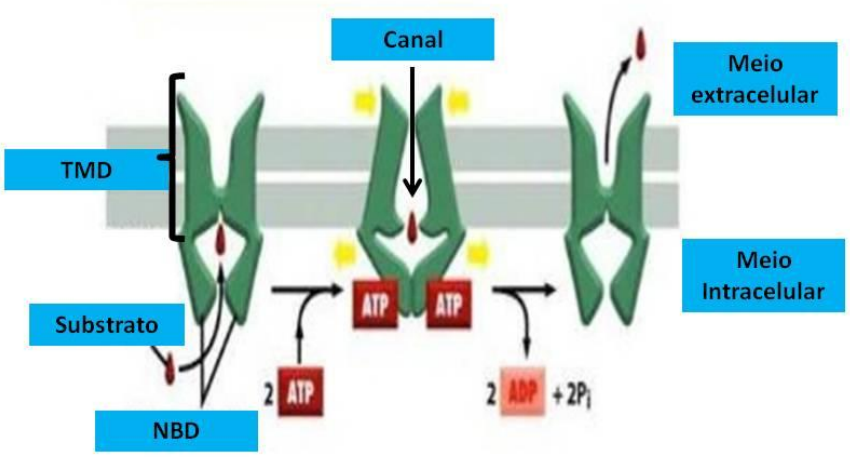

Figura 2: Principais estruturas de um transportador ABC (ATP *binding* cassete) envolvidas na extrusão de substratos. Adaptado do endereço eletrônico: https://www.slideshare.net/AmitKumar2325/cell-membrane-and-transport-52246921.

A superfamília ABC é dividida em quatro grupos principais quanto à função: 1) importadores de nutrientes (principalmente transportadores bacterianos); 2) exportadores de compostos (peptídeos, metabólitos ou xenobióticos/subfamílias ABCA-D e ABCG); 3) canais iônicos (por exemplo, o ABCC7) ou reguladores de canais iônicos (por exemplo, ABCC8 e ABCC9) e 4) proteínas ABC que não possuem TDM e estão envolvidas no reparo de DNA e tradução (subfamílias E e F) (Bryan *et al.,* 2007; Barthelme *et al.,* 2011; Boel *et al.,* 2014), sendo a principal diferença estrutural entre os transportadores com TMD o número de segmentos transmembrana (STM) através da membrana plasmática, por exemplo ABCB1 possui 12 STM; ABCC1 17 STM e ABCG2 6 SMT (Choudhurin e Klaassen, 2006) (Figura 3). Os transportadores da família ABC que possuem TMD possuem especificidades por substrato parcialmente sobrepostas (Nerada *et al.,* 2016), contudo existem predominantes: o transportador ABCB1 realiza efluxo de substâncias hidrofóbicas e catiônicas; o ABCC1 transporta substratos livres e conjugados a glutationa ou glicídes e o transportador ABCG2 carreia substratos conjugados a sulfato e glicídeos (Choudhurin e Klaassen, 2006).

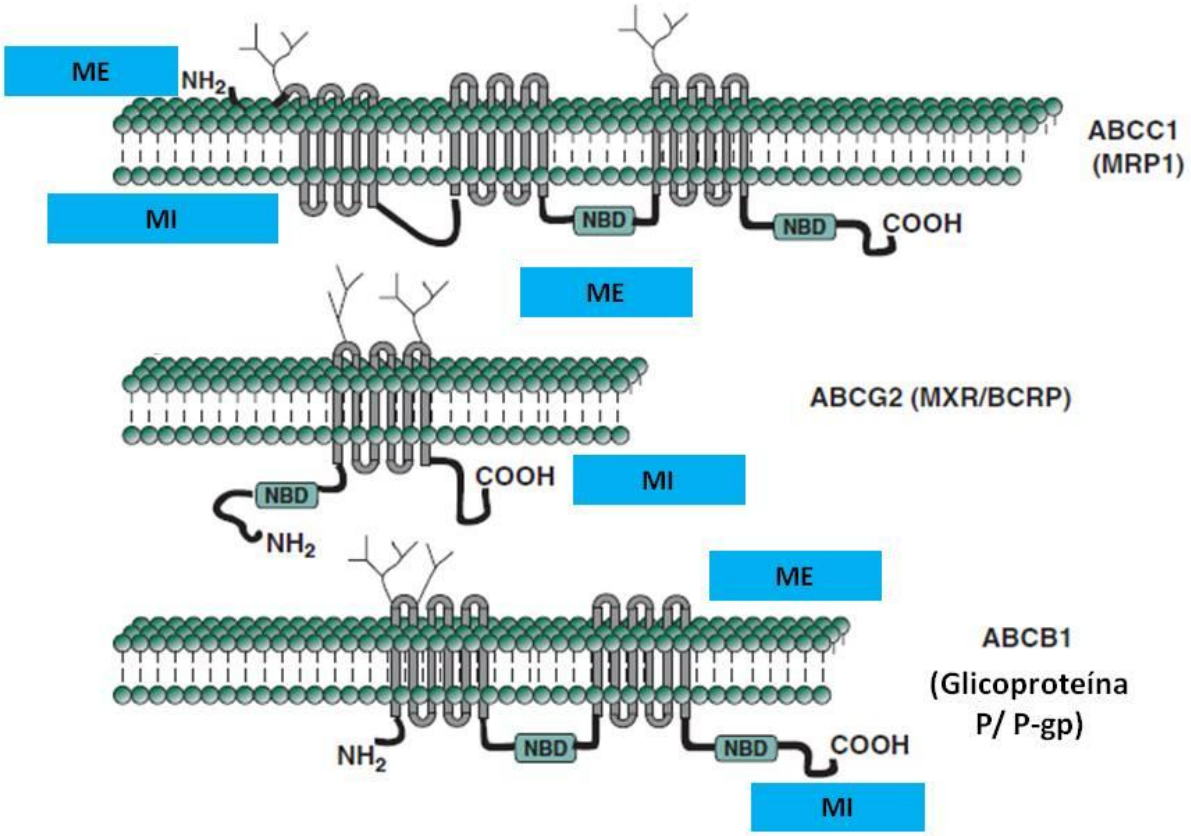

Figura 3: Diferenças estruturais entre alguns representantes das subfamílias (C, B e G) dos transportadores ABC. ABCC1 possui 17 segmentos transmembrana (STM); ABCB1 possui 12 STM e ABCG2 6 STM. Abreviações: ME – meio externo; MI – Meio interno; NBD: Domínio de ligação a nucleotídeos (Adaptado de Vatier *et al.*, 2006).

Considerando a superexpressão de transportadores ABCB1 e ABCC1 (que atuam na exportação de quimioterápicos) pelas células que foram modelo de estudo da tese, serão abordados apenas os modelos de funcionamento desses subtipos de transportadores (tendo o transportador ABCB como modelo). O "modelo de alteração de acesso" é bastante genérico e foi o primeiro a ser proposto. Nesse modelo o substrato é enviado para o meio extracelular por mudança na afinidade por esse substrato (Jardetzky, 1966). Um modelo baseado no anterior, porém mais detalhado é o chamado "aspirador hirofóbico" sendo proposto que os substratos (que apesar de diversos são predominantemente hidrofóbicos para ABCB) acumulam-se na bicamada lipídica e então o transportador recupera o substrato do folheto interior da membrana e, em seguida, envia-o para o folheto exterior para expulsá-lo diretamente para o meio extracelular (Higgins e Gottesman, 1992). Atualmente, estão sendo estudados diversos modelos para uma compreensão mais detalhada do mecanismo de transporte passo a passo (Szöllősi et al., 2018).

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b) Inibição do influxo de drogas: Certos medicamentos solúveis em água ou que entram na célula por meio de endocitose podem deixar de se acumular sem que haja o aumento do efluxo e sim a diminuição do influxo. Um dos mecanismos envolvidos nesse processo trata-se da expressão reduzida de proteínas ligantes dessas drogas. Algumas drogas que tem seu influxo reduzido nas células resistentes incluem o metotrexato, análogos de nucleotídeos, tais como 5-fluorouracil e 8-azaguanina e cisplatina (Shen *et al.*, 1998; Shen *et al.*, 2000).

c) Reparo do DNA: Uma célula resistente possui a capacidade de fazer a excisão de lesões letais no seu DNA, conferindo assim resistência principalmente a fármacos cujo mecanismo de ação concentra-se na desestabilização do DNA (Casorelli *et al.*, 2012).

d) Resistência a apoptose: A capacidade das células tumorais resistirem à morte celular programada (apoptose) contribui para a resistência. A transformação de uma célula normal em maligna carrega consigo alterações que levam a uma resistência a apoptose, como, por exemplo, mutações ou a não funcionalidade da proteína p53 (Lowe *et al.*, 1993). Além disso, durante a exposição a quimioterápicos, as células podem adquirir mudanças nas vias apoptóticas, como a alteração nos níveis de ceramida (Liu *et al.*, 2001) e mudanças na maquinaria envolvida no controle do ciclo celular.

e) Regulação epigenética: As alterações epigenéticas (como, por exemplo, demetilação ou baixa metilação do DNA) em uma célula resistente são capazes de regular o fenótipo MDR. Assim, o padrão de metilação de promotores dos genes da família ABC influencia na regulação da expressão desses transportadores contribuindo assim para o efluxo de drogas (Martin *et al.*, 2013).

f) Autofagia: Em uma célula saudável a autofagia é o processo pelo qual os componentes citoplasmáticos são degradados em massa pelos lisossomos devido a sinalizações intra e extracelulares (Vinod *et al.*, 2013). Em uma célula resistente a autofagia torna-se responsável por degradar moléculas de drogas, ajudando as células cancerosas a evadir a apoptose (Vinod *et al.*, 2013).

g) Células-tronco tumorais: As células-tronco tumorais são uma subpopulação de células com capacidade de auto-renovação e propriedades de diferenciação. Essas células são capazes de desenvolver o fenótipo MDR, contribuindo para a resistência (Xue *et al.*, 2012).

h) MicroRNAs: São uma classe de RNAs endógenos de aproximadamente 22 nucleotídeos, que atuam como silenciadores pós-transcricionais, inibindo a tradução de RNAs mensageiros-alvo (Ricarte Filho e Kimura, 2006). Esses RNAs regulam as funções de fenótipos malignos como o MDR. Assim, um exemplo da relação miRNAs e MDR é o fato da expressão da proteína ABCB1 ser regulada por miRNAs (Lopes-Rodrigues *et al.*, 2014).

i) Hipóxia: O fator induzido por hipóxia (diminição das taxas de oxigênio) – HIF-1 (do inglês hypoxia-inducible factor 1) pode regular o fenótipo MDR, uma vez que o HIF-1α é capaz de ativar a expressão de ABCB1 e impedir a apoptose pela regulação de c-Myc e p21 em células tumorais (Roncuzzi *et al.*, 2014).

1.4. Linhagens celulares eritroleucêmicas humanas K562, K562-Lucena e FEPS

As linhagens celulares K562, K562-Lucena e FEPS que serviram de modelo de estudo para a presente tese são eritroleucemias humanas. O termo eritroleucemia relaciona-se ao tipo de célula que está em divisão descontrolada nessa patologia. Resumidamente, a hematopoiese (Figura 4) conta com uma célula tronco hematopoiética que dá origem a um progenitor celular multipotente (MPP). O MPP se diferencia em dois tipos de precursores, o linfoide e o mieloide, sendo que cada precursor dá origem a grupos celulares distintos (Antoniani *et al.*, 2017). No caso da eritroleucemia o precursor mieloide deu início à formação de um eritrócito, assim, diferenciou-se em eritroblasto (eritrócito jovem, ainda com núcleo) e estes eritroblastos passaram a realizar as mitoses de modo descontrolado (Figura 4). A linhagem celular K562 foi estabelecida *in vitro* na década de 70 a partir de uma efusão pleural de um paciente com LMC em crise blástica (Lozzio e Lozzio, 1973).

Através da exposição das células K562 a concentrações crescentes de vincristina, Rumjanek e colaboradores (1994/2001) criaram a linhagem celular MDR K562-Lucena. A vincristina trata-se de um quimioterápico que interrompe a polimerização dos monômeros de β –tubulina para formação dos microtúbulos, impedindo assim a mitose (Gagné-Boulet *et al.*, 2015; Verma e Kannan, 2017). A linhagem K562-Lucena apresenta as seguintes

características: a expressão da proteína de efluxo de drogas P-gp ou ABCB1 (Gottesman e Pastan, 1993); resistência a medicamentos não relacionados (Kartner e Ling, 1989; Tiirikainen e Krusius, 1991); extrusão de corante de rodamina (Neyfakh, 1988) e reversão da resistência induzida por agentes como trifluoperazina, verapamil e ciclosporina A (Ford e Hait, 1990; Sikic, 1993).

Posteriormente, o mesmo grupo de pesquisa criou a linhagem celular MDR FEPS (Daflon-Yunes *et al.*, 2013) pela exposição da linhagem parental K562 a concentrações crescentes de daunorrubicina, um quimioterápico que se intercala na dupla fita de DNA impedindo a síntese de DNA e RNA (Avendano e Menendez, 2015; Krzak *et al.*, 2017). Na linhagem FEPS pode-se verificar características distintas em relação à K562-Lucena, como a menor expressão do receptor de morte celular CD95, expressão mais elevada (em relação à linhagem K562-Lucena) de proteínas de efluxo de drogas P-glicoproteína (P-gp/ABCB1) e a presença da proteína de resistência a múltiplas drogas-1 (MRP1/ABCC1).

Mesmo que a diferença mais marcante entre as linhagens eritroleucêmicas MDR e não MDR seja a presença de proteínas de efluxo de drogas, outros fatores podem estar envolvidos com o fenótipo MDR como, por exemplo: Uma maior capacidade antioxidante (Trindade *et al.,* 1999; Votto *et al.,* 2007; Votto et al., 2010) da linhagem K562-Lucena em relação a K562, um maior nível de α-tubulina da linhagem K562-Lucena em relação a K562 (Votto et al., 2007), a superexpressão do marcador de célula tronco *Oct-4* das linhagens K562-Lucena e FEPS em relação a K562 (Marques et al., 2010; Carrett-Dias et al., 2016) e a superexpressão de *ALOX5* das linhagens K562-Lucena e FEPS em relação a K562 (Carrett-Dias et al., 2016) e *PTGS2* da linhagem K562-Lucena em relação a K562, que são genes relacionados a resposta inflamatória (Carrett-Dias et al., 2011).

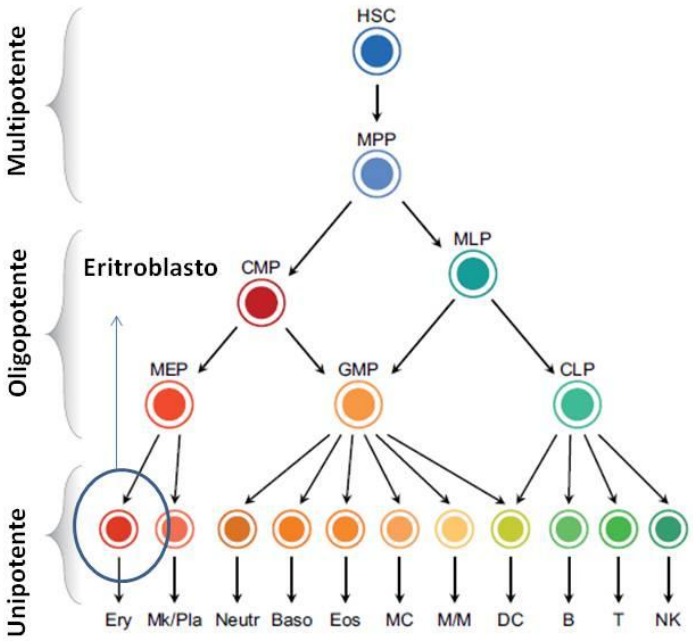

Figura 4: A hematopoiese humana, destacado com o círculo azul o tipo de célula que se divide descontroladamente em uma eritroleucemia. Abreviações: HSC: célula tronco hematopoiética; MPP: progenitor celular multipotente; CMP: progenitor mieloide comum; MLP: progenitor multilinfóide MEP: progenitor megacariócitos/eritróide; GMP: progenitor de granulócitos/macrófagos; CLP: progenitor linfóide comum; Ery: eritrócitos; Mk/Pla: megacariócitos/plaquetas; Neutr: neutrófilos; Baso: basófilos; Eos: eosinófilos; MC: mastócito; M/M: monócitos/macrófagos; DC: células dendríticas; B: células B; T: células T; NK: células natural killer (Adaptado de Antoniani *et al.*, 2017).

1.5. C-ficocianina

As cianobactérias, um grupo de procariotos fotoautotróficos Gram-negativos, são capazes de realizar fotossíntese pela utilização de pigmentos como a C-ficocianina (C-FC) (Singh *et al.*, 2011). A C-FC é uma heteroproteína de intensa cor azul esverdeada (Singh *et al.*, 2011) relatada pela primeira vez em 1928 (Lemberg, 1928) pertencente à família das ficobiliproteínas (PBP) (Patel *et al.*, 2005). As PBPs são divididas em dois grandes grupos: ficoeritrinas que são vermelhas e ficocianinas que são azuis (Killilea *et al.*, 1980; Kuddus *et al.*, 2013). Dentre as ficocianinas existem três tipos: C-FC, R-FC e Alo-FC (Brown *et al.*, 1979; Kuddus *et al.*, 2013), sendo a C-FC a principal PBP encontrada em cianobactérias do gênero *Nostoc*, *Spirulina*, e *Aphanizomenon*, entre outros (Kuddus *et al.*, 2013).

As PBPs/C-FC compõem grânulos (ficobilissoma) localizados na membrana dos tilacóides capazes de absorver luz (De Marsac e Cohen-Bazire, 1977; Benedetti *et al.*, 2006). A C-FC é formada pela associação de dois hexâmeros compostos por uma parte proteica (formada por subunidades α e β) e uma parte não proteica (um grupo prostético cromóforo, as ficocianobilinas, responsáveis pela cor azul, sendo uma ficocianobilina associada à cadeia α e

duas associadas à cadeia β) (Figura 5) (Wang *et al.*, 2001; Li *et al.*, 2006; Fernández-Rojas *et al.*, 2014).

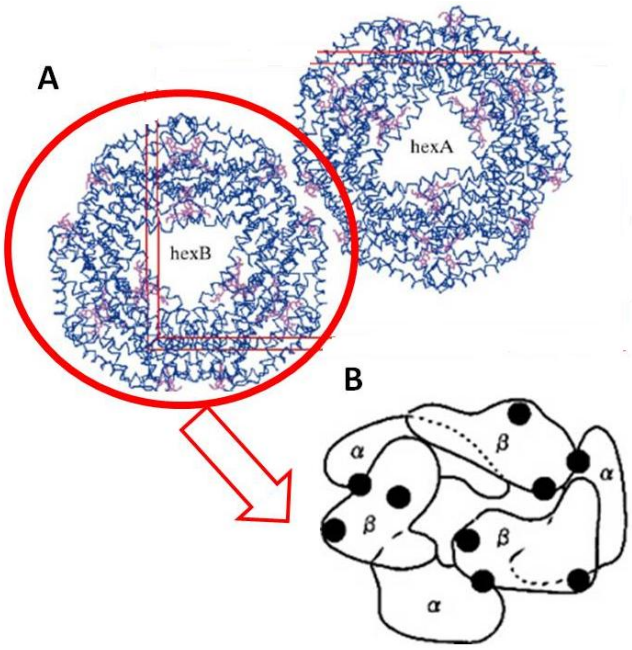

Figura 5: Estrutura da C-Ficocianina de *Spirulina platensis*: (A) Os dois hexâmeros que compõem a C-FC (B) Cada hexâmero é formado por subunidades alfa e beta sendo um grupo cromóforo (pontos pretos) associado à subunidade alfa e dois associados à subunidade beta (Adaptado de Wang et al., 2001; Li et al., 2006).

1.6. C-ficocianina, câncer e o fenótipo MDR

Apesar da C-FC ainda não ser utilizada clinicamente, a mesma possui propriedades antitumorais em diversas linhagens celulares, sem afetar células saudáveis, demonstrando a especificidade desta molécula para as células tumorais (Wang *et al.*, 2007; Li *et al.*, 2010). Dentre as propriedades antitumorais são observadas basicamente: 1) a diminuição de viabilidade e/ou proliferação, 2) o aumento de apoptose e necrose, 3) fragmentação de DNA e 4) redução da expressão de proteínas anti-apoptóticas, para diversas linhagens (AK-5/ histiocitoma de rato; K562/ Leucemia mieloide crônica humana; HeLa/ adenocarcinoma cervical humano; 686LN e 686LN-M4C1/ Carcinoma escamoso humano de cabeça e pescoço; HT-29/ adenocarcinoma colorretal humano; MCF-7/ câncer de mama responsivo ao estrógeno; HepG2/ carcinoma hepatocelular; A549/ adenocarcinoma de pulmão; SKOV-3/ adenocarcinoma de ovário) (Pardhasaradhi *et al.*, 2003; Subhashini *et al.*, 2004; Li *et al.*, 2006; Wang *et al.*, 2007; Li *et al.*, 2010; Wang *et al.*, 2012; Thangam *et al.*, 2013; Pan *et al.*, 2015).

Talvez o principal motivo para que a C-FC não seja utilizada clinicamente deva-se ao fato de que até o presente momento, os mecanismos antitumorais da C-FC permanecem pouco compreendidos (Gupta e Gupta, 2012). Apesar disso, no primeiro artigo que compõe esta tese tem-se a hipótese de que, na verdade, a C-FC possui mais de um alvo celular e consequentemente desencadeia diversos mecanismos que culminam na morte celular e/ou inibição de proliferação. Essa hipótese foi formulada uma vez que os relatos da literatura mostram que a C-FC interage com praticamente todas as estruturas das células, desde a membrana, citoplasma, até o núcleo (Silva *et al.*, 2018). Em contato com as estruturas celulares (membrana plasmática, β-tubulina, COX-2, enzimas antioxidantes, genes pró-apoptóticos e gene para a proteína ABCB1) a C-FC pode gerar mecanismos diversos como: Parada do ciclo celular em fases específicas (Wang *et al.*, 2012), modificação do estado redox (Pardhasaradhi *et al.*, 2003; Wang *et al.*, 2012; Thangam *et al.*, 2013; Pan *et al.*, 2015), indução de apoptose e necrose (Pardhasaradhi *et al.*, 2003; Subhashini *et al.*, 2004; Li *et al.*, 2006; Wang *et al.*, 2007; Wang *et al.*, 2012; Thangam *et al.*, 2013; Pan *et al.*, 2015), indução de dano de DNA (Thangam *et al.*, 2013), translocação de Gliceraldeído-3-fosfato-desidrogenase do núcleo para o citoplasma (Wang *et al.*, 2007).

Dentre os diversos alvos celulares da C-FC alguns demonstram o potencial da C-FC de agir sobre células resistentes. Especificamente, a interação da C-FC com β-tubulina, COX-2 e o gene MDR1 demonstram o potencial anti-MDR da C-FC, uma vez que linhagens resistentes como a K562-Lucena possuem maior quantidade desses alvos, podendo assim sofrer ação da C-FC (Silva *et al.*, 2018).

Devido à heterogeneidade de características dos diferentes tipos tumorais, a expressão do fenótipo MDR em um tumor hematopoiético, por exemplo, conta com características inerentes a estes tipos tumorais, ao passo que o fenótipo MDR expresso em um tumor hepático, por exemplo, conta com características inerentes distintas. Nesse contexto, por existirem apenas artigos explorando o fenótipo MDR de tumores hepáticos associados a C-FC (Roy *et al.*, 2007; Roy *et al.*, 2008; Nishanth *et al.*, 2010; Huang *et al.*, 2017), torna-se difícil afirmar o potencial anti-MDR da C-FC para outras linhagens. Assim, a abordagem desta tese visa compreender o papel anti-MDR da C-FC em eritroleucemias humanas (sensível e resistentes com diferentes origens), pelo fato das leucemias serem um dos mais comuns tipos de câncer, contribuindo assim para uma compreensão mais abrangente do papel da C-FC na resistência.

2. Objetivos

2.1. Objetivo geral

Verificar o potencial antineoplásico da C-FC extraída da cianobactéria *Spirulina platensis*, bem como os mecanismos para geração deste efeito, sobre linhagens eritroleucêmicas humanas com (K562-Lucena, FEPS) e sem o fenótipo MDR (K562).

2.2. Objetivos específicos

- Realizar uma revisão de literatura acerca dos temas: C-ficocianina, câncer e fenótipo MDR;
- Avaliar a sensibilidade das linhagens eritroleucêmicas humanas K562, K562-Lucena e FEPS a diferentes concentrações de C-FC;
- Verificar a sensibilidade de macrófagos peritoneais não tumorais a C-FC;
- Determinar a marcação por C-FC nas linhagens eritroleucêmicas através de citometria de fluxo;
- Avaliar a interação da C-FC com as proteínas de extrusão ABCB1 para as linhagens K562-Lucena e FEPS e ABCC1 para linhagem FEPS através de análises de atividade de efluxo (*in vitro*), e análise *in silico* de *docking*;
- Examinar a viabilidade celular das linhagens MDR na co-exposição à C-FC e aos quimioterápicos VCR (K562-Lucena e FEPS) e DNR (FEPS);
- Verificar a interação da C-FC com os quimioterápicos Vincristina (VCR) e Daunorrubicina (DNR) através de análise *in silico* de *docking*;
- Examinar a capacidade de indução de apoptose e/ou necrose da C-FC nas linhagens eritroleucêmicas humanas;
- Avaliar os níveis de espécies reativas de oxigênio nas três linhagens submetidas aos tratamentos;

- Examinar a capacidade da C-FC de alteração da expressão gênica dos transportadores ABCB1 e ABCC1 e dos genes relacionados ao processo inflamatório *PTGS2* e *ALOX5* nas três linhagens.

Artigo I

C- Phycocyanin: Cellular targets, mechanisms of action and multi drug resistance in cancer

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Review article

C-Phycocyanin: Cellular targets, mechanisms of action and multi drug resistance in cancer

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ABSTRACT

C-Phycocyanin (C-PC) has been shown to be promising in cancer treatment; however, although several articles detailing this have been published, its main mechanisms of action and its cellular targets have not yet been defined, nor has a detailed exploration been conducted of its role in the resistance of cancer cells to chemotherapy, rendering clinical use impossible. From our extensive examination of the literature, we have determined as our main hypothesis that C-PC has no one specific target, but rather acts on the membrane, cytoplasm, and nucleus with diverse mechanisms of action. We highlight the cell targets with which C-PC interacts (the *MDR1* gene, cytoskeleton proteins, and COX-2 enzyme) that make it capable of killing cells resistant to chemotherapy. We also propose future analyses of the interaction between C-PC and drug extrusion proteins, such as ABCB1 and ABCC1, using *in silico* and *in vitro* studies.

Contents

Introduction

Cancer is among the leading causes of mortality globally, the agent of 4.3 million premature deaths in 2012. The premature deaths it causes are expected to increase 44% from 2012 to 2030, which demonstrates the need to investigate new substances able to control and/or fight it. The drugs currently available for treatment, such as vinca alkaloids and taxanes, are not satisfactory in their effectiveness, because of the resistance developed by cancer cells, a major factor in the failure of treatment [1–16].

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1734-1140/

Natural products have been the basis of cancer chemotherapy for the last 30 years and cyanobacteria are a rich source of natural products. Among the natural compounds produced by cyanobacteria, the use of C-phycocyanin (C-PC), a phycobiliprotein (PBP), has led to interesting results in the fight against cancer in several studies [1–16].

In spite of these promising results, there remains no C-PC-based chemotherapy in the pharmaceutical market, perhaps because the mechanism of the action of this substance is not yet fully understood. This review will bring together the main cellular targets and mechanisms of action of C-PC proposed by the scientific literature and propose possible targets and mechanisms related to the reversal of the multidrug resistance (MDR) phenotype [10,17].

Cancer and multidrug resistance

Cancer is characterized by the uncontrolled growth and spread of abnormal cells. It can be caused by external factors (tobacco, infectious organisms, inadequate nutrition, chemical agents, and radiation) and internal factors (hereditary mutations). However, cancer is essentially a genetic disease, arising from nonlethal changes in DNA that can occur in specific genes, namely, protoconcogenes and tumor-suppressor genes, which cause uncontrolled cell proliferation when mutated. Mutated protoncogenes become oncogenes and act in the uncontrolled stimulation of cell division, and mutated tumor suppressor genes become unable to block excessive cell growth [18].

Hanahan and Weinberg [19,20] suggested eight essential alterations in cell physiology that collectively dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, the evasion of programmed cell death (apoptosis), limited replicative potential, sustained angiogenesis, tissue invasion and metastasis, reprogramming of energy metabolism to survive with anaerobic metabolism, and evasion of immune destruction, in particular by T and B lymphocytes, macrophages, and natural killer cells. In addition to cancer cells, tumors exhibit another dimension of complexity: they contain a repertoire of recruited, ostensibly normal cells that contribute to the acquisition of hallmark traits by creating the "tumor microenvironment" [20].

A main cause of failures in current chemotherapy treatment is the acquisition of resistance. The MDR phenotype is defined as the resistance of cancer cells to a specific chemotherapeutic drug, accompanied by resistance to other chemotherapeutic drugs that may have different mechanisms of action and structures. Thus, one cancer cell acquires resistance to drugs that are structurally and functionally unassociated, even to drugs to which the patient has not been exposed [21,22]. The mechanisms that make a cell resistant are diverse, complex, and not fully understood. The main factors follow [23,24]:

I) Epigenetic regulation: MDR cancer cells have epigenetic changes, such as DNA methylation (demethylation or low methylation) and histone modification, which can regulate malignant phenotypes [24].
II) Resistance to apoptosis: Several mechanisms of resistance to apoptosis can occur, e.g., epidermal growth factor receptor 1, frequently overexpressed in breast, colon, and ovarian cancers; this suppresses apoptosis and controls cancer cell proliferation and migration [25].
III) Resistance induced by hypoxia: Hypoxia-inducible factor 1 (HIF-1) is over-expressed in gastric vincristine-resistant SGC7901/VCR cells even under normoxic conditions; it increases the levels of HIF-1a and HIF-2a transcription factors, which can upregulate glycolysis (important for generating energy in cancer cells) [26–29].
IV) Resistance induced by autophagy: Autophagy has a cytoprotective effect, degrading drug molecules and helping cancer cells evade apoptosis [30].
V) Transporters of the ABC family (drug extrusion proteins): ABC transporter pumps are part of the ABC transporter family, which is known to have at least 48 members in humans, including the well-known P-glycoprotein (Pgp, encoded by the *ABCB1* gene), MDR-associated protein 1 (MRP1, encoded by the *ABCC1* gene), and ABC subfamily G member 2, also known as breast cancer resistance protein, BCRP, which is encoded by the *ABCG2* gene. These pumps are located on the cytoplasmic side of the resistant cell membrane, resulting in increased drug efflux [22,31].
VI) Cancer stem cells: Cancer stem cells are a subpopulation of cancer cells with self-renewal and differentiation properties; these are the origin of cancer and the basis of malignant cancer phenotypes such as MDR. Marques et al. [32] belonging to our research group demonstrated a relationship between the MDR phenotype and stem cells and found that the expression of the transcription factor Oct-4, a stem cell marker, was three times higher in the MDR line of human erythroleukemia K562-Lucena 1 (vincristine resistant) than in the non-MDR K562 parental cell line. That study also found that the promoter region of the *ABCB1* gene (drug efflux protein) has Oct-4 binding sites, which reinforces the relationship between MDR and stem cells.
VII) DNA repair capacity: Cancer cells are capable of excising lethal DNA lesions, contributing to chemoresistance [33].
VIII) Existence of miRNAs: The miRNAs of MDR cancer cells tend to regulate cell functions by modulating the aberrant functions of their target genes. For example, miR-19a and miR-19b, members of the miR-17–92 cluster, are upregulated in MDR cell lines and modulated MDR in gastric cancer cells [34].

C-Phycocyanin

PBPs are cyanobacteria/microalgae proteins present in the granules that capture light and transfer energy, the phycobilissomas; these are analogous to light-collecting complexes of green plants containing a and b chlorophyll. All PBPs have a similar three-dimensional structure. Each PBP is composed of α and β subunits (polypeptides), occurring generally in equal amounts. Theirmolecular weights differ depending on the organism of origin, ranging from 12,000 to 20,000 Da for the α subunit, and from 15,000 to 22,000 Da for the β subunit.

PBPs exist as oligomers, where the trimer $(\alpha\beta)_3$ is the smallest stable aggregate and the hexamer $(\alpha\beta)_6$ is their basic building block. From one to four open-chain tetrapyrrole chromophores, known as phycocyanobilins, bind covalently to the apoprotein. This occurs through a thioether bond between a cysteine residue and the A ring of tetrapyrrole [35–38]. Each α and β subunit contains eight α helices, six of which are folded into a globin-like structure (the protein part of hemoglobin), covalently binding to phycocyanobilins, in an analogous position to that of porphyrin in globin proteins. The two complementary helices form the domain of association between the two subunits in the formation of the αβ monomer.

PBPs are divided into two major groups according to their colors: phycoerythrins (red) and phycocyanins (blue). There are three types of phycocyanin: C-PC, R-phycocyanin, and allophycocyanin. C-PC, a water-soluble protein, was first reported in 1928 by Lemberg [39] and is the main PBP found in the cyanobacteria/microalgae of the genera *Nostoc*, *Spirulina*, and *Aphanizomenon*,

among others. It can constitute up to 20% of the dry weight of the *Spirulina platensis* cyanobacterium [12,38–42]. The α subunit of C-PC contains a phycocyanobilin covalently attached to α-84 cysteine, while the β-subunit contains two phycocyanobilins linked respectively to the β-84 and β-155 cysteine of the apoprotein.

C-PC is widely used in the food and pharmaceutical industries and as a fluorophore in clinical and immunological analyses. Its high commercial interest is due to its high protein yield and relatively simple extraction procedures. Because its cultivation is independent of organic substrates, it may have an economic advantage over other microorganisms [38,43,44].

Tumor specificity of C-PC

The nonspecificity of numerous chemotherapies induces toxicity in healthy tissues, impairing the quality of life, weakening the immune system, and sometimes generating irreversible damage to the recovery power of the patient. C-PC specificity has been verified to have significant effects on the multiplication of uterine cervix carcinoma cells (HeLa) without such an effect on non-cancer cells (details in Table 1) [3,45]. Photodynamic therapy (PDT) can induce the accumulation of C-PC in tumor tissue; moreover, with the direction of light into tumor tissue, damage to surrounding normal tissues is reduced, increasing the specificity of treatment. In addition, the recombinant β-subunit of C-PC has a smaller effect on proliferation in non-cancer cells (details in Table 1) as well as greater toxicity in metastatic cells. Hence, C-PC could be an agent for the prevention of metastasis [5,6].

Cellular targets of C-phycocyanin and mechanisms of action

Several authors have reported the antiproliferative and cytotoxic capacity of C-PC both *in vitro* (Table 1) and *in vivo* (Table 2). However, unlike other natural substances with antitumoral potential, such as acetylsalicylic acid (which irreversibly inhibits the two isoforms of the enzyme cyclooxygenase, COX-1 and COX-2, avoiding the formation of the prostaglandins that are involved in the initiation and promotion of cancer), C-PC does not have a known mechanism of action [46,47]. Nevertheless, based on existing evidence, we hypothesize that C-PC has more than one specific target; therefore, it has a diversity of effects. Thus, due to its several targets, the mechanisms by which C-PC generates its antitumor effects would be as follows: cell cycle arrest in specific phases, the modification of the cellular redox state through the modulation of enzymatic and non-enzymatic antioxidants and

Table 1
Summary of *in vitro* investigations involving C-PC as antitumoral.

Cancer Type/Cell Line	Results or effects of C-PC	Reference
Rat histiocytic tumor – maintained as ascites in Wistar rats/AK-5	Apoptosis induction by caspase-3 and ROS activation ROS inhibition by Bcl-2 overexpression Cells transfected with Bcl-2 gene resisted to C-PC-induced death	[1]
Human Chronic Myeloid Leukemia/K562	↓ 49% Cell proliferation Apoptosis *Downregulation* of antiapoptotic protein Bcl-2 None changes in pro-apoptotic protein Bax	[2]
Human Adenocarcinoma/HeLa and Chinese hamster ovary/CHO	↓ HeLa proliferation, any changes CHO proliferation (selectivity) Apoptosis ↓ Bcl-2 expression Activation of Caspases-2, −3, −4, −6, −8, −9 and −10 Release of cytochrome c from mitochondria	[4]
Squamous carcinoma of the head and neck/686LN and 686LN-M4C1 Human Chronic Myeloid Leukemia/K562 Colorectal Adenocarcinoma/HT-29 Controls (normal cell): Lymphocytes B and T/RPMI1788 and C5/MJ, respectively	Greatest inhibitory proliferation with 686LN-M4C1cells and minor inhibitory effects on the growth of non-cancer cells Interaction of C-PC/β subunit with β-tubulin and GAPDH Microtubules and actin filaments depolymerization ↑ Apoptosis ↑ Caspase-3 and caspase-8 ↓ Nuclear levels of GAPDH	[5]
Estrogen- responsive breast cancer/MCF-7	↓ Cell proliferation Caspase-9 expression Cytochrome c release *Downregulation* of Bcl-2expression Laser irradiation intensified the effects	[6]
Hepatocellular Cancer/HepG2	↓Cell proliferation Apoptosis and necrosis ↑ ROS Mitochondrial damage Cytochrome c release Caspase-3 activation Cell cycle arrest (G2/M)	[11]
Colorectal Adenocarcinoma/HT-29 Lung adenocarcinoma/A549	Apoptosis Antioxidant and antiproliferative activity DNA fragmentation	[12]
Ovary Adenocarcinoma/SKOV-3	↑ Apoptosis to 1,6% for 19,8% ↑ Caspases-3, −8 and −9 activity ↑ ROS ↓ HSP60 proteins, nucleolin, PSME3, Prdx-4, PPase, ↑ mtSSB protein	[15]

Abbreviations: DNA: Deoxyribonucleic acid; ROS: Oxigen-reactive species; Bcl-2 (B cell lymphoma 2); GAPDH: glyceraldehyde-3-phosphate dehydrogenase; PPase: Cytoplasmic inorganic phosphatase; Prdx-4: Peroxirredoxin-4; PSME3: Subunit of the human proteasome activator complex-3; MDR1: Multi-drug resistance-1 gene; HSP60 Heat shock protein-60; mtSSB: mitochondrial single-stranded binding protein; C-PC: C-phycocyanin.

Table 2
Summary of *in vivo* investigations involving C-PC as antitumoral.

Animal model	Cell line	Results or effects of C-PC	Reference
Tumorigenesis induced in BALB/c mice by MCF-7 injection	MCF-7	↑ weight of immune organs ↑ proliferation of immunocytes ↑ expression of pro-apoptotic Fas protein ↓ tumor weight ↓ expressions of anti-apoptotic proteins and CD44	[6]
TPA-Induced Skin Tumorigenesis	–	TPA induced: *upregulation* of ODC and *downregulation* of TG2 (for mRNA and protein) and C-PC reversed this profile, thus C-PC induced *downregulation* of ODC and *upregulation* of TG2. TPA induced: *upregulation* of COX-2, IL-6 e pSTAT3 (for mRNA and protein) and C-PC reduced this *upregulation* TPA-induced.	[10]
Tumorigenesis induced in adults rats by A549 injection	Lung cancer (A549)	Inhibition of cellular growth *in vitro* and *in vivo* by C-PC or ATRA; Increased of antitumoral effects by combination C-PC and ATRA. C-PC + ATRA: ↓Expression of CDK-4; ↑ Expression of caspase-3; Apoptosis; Immune stimulus (↑ viability of T cells and spleen); *upregulation* of TNF gene; *downregulation* of Bcl-2 and Cyclin genes.	[63]

Abbreviations: TG2: Transglutaminase2; ODC: ornithine decarboxylase; TPA: 12-O-tetradecanoyl-phorbol-13-acetate; pSTAT-3: Transcription Activator-3;CD44: cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration; COX-2: Cyclooxygenase-2; IL-6: Interleukin-6; Bcl-2 (B cell lymphoma 2); TNF: tumor necrosis factor; pSTAT3: Signal transducer and activator of transcription 3; CDK-4: Cyclin-dependent kinase 4; ATRA: all-trans retinoic acid.

COX-2, the induction of apoptosis and necrosis, and the intracellular accumulation and potentialization of the effects of doxorubicin (a traditional anticancer drug).

The recombinant β-subunit of C-PC induces apoptosis in the erythroleukemic (K562), colorectal adenocarcinoma (HT-29), and head and neck carcinoma with basal metastatic capacity (686LN) cell lines. It also presents a high rate of inhibition of proliferation (about 60%) in the head and neck carcinoma cell line with high metastatic capacity (686LN-M4C1) [2,5] (Table 1).

Moreover, C-PC significantly reduces the viability of rat myeloma cells after PDT. This effect is mediated by apoptosis, because C-PC-mediated PDT may preferentially induce the activation of the pro-apoptotic genes Fas and p53 and the downregulation of antiapoptotic proteins, such as Bcl-2, NF-kB, and CD44, in addition to inducing the release of cytochrome c from mitochondria. The Fas protein ultimately activates caspases that facilitate the transduction of apoptotic signals, leading to the apoptosis of MCF-7 cells [6]. HT-29 (colon carcinoma) and A549 (lung epithelial adenocarcinoma) cells submitted to C-PC have a decreased G2/M phase than untreated cells. Furthermore, there is an increase in the percentage of cells in the G0/G1 phases, accompanied by the interruption of the cell cycle. Thus, C-PC acts as a "G1 checkpoint" that controls entry into the S phase and prevents DNA replication [12,48].

It is likely that mechanisms related to the antitumor capacity of C-PC stem from its interactions with the plasma membrane, cytoplasmic enzymes, mitochondria, and DNA. In interacting with the b-tubulin at the cytoplasm level, C-PC may be able to inhibit mitosis by depolymerizing microtubules, preventing the formation of the mitotic spindle. Another target for C-PC is GAPDH, an enzyme with important antineoplastic roles, as the biological functions of this enzyme depend on its subcellular localization [9,49,50], and C-PC is capable of altering this location, recruiting it from the nucleus to the plasma membrane (more details in Wang et al. [5]). Nuclear GAPDH acts as a necessary signal for the entry into S phase, and its decrease may cause cell cycle arrest and subsequently the inhibition of proliferation [5,51,52].

The COX-2 enzyme catalyzes the conversion of arachidonic acid into prostaglandins (PGs) and other eicosanoids. The levels of COX-2 and PGs are elevated in several types of cancer, indicating that these molecules play a role in cancer cell survival and are important targets for therapies. In this context, an important antineoplastic mechanism of C-PC is the ability to inhibit COX-2, which generates a reduction of PG levels activating proapoptotic signaling pathways [17,53,54].

When C-PC targets antioxidants (enzymatic and nonenzymatic) it can alter the redox state of the cell and induce signaling pathways related to antiproliferative effects. An antiproliferative effect is observed when C-PC generates increased production of reactive oxygen species (ROS) and activation of caspases that culminates in the activation of pro-apoptotic pathways in cancer cells [1,15]. C-PC, acting as an antioxidant, aides effects such as the induction of apoptosis and cell cycle arrest [12].

C-Phycocyanin and multidrug resistance

The anti-MDR role of C-PC remains poorly explored, with few studies of it having been conducted (Table 3). Considering the role of the MDR phenotype in chemotherapy failures, extensive research is needed in this field, and our review demonstrates that C-PC is a strong candidate for overcoming chemoresistance. Some previously cited mechanisms of action of C-PC may also be related to its anti-MDR potential. The mechanisms for the chemoresistance of tumor cells are not fully understood, but some studies have shown that the increased expression and/or activity of certain proteins may contribute to this phenotype (Table 4).

Thus, its interaction with tubulin may be key for the ability of C-PC to reverse the MDR phenotype, as previously found by our research group [55]; that study demonstrated that the MDR K562-Lucena 1 cell line has a higher concentration of α-tubulin

Table 3
Investigations involving C-PC in cancer chemoresistance.

Cancer Type/Cell Line	Results or effects of C-PC	Reference
Hepatocellular Cancer/HepG2	*Downregulation* of *MDR1* gene by ROS, COX-2, NF-kB and AP-1 ↑ Accumulation and sensitivity to doxorubicin	[7]
Mouse macrophage cell line/RAW 264.7 with MDR1 expression induced by 2-AAF	Inhibition of 2-AAF-induced expression of *MDR1* by reduction of ROS	[61]
Liver of albino mice	Inhibition of 2-AAF-induced expression of *MDR1* by reduction of ROS	[62]

Abbreviations: ROS: Oxigen-reactive species; COX-2: Cyclooxygenase-2; NF-kB: Nuclear factor kappa B; AP-1: Activating Protein-1; 2-AAF: 2-acetylaminofluorene.

Table 4
Mechanisms of chemoresistance in MDR cancer cells: Targets for C-PC.

Mechanisms	Reference
↑ α-Tubulin monomers	[5,55]
↑ COX −2	[17,53,54,57]
Transporters of the ABC family (drug extrusion proteins)	[22,31]

Abbreviations: COX-2: Cyclooxygenase-2.

monomers and greater capacity for microtubule polymerization than the non-MDR K562 cell line. Thus, C-PC can induce mitotic arrest through the disruption of the dynamics of tubulin in MDR cells [56].

In addition, the ability to reduce COX-2 levels may be important for the generation of effects by C-PC on cells with the MDR phenotype, as shown in a study by our research group, where Carrett–Dias et al. [57] verified the higher gene expression of COX-2 in the MDR K562-Lucena 1 cell line than in the non-MDR K562 cell line. As mentioned previously, the expression of COX-2 is related to the increase in inflammatory mediators such as prostaglandins, so the inhibition of COX-2 may reduce the inflammatory process in the tumor microenvironment, reducing the potential for chemoresistance [17,53,54,58,59].

Further, one of the best-understood features that gives a cell MDR is the overexpression in the plasma membrane of a protein of 170 kDa called P-glycoprotein (P-gp) or ABCB1, encoded by the *MDR1* or *ABCB1* gene [7,60]. C-PC can influence the activity of this pump directly and indirectly. Indirectly, it can regulate the expression of the *MDR1* gene; this can be seen in hepatocellular carcinoma (HepG2), where C-PC activates pathways involving ROS, COX-2, NF-kB, and AP-1 and decreases the expression of the *MDR1* gene, increasing sensitivity to doxorubicin [7,61,62]. It is also possible that C-PC directly influences P-gp activity. We support this hypothesis based on evidence from previous studies. The accumulation of doxorubicin may be due not only to the decrease in MDR1 expression but also to pump inhibition directly on the plasma membrane. Because C-PC is hydrophilic and its accumulation has been found on plasma membranes, this accumulation should occur on portions of the plasma membrane protein such as P-gp or other ABC family transporters. It would be interesting to carry out *in silico* simulations associated with laboratory tests to verify the possibility that C-PC could be a substrate for P-gp or other ABC family transporters [5,42].

Finally, the great potential of C-PC to overcome chemoresistance has been verified, due to its ability to interact with various cellular targets and the fact that some targets are more prominent in MDR cells (Table 4).

Perspectives and conclusion

The studies mentioned in this paper have undeniably shown the chemotherapeutic potential of C-PC. In addition to its inhibition of cell proliferation, which is a basic requirement, it has the important ability to circumvent MDR and the lack of specificity of current anticancer drugs. C-PC demonstrates interesting effects when isolated; however, its interaction with traditional drugs could potentiate the effects of C-PC, mainly by decreasing the extrusion of drugs *via* the ABC transporter family and/or increasing the specificity of standard drugs. However, it is necessary to continue to research the molecular mechanisms of C-PC and to conduct further *in vivo* research for future use in clinical trials.

Conflict of interest

The author(s) confirm that this article content has no conflict of interest.

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Manuscrito I

C-phycocyanin overcome the multidrug resistance phenotype in human erythroleukemias with or without interaction with ABC transporters

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C-phycocyanin overcome the multidrug resistance phenotype in human erythroleukemias with or without interaction with ABC transporters

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Abstract

The phenotype of multidrug resistance (MDR) is one of the main causes for failures in chemotherapy. Our study investigated the effect of C-phycocyanin (C-PC) in three human erythroleukemic cell lines with or without MDR phenotype: K562 (non-MDR and without overexpression drug efflux proteins), K562-Lucena (MDR; with ATP-binding cassette, sub-family B/ ABCB1 overexpression), and FEPS (MDR; with / ABCB1 and ATP-binding cassette, sub-family C/ ABCC1 overexpression). MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay showed that C-PC at 20 and 200 μg/mL decreased K562 cell viability after 24 h and C-PC at 200 μg/mL decreased K562-Lucena cell proliferation after 48 h. For FEPS, C-PC did not decrease cell viability. In other hand, MTT assay showed that exposure of C-PC at 2, 20 and 200 μg/mL for 24 or 48 h was not cytotoxic to peritoneal macrophages. In 72h trypan blue exclusion assay showed that C-PC at 20 μg/mL decreased K562 and K562-Lucena proliferation and for FEPS only C-PC at 200 μg/mL decreased the proliferation. In addition, protein-protein docking showed differences of energy and binding site of ABCB1 and ABCC1 for C-PC. This was confirmed by efflux protein activity assay. Only ABCC1 activity was altered in C-PC presence and FEPS showed lower C-PC accumulation, suggesting C-PC remotion by ABCC1, conferring C-PC resistance. In combination with chemotherapeutic (vincristine/VCR and daunorubicina/DNR), K562-Lucena sensitivity for C-PC plus VCR did not increase, whereas FEPS sensitivity for C-PC plus DNR increased. In molecular docking, estimated free energies of binding (FEB) for C-PC associated with chemotherapeutic were similar (VCR: -6.9 kcal/mol and DNR: -7.2 Kcal/mol) and these drugs were located within C-PC cavity. However, C-PC has specificity for tumor cells and K562 were more sensitive than K562-Lucena, followed by FEPS. Thus, C-PC is a possible chemotherapeutic agent for MDR cells, both isolated in cells such as K562-Lucena (ABCB1 resistance), or in combination with other drugs for cells similar to FEPS (ABCC1 resistance), without damages for health cells (peritoneal macrophages of *Mus musculus*).

Keywords: Antiproliferative; Docking; ABCB1; ABCC1.

1. Introduction

The number of new cases of cancer increased largely because growth and aging in world population [1]. In 2030, it is expected about 5.76 million deaths by cancer around the world [2,3]. Cancer treatment usually uses chemotherapeutic agents for reducing mortality/morbidity and increasing patient life quality [4]. However, several chemotherapeutic agents have low therapeutic index, generating serious problems, such as multidrug resistance (MDR) phenotype [5]. MDR phenotype is the most significant reason of cancer chemotherapeutic failures and it plays a central role in cancer metastasis and recovery [6].

MDR is a well known phenomenon that results in cancer cells resistance to one chemotherapeutic drug accompanied by resistance to other chemotherapeutic drugs that may have different structures and action mechanisms [7]. The mechanisms that cancer cells use and/or develop to evade chemotherapy treatment are complex and remain not fully understood. However, one of the most prominent mechanisms is overexpression of ATP-binding cassette (ABC) transporters [8]. Resistant cancer cells have high expression of several ABC transporter pumps, resulting in drug efflux increase [9]. ABC transporters including well known P-glycoprotein (P-gp, encoded by the *ABCB1* gene), MDR-associated protein 1 (MRP1, encoded by the *ABCC1* gene), and ABC subfamily G member 2, also known as breast cancer resistance protein, BCRP, which is encoded by *ABCG2* gene [10].

Although drug efflux proteins overexpression is often associated with MDR phenotype, it is composed by several other mechanisms. Thus, substances with different action mechanisms would be an alternative to overcome MDR phenotype. In this context, C-phycocyanin (C-PC) seems promising, with several cellular targets and action mechanisms [11].

C-PC is a water-soluble protein, first reported by Lemberg in 1928 [12] is the main phycobiliprotein found in cyanobacteria/microalgae of genus *Nostoc*, *Spirulina*, *Aphanizomenon*, and more. According to Vonshak (2002) [13] the protein fraction of *Spirulina platensis* cyanobacterium may contain up to 20% of C-PC [12,14–17]. C-PC is widely used in food and pharmaceutical industries and it has other applications, such as fluorophore in clinical and immunological analyzes [18–20]. It has been reported anti-cancer properties of C-PC by cytotoxicity induction and inhibition of cell proliferation [17,21–25].

Studies that explore the potential of C-PC to inhibit cancer-resistant cells (with MDR phenotype) growth remain poorly explored. However, C-PC can increase intracellular

accumulation of doxorubicin in HepG2 cell line (resistant to chemotherapy) and increase anti-cancer effects of doxorubicin, showing ability of C-PC to act on cells with MDR phenotype [25]. Here, to understand effects of C-PC on MDR phenotype, three human erythroleukemia cell lines were used: one non-MDR: K562 that was isolated and culturing firstly by Lozzio and Lozzio (1975) [26] and two MDR cell lines: K562-Lucena and FEPS.

K562-Lucena cell line was established from the K562 (parental cell line) by Rumjanek et al. [27,28], using vincristine, an anti-cancer drug with cytoskeletal components as target [29,30] according to Tsuruo et al. [31]. K562-Lucena cells possess characteristics like the resistance to non-related drugs [32,33] and the P-gp overexpression [34].

FEPS cell line was selected from K562 with daunorubicin, an anti-cancer drug that inhibits DNA synthesis [35,36], and it has different characteristics from K562-Lucena, such as lower expression of cellular death receptor CD95, overexpression of P-glycoprotein (P-gp), and multidrug resistance related protein 1 (MRP1) [37]. The aim of this study is to compare anti-cancer properties of C-PC in one non-MDR (K562) and two MDR (K562-Lucena and FEPS) cell lines, as well as to verify ability of C-PC to increase action of traditional chemotherapeutic agents (vincristine and daunorubicin).

2. Materials and methods

2.1. Human erythroleukemic cells culture

K562, K562-Lucena, and FEPS cell lines were obtained from Laboratório de Imunologia Tumoral of Instituto de Bioquímica Médica Leopoldo de Meis (Universidade Federal do Rio de Janeiro, Brazil). K562 parental cells were grown in RPMI 1640 medium (Sigma), supplemented with sodium bicarbonate (0.2 g/l) (Synth), L-glutamine (0.3 g/l) (Vetec), with 10 % fetal bovine serum (FBS) (Gibco) and 1% antibiotic and antimycotic (penicillin [100 U/mL], streptomycin [100 μg/mL], and amphotericin B [0.25 μg/mL]) (Gibco), in disposable plastic flasks at 37 °C and 5% of CO_2. K562-Lucena and FEPS cells were grown under the same conditions above, but with concentrations of chemotherapeutic agents: 60 nM vincristine (VCR) [27,28] or 532 nM daunorubicin (DNR) [37], respectively, in order to preserving MDR phenotype. In the experiments VCR or DNR was not added to K562-Lucena and FEPS cells only in the assays of combination chemotherapeutic plus C-PC.

2.2. C-phycocyanin extraction and purification

Spirulina platensis LEB 52 was cultivated [38] and supplied by Laboratório de Engenharia Bioquímica (Universidade Federal do Rio Grande – FURG, Brazil).

C-phycocyanin extraction process occurred according to Moraes et al. [39]. Briefly, crude broth containing cells was centrifuged (1890Xg, 20 min) and pH was adjusted at 6.5. Purification process was by ultrafiltration performed in dead-end ultrafiltration cell with 50 kDa polyethersulfone membrane (UH050 P, Nadir, Germany) at 25 ºC and 1.0 kgf/cm² in diafiltration/ultrafiltration mode [40]. Purity of C-PC extract was calculated according to Abalde et al., [41], using absorbance ratio OD_{620}/OD_{280}. Treatments were constructed using purified extract with purity degree of 1.00, considered as food grade [42]. C-PC concentration (mg/mL) was calculated according to Bennet and Bogorad [43] by following equation, with changes in the wavelength:

$$\text{C-PC} = \frac{(OD620 - 4.74 \times (OD652))}{5.34}$$

2.3. MTT assay with human erythroleukemic cells

Cells were centrifuged (197Xg; 2 minutes), suspended (2.10^4 cells/mL) in RPMI 1640 medium (Sigma), plated in 96-well culture plates, and treated with different concentrations of C-PC (2.0, 20.0 and 200.0 µg/mL of C-PC). In addition, a control group (without C-PC), receiving the same volume of sterile water was used. Cells were incubated at 37 °C.

MTT assay was performed after 24 and 48h of C-PC exposure. Briefly, after incubation with C-PC the cells were centrifuged (524xg, 5 minutes) for removal of the culture medium and washed with phosphate buffer saline (PBS). Subsequently, the cells were centrifuged again (524xg, 5 minutes), the PBS was removed and added 200µl of RPMI 1640 medium and 20 µl of MTT (5 mg/mL) in each well. Cells were incubated for 3 h at 37 ºC. Supernatant was removed after centrifugation (524xg, 5 minutes) and formazan crystals were dissolved in 200 µl of dimethylsulfoxide (DMSO, Sigma), shaking gentle. Absorbance values at 490 nm were determined on multiwell plate reader (ELX 800 Universal Microplate Reader, Bio-TEK).

2.4. MTT assay with peritoneal macrophages

Animals: Five female Swiss albino mice (6-8 weeks) were obtained from Thomas George animal house of Instituto de Pesquisa em Fármacos e Medicamentos (Universidade Federal da Paraíba). Animals were kept under standard laboratory conditions on a constant 12

h light/dark cycle with controlled temperature (21 ± 1 ºC). Food and water were given *ad libitum*. After manipulation, euthanasia was employed by cervical dislocation. All procedures adopted in this study were approved by Institutional Ethics Committee of Biotechnology Center/UFPB (protocol number: 111/2016).

Peritoneal macrophage elicitation: Peritoneal inflammation was induced by injection of 4 mL of 4% thioglycollate (Sigma Aldrich). Four days after the i.p. thioglycolate injection, animals were euthanized by cervical dislocation and the peritoneal cavity was washed with 8 mL of PBS (phosphate buffer saline), supplemented with 3% fetal bovine serum (FBS) (Gibco). Cell suspension obtained from peritoneal lavage was centrifuged (390Xg; 5 minutes; 4 ºC). After that, supernatant was discarded and the pellet was resuspended in 1 mL of complete RPMI medium. Viable cells were counted with a Neubauer chamber using Trypan blue solution.

Macrophages culture: Macrophages were enriched by adherence to plastic. For that, viable peritoneal cells were seeded in 96-well plates at a concentration of 4×10^5 cells/well and incubated for 24 h with supplemented fetal bovine serum (FBS) culture medium in an atmosphere of 5% CO_2 at 37 ºC. Then, nonadherent cells were removed by aspiration. Remaining cells were further incubated for 24 h with complete RPMI medium in the presence and absence of different C-PC concentrations: 0 (control group); 2.0; 20.0; and 200.0 μg/mL). After 24 and 48 hours of culture, viability was analyzed.

MTT assay: Supernatant was discarded and was added 90 μl of complete RPMI culture medium and 10 μl of 5 mg/mL MTT. Cells were incubated for 4 h at 37 ºC. Supernatant was removed and formazan crystals were diluted with DMSO. Absorbance values at 570 nm were determined on multiwell plate reader (EL 800 Universal Microplate Reader, Bio-TEK) [44].

2.5. Sensitivity of tumoral cells to C-PC by Trypan blue exclusion assay

Cells were centrifuged (197Xg; 2 minutes) and suspended in RPMI 1640 medium (2.10^4 cells/mL). Next, cells were treated with different concentrations of C-PC (2.0, 20.0, 50.0, 100.0, and 200.0 μg/mL). In addition, a control group (without C-PC), receiving the same volume of sterile water, was used. Cells were incubated at 37 °C and cell viability was performed immediately (0 h), 24, 48, 72, and 96 h after treatment with C-PC by Trypan blue exclusion assay. Trypan blue gets into the non-viable cells and provided two variables:

number of viable cells and cell viability (given by the ratio of the number of viable cells to total cells).

2.6. Protein-protein docking

Protein-protein docking was performed with PRISM web server, (available on http://cosbi.ku.edu.tr/prism/) considering C-PC and efflux proteins (ABCB1 or ABCC1). Proteins structures were obtained from Protein Data Bank (PDB) [45], C-PC from *Spirulina platensis*, PDB ID: 1GH0; ABCB1 from *Mus musculus*, PDB ID: 4Q9I; ABCC1 from *Bos taurus*, PDB ID: 5UJ9. Since there are no human efflux proteins available on PDB, we performed a protein sequence alignment, using *Blastp* tool (available on https://www.ncbi.nlm.nih.gov/) considering as input the protein sequence accession number: ABCB1 from *Homo sapiens*, NP_001335874.1; ABCB1 from *Mus musculus,* NP_035206.2; ABCC1 from *Homo sapiens*, NP_004987.2; ABCC1 from *Bos taurus*, NP_776648.1, where we obtained results with high identity of alignment: ABCB1 from *Mus musculus* with ABCB1 *from Homo sapiens* 87% of identify and ABCC1 from *Bos taurus* with ABCC1 *from Homo sapiens* 91% of identity.

Protein-protein docking simulations were performed with PRISM, a web server for prediction of protein-protein interactions by structural matching. The algorithm of PRISM provide a large-scale prediction that combines structural similarity and accounts for evolutionary conservation in template interfaces. PRISM rationale follows our observation that globally different protein structures can interact via similar architectural motifs. Given two protein structures, PRISM will provide a structural model of their complex if a matching template interface is available. Then the user can obtain this complex structure and visualize the residues that are interacting between the two proteins. The method consists of two components: (1) rigid-body structural comparisons of target proteins to known template protein-protein interfaces and (2) flexible refinement using a docking energy function. Energy function is based on Fiberdock energy scoring and how much more negative is better, showing greater stability of protein-protein complex [46,47].

First was performed a protein-protein docking with C-PC and ABCB1 and only chain F of C-PC interacted with ABCB1. Then, was performed a new protein-protein docking only with chain F of C-PC, which demonstrated better interaction (more negative score). Subsequently, was performed a protein-protein docking with C-PC and ABCC1 and PRISM

was not able to generate prediction of complex. Thus, we performed a new protein-protein docking between C-PC chain F and ABCC1.

2.7. Protein-ligand docking

C-PC structure was obtained from PDB (PDB ID: 1GH0) and vincristine and daunorubicin structure (ligands) were obtained from ZINC database (Zinc IDs: 85432549 and 3917708, respectively) [48,49]. Before performing molecular docking simulation, it was necessary to prepare receptor and ligand input files using AutoDockTools 4 software for AutoDockVina [50]. For preparing receptor input PDB file, we remove hetero atoms, ligands and cristalographic water molecules. Besides, polar hydrogen atoms were added to protein receptor and partial atomic charges were calculated. Processed receptor structure file was saved in PDBQT file format, which contains a protein structure with hydrogen's in all polar residues. For preparing ligand input file, we added polar and non polar hydrogen atoms and calculate Gasteiger charges for entire ligand, saving ligand also in PDBQT format. To perform docking simulations, we configure grid box as: size x = 52Å; size y = 42Å; and size z = 54Å; and center box coordinates are x = 76.071Å center; y = 14.983Å center; z = 169.621Å; considering exhaustiveness as 128.

Molecular docking simulations were performed with Autodock Vina [50]. Protein was kept as rigid and ligand molecules were kept flexible throughout docking process. Then, Free Energy of Binding (FEB) of docked ligand-receptor was estimated in Kcal/mol. The more negative FEB indicates the greater stability of ligand-receptor complex. Visual analysis of docking results was performed with VMD. Interaction analysis between protein-ligand were performed by LigPlot+ [51].

2.8. Activity of efflux proteins in presence of C-PC

Analysis of the interaction between efflux proteins and C-PC was investigated with fluorescent probe Rhodamine 123 (Rho 123) for ABCB1 and 5(6)-Carboxyfluorescein diacetate (CF-DA) for ABCC1 in an efflux assay. Cells were centrifuged (197Xg; 2 minutes),

suspended in RPMI 1640 medium (2.10^5 cells/mL), plated in 24-well plate, and treated according to following experimental groups: control, cells and 100 μg/mL C-PC. For ABCB1 inhibition were used 10 μM of verapamil (VP) with 48h of exposure, and for ABCC1 inhibition were used 300 μM of indomethacin (IM) with immediate exposure (during protocol execution), After 48h at 37°C, efflux assay was executed. The K562-Lucena cells were incubated with Rho 123 (300 ng/mL) for 1 h at 37ºC in presence or absence of inhibitor (VP). The FEPS cells were incubated for 30 minutes with Rho 123 or CF-DA (230 μg/mL) at 37ºC in presence or absence of inhibitor (VP or IM). After incubation, cells were washed with PBS and re-incubated in presence or absence of inhibitors (VP or IM) for 1 h (K562-Lucena) or 30 minutes (FEPS), in RPMI 1640 medium without fluorescent probes, to analyze its extrusion. After washed with PBS, Rho 123 or CFDA accumulation was assessed by fluorimeter (FilterMax F5 Multi-Mode Microplate Reader, Molecular Devices) with excitation and emission wavelength (485/590 nm). The data are presented as the difference between the fluorescence of the cells treated with fluorescent probes and the autofluorescence of each treatment.

2.9. C-PC cell label by flow cytometry

K562, K562-Lucena, and FEPS cell lines were suspended in RPMI 1640 medium (2.10^4 cells/mL) and incubated with 200.0 μg/mL of C-PC for one hour at 37°C (the highest concentration was chosen to assure the labeling, since only unlabeled C-PC fluorescence was measured without use of antibody). Untreated cells were used as a control group. 10,000 events were assessed, discriminating cellular populations in FSC vs. SSC density plot graphic. C-PC NIR fluorescence was analyzed using a FL4-H histogram. Measurements were performed using Accuri C6 flow cytometer and analyses were performed using BD C6 Accuri Software.

2.10. Sensitivity of K562-Lucena and FEPS to C-PC plus chemotherapeutic drugs

This analyze occurred by Trypan blue exclusion assay immediately (0h), 24, 48, 72 and 96 h after incubation with 100 μg/mL C-PC isolated or in combination with 60 nM of

VCR (for K562-Lucena and FEPS) or 532 nM DNR (for FEPS). Cells were centrifuged and suspended in RPMI 1640 medium (2.10^4 cells/mL). After treatment, cells were plated in 96-well culture plates and incubated at 37 °C.

2.11. Statistical analyzes

All experiments occurred at least three replicates of the samples. Normality of the data was verified by the Shapiro-Wilk test. Parametric data were compared by analysis of variance ANOVA followed by Tukey post-test. Non-parametric data were compared by the Kruskal-Wallis test. The data were expressed as mean and standard error of the mean, being $p < 0.05$ utilized.

3. Results

3.1. Human erythroleukemic cells and peritoneal macrophages viability by MTT assay

By MTT analysis for K562 cells, it was verified that C-PC at 20 or 200 µg/mL caused decrease in viable cells compared to control after 24 h (P-value, 24h =0.0025; 48h =0.0000). For K562-Lucena cells, effects are perceived only with C-PC at 200 µg/mL after 48h (P-value, 24h=0.3478; 48h=0.0096). For FEPS cells, no effect was observed (P-value, 24h=0.7408; 48=0.1335). For peritoneal macrophages, no cytotoxic effect was also observed, demonstrating safety for healthy cells (P-value, 24h=0.5828; 48= 0.1268) (Figure 1).

3.2. Human erythroleukemic cells viability by Trypan blue exclusion assay

Concentration-response curves demonstrated ability of C-PC to inhibit cell proliferation (cytostatic role) (P-value for K562: 0h=0.3499; 24h=0.2363; 48h=0.0021; 72h=0.0000; 96h=0.0000/ P-value for K562-Lucena: 0h=0.2875; 24h=0.1002; 48h=0.0002; 72h=0.0000; 96h=0.0000/ P-value for FEPS: 0h=0.0047; 24h=0.4949; 48h=0.0818; 72h=0.0004; 96h=0.0000). The C-PC was not cytotoxic, because there was no reduction cell viability results (P-value for K562: 0h=0.3967; 24h=0.5044; 48h=0.0578; 72h=0.1757; 96h=0.0823/ P-value for K562-Lucena: 0h=0.5873; 24h=0.8469; 48h=0.9850; 72h=0.1147; 96h=0.0842/ P-value for FEPS: 0h=0.7710; 24h=0.6747; 48h=0.7453; 72h=0.0888; 96h=0.0306) (Fig. 2B, D and F). For K562 and K562-Lucena cells, this inhibition is observed

from 20 μg/mL C-PC exposure for 72h. For FEPS, in this same experimental time only 200 μg/mL C-PC is capable of inhibiting cell proliferation (Fig. 2 A, C and E).

3.3. Protein-protein docking

Binding energy attributed to C-PC and ABCB1 complex was -10.15, while binding energy to C-PC chain F and ABCB1 was -87.1. Twenty amino acid residues participated in the interaction between full structure of C-PC and ABCB1 with residues detailed in supplementary data (Figure 3 and Sup. 1). Thirty-three amino acid residues participated in the interaction between the C-PC chain F and ABCB1 (Figure 4 and Sup. 2). PRISM server was not able to predict interactions between C-PC and ABCC1, it only generated complex for C-PC chain F and ABCC1 (Figure 5). Binding energy attributed to C-PC chain F complexed with ABCC1 was -55.33. Twenty-five amino acids residues participated in the binding between the F-chain of C-FC and ABCC1 with residues detailed in supplementary data (Figure 5 and Sup. 3). When comparing binding energies between C-PC chain F and ABCB1 or ABCC1, binding energy is better for ABCB1 (-87.1) than for ABCC1 (-55.33). Chain F binds with 33 amino acids to ABCB1 and to 25 amino acids in ABCC1, resulting in a different binding conformation to ABCB1 and e ABCC1 (Figure 4 A and B; Figure 5 A and B).

3.4. Activity of efflux proteins in presence of C-PC

ABCB1 activity, assessed by Rho 123 extrusion, was not altered by C-PC for both K562-Lucena and FEPS cells (Figure 6A and B) (P-value for K562-Lucena=0.0075; P-value for FEPS= 0.0068). However, ABCC1 activity was modified because fluorescence in FEPS cells incubated with C-PC was higher than control cells or cells treated with indomethacin (ABCC1 inhibitor) (Figure 6C) (P-value= 0.0003).

3.5. C-phycocyanin presence

K562 and K562-Lucena cells labeled with C-PC presented similar fluorescence index (mean ± S.E.M. of fluorescence index: 10091.44 ± 584.30; 9201.86 ± 1421.70, respectively), while FEPS cell presented lower fluorescence index than K562 and K562-Lucena (mean ± S.E.M. of fluorescence index 1139.02 ± 599.19) (p=0.001); however with higher fluorescence than unlabeled cells (Figure 7).

3.6. Protein-ligand docking and erythroleukemic cells viability for C-PC plus chemotherapeutic drug

Molecular docking estimated free energy of binding (FEB) for C-PC and chemotherapeutics were similar (VCR: -6.9 kcal/mol and DNR: -7.2 Kcal/mol) (Tab. 1) and the final pose predicted for both drugs were located within a same "binding site" cavity of C-PC (Fig. 8). Interaction between both drugs and C-PC residues was through van der Waals forces. VCR interacted with eleven amino acid residues of C-PC: Gln; Ala (two residues); Ile; Asp (two residues); Asn; Glu; Phe; Thr; Gly. DNR interacted with nine amino acid residues of C-PC: Ala; Asp; Asn; Glu; Arg; Thr; Leu; Val and Ser. Amino acids of C-PC interacting with VCR and DNR (shared) were: Ala; Asp; Asn; Glu (Tab. 5 and Fig. 8). Sensitivity analysis demonstrates that both C-PC and traditional drugs were predominantly cytostatic, decreasing viability only for C-PC + DNR for FEPS in 96h (P-value for viability of K562-Lucena with C-PC + VCR 0h=0.6356; 24h=0.4235;48h=0.1383;72h=0.3405;96h=0.1912/ P-value for viability of FEPS with C-PC + VCR 0h=0.3385; 24h=0.4235;48h=0.5247;72h=0.4235;96h= nonexistent / P-value for viability of FEPS with C-PC + DNR 0h=0.9976; 24h=0.9994;48h=0.4616;72h=0.1738;96h=0.0004). Sensitivity to combination of C-PC and chemotherapeutic was different between erythroleukemic cells. For K562-Lucena cells (Fig. 9 A, B), combination was more potent only in relation to VCR, not differing from isolated C-PC. For FEPS cells, combination was able to potentiate cell sensitivity both in relation to DNR and in relation to C-PC (Fig. 9 C, D) (P-value for number of viable cells of K562-Lucena with C-PC+VCR: 0h=0.7723; 24h=0.2735; 48h=0.0672; 72h=0.0000; 96h=0.0000/ P-value for number of viable cells of FEPS with C-PC+VCR: 0h=0.2687; 24h=0.2368; 48h=0.6640; 72h=0.0843; 96h=0.0017/ P-value for number of viable cells of FEPS with C-PC+DNR: 0h=0.2393; 24h=0.8535; 48h=0.1794; 72h=0.0000; 96h=0.000). Therefore, there were no differences between C-PC + VCR and C-PC only. To test this hypothesis, C-PC + VCR combination exposure on FEPS was performed and the same result for K562-Lucena was verified (Fig. 9 E, F).

4. Discussion

Our work investigated antitumoral and anti-MDR effect of C-PC in one non-MDR (K562) and two MDR (K562-Lucena and FEPS) cell lines. K562-Lucena cells became resistant through a drug acting on cytoskeleton [29,30] and FEPS cells become resistant through a drug acting on DNA [35,36].

It is increasingly necessary to understand potential of substances that act against MDR phenotype because challenge that this phenotype represents for chemotherapy [6]. To our knowledge there were only articles exploring role of C-PC on MDR phenotype with hepatocytes [25]. Thus, study with different cell lines is necessary.

Cell viability by MTT assay for three erythroleukemic cell lines demonstrated a decrease in sensitivity to C-PC in function of MDR phenotype. The K562 cell line was the most sensitive (demonstrating decrease in viable cells compared to control from 24h for the intermediate concentration), the K562-Lucena cell line was moderately sensitive (decrease in viable cells compared to control only in 48 hours at the highest concentration) and the FEPS cell line the most resistant (without decreasing viable cells compared to control).

After checking the anti-MDR characteristic, we decided to confirm tumor specificity of C-PC. We evaluated cell viability by MTT using mouse peritoneal macrophages, which did not demonstrate citoxicity to C-PC, confirming that C-PC can be antitumoral. This antitumoral character is in agreement with literature, demonstrating specificity of C-PC not only for tumor cells, but also for different tumor cell types, since C-PC can act more effectively in cells with metastatic capacity [11,23,24,52,53].

For a more detailed analysis (with more concentrations and experimental times) of the role of C-PC on erythroleukemic cells, we constructed viability concentration-response curves by Trypan blue exclusion assay. C-PC was predominantly cytostatic (inhibited proliferation), being little cytotoxic for three cell lines. The loss effect of C-PC as a function of the MDR phenotype (mainly for FEPS) verified in the MTT assay was not verified in the concentration-response curves. Thus, K562 and K562-Lucena similarly underwent the action of C-PC, being verified inhibition of proliferation for both cell lines from 20 μg/mL at 72h. For FEPS, only 200 μg/mL (the highest concentration tested) C-PC is capable of inhibiting proliferation in this same experimental time.

It is known that most striking difference between K562, K562-Lucena, and FEPS is presence of efflux proteins: K562 does not overexpress efflux proteins; K562-Lucena

overexpresses only ABCB1; and FEPS overexpresses ABCB1 and ABCC1 [27,28,37]. Considering that FEPS was least sensitive cell line, it would be possible that efflux proteins would be main factor involved in lower sensitivity. To verify possible relationship between C-PC and efflux proteins, we first performed molecular docking (protein-protein).

Molecular docking performed between C-PC and ABCB1 demonstrated that only "F" chain of C-PC participated in binding, with interaction only of "F" chain with ABCB1 being about 8 times greater than complete C-PC. In addition, PRISM was not able to predict the interaction of C-PC in its complete form with ABCC1, only generated a complex between "F" chain of C-PC and ABCC1. Together, these results demonstrate that "F" chain of C-PC can be an important site of interaction with efflux proteins. Binding energy attributed to "F-chain of C-PC-efflux protein" complex was little different for the two efflux proteins. However, location of "F" chain of C-PC in efflux protein was quite different, perhaps because the linkage between the F-chain of C-PC occurred with 33 amino acids for ABCB1 and 25 amino acids for ABCC1 resulting in a different binding conformation. Through these results: slight difference between binding energies and different binding conformation for ABCB1 and ABCC1, we hypothesized that biologically there was interaction between C-PC and efflux proteins with different standards (i.e. that final biological response could be different).

To complement hypothesis generated by protein-protein docking, we performed fluorescent probes extrusion assay (substrates for the efflux proteins) to verify if C-PC was able to modulate activity of efflux proteins. It was found that C-PC did not alter ABCB1 activity (for both K562-Lucena and FEPS). It only altered ABCC1 activity. Thus, differential interaction pattern of molecular docking (slightly different binding energy and rather different binding site between C-PC F chain and ABCB1 or ABCC1) generates different biological responses, since C-PC can interact with ABCB1, but not to point of modifying its activity, whereas interaction of C-PC with ABCC1 is capable of modifying activity of this pump.

When we verified that C-PC altered activity of only ABCC1, our hypothesis was that alteration of activity occurred for extrusion of C-PC, since only FEPS has ABCC1 and this was less sensitive cell line. In order to verify labeling of cells with C-PC, we performed an analysis using cytometer. It is suggested that C-PC was expelled from cell via ABCC1 by cytometry results, since FEPS (single cell with ABCC1) was least accumulated C-PC.

There are some theories about the functioning of efflux proteins, however in both there is the involvement of a channel in the protein [54]. Considering that the dimensions of the

total structure of the C-PC are slightly larger than the channel of the ABCC1 and the fact that the F chain is the only one capable of interacting with ABCC1 through the docking: It is possible that the C-PC is not in the native form when it is expelled from the cell. Besides, only ABCC1 has the ability to carry conjugates and when considering the hydrophobic character of the C-PC it is possible that ABCC1 conveys reduced glutathione-conjugated C-PC chains to increase its hydrophilicity [55].

As C-PC interacts with several chemical structures (that could include chemotherapy agents) [11] but C-PC cannot be expelled by ABCB1 (result of our protein activity assay) we come to believe that when the C-PC interacting with a chemotherapeutic it could not being expelled by ABCB1. Thus, C-PC could be a candidate for a combination therapy with traditional chemotherapeutics, which have become resistant by extrusion via ABCB1. Molecular docking between C-PC and VCR or DNR demonstrated that the two drugs have the same potential to be sequestered. To verify if combination of C-PC and chemotherapeutic drugs (which could possibly be sequestered) would be able to potentiate effects in relation to molecules isolated, we performed a cell viability assay. An interesting result was found, since combination of C-PC and VCR was not able to potentiate effects on C-PC isolated for both K562-Lucena and FEPS. However, for combination of C-PC and DNR (for FEPS), there was an increase in effects for combination in relation to C-PC or chemotherapy alone.

The results were surprising, since K562-Lucena, which was more sensitive that FEPS, was not affected by combination of C-PC and VCR. This may have occurred because C-PC isolated could generate effects on K562-Lucena, so despite tendency for combination, C-PC and VCR did not potentiate effect to point of generating statistical difference. For FEPS it is likely that C-PC and DNR complex will cause a conformational change in C-PC, impeding efficient interaction with ABCC1 and avoiding expulsion of complex. Another hypothesis for potentiated effect of C-PC and DNR combination may be a competitive inhibition, that is, ABCC1 is concerned with expelling C-PC and no longer expels DNR or vice versa.

5. Conclusion

Our data demonstrate that C-PC is a promising substance for chemotherapy of resistant cells, be isolated in cells which resistance is mainly due to ABCB1 transporter, or in combination with other drugs for cells which resistance is due in part to ABCC1. Besides, the C-PC didn’t present cytotoxicity for non tumoral cells in this study.

6. Conflict of interest

The author(s) confirm that this article content has no conflict of interest.

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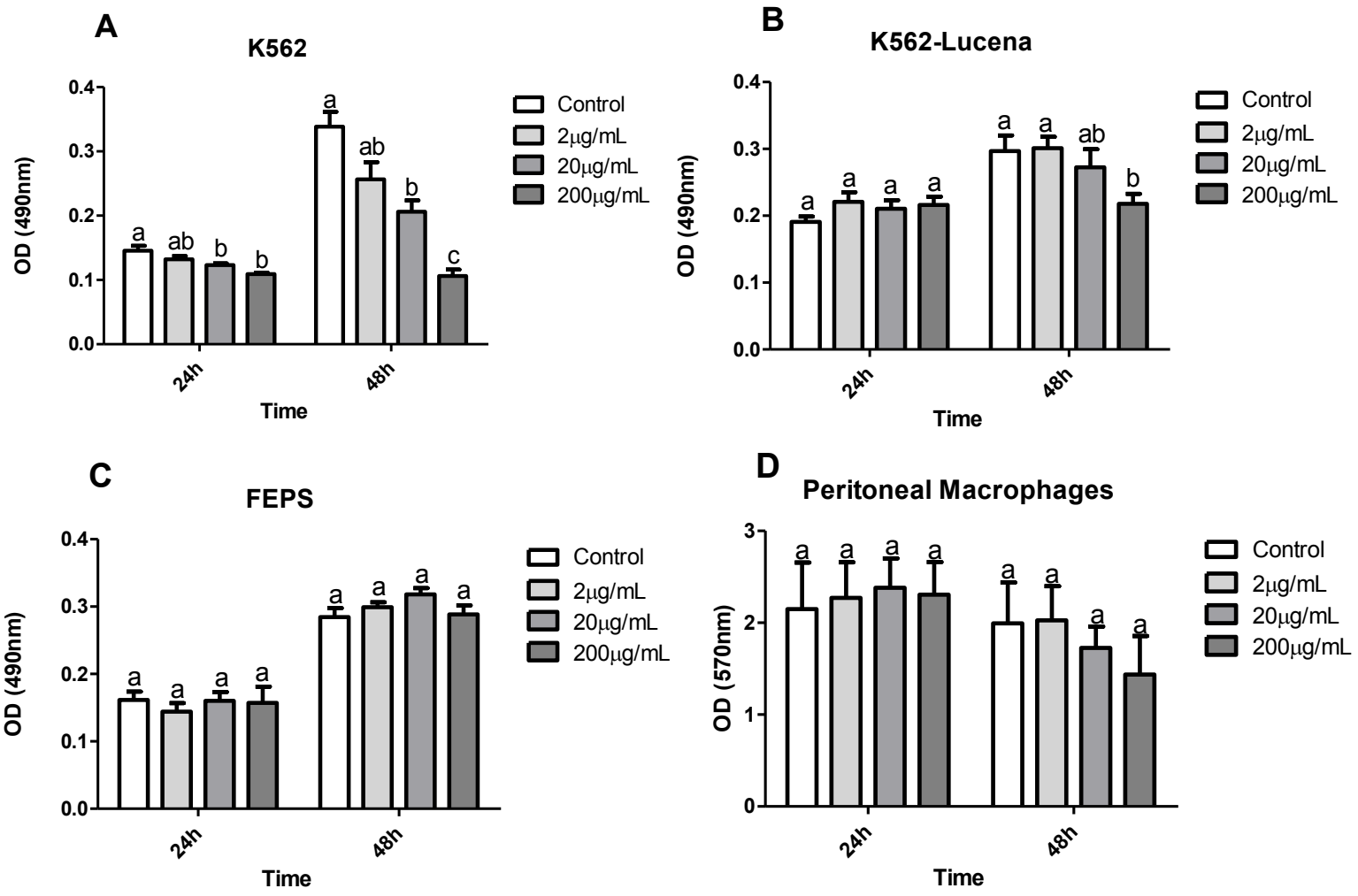

Figure 1 – Sensitivity of cells to C-PC by MTT assay: Optical density (OD) of K562 (A), K562-Lucena (B), FEPS (C) and *Mus musculus* peritoneal macrophages (D) viable cells by the MTT assay, 24 and 48h after exposure to different concentrations of C-PC. Results are expressed as means , ±S.E.M, different letters indicate statistical difference by the ANOVA test followed by *Tukey* in each time separately ($p<0.05$).

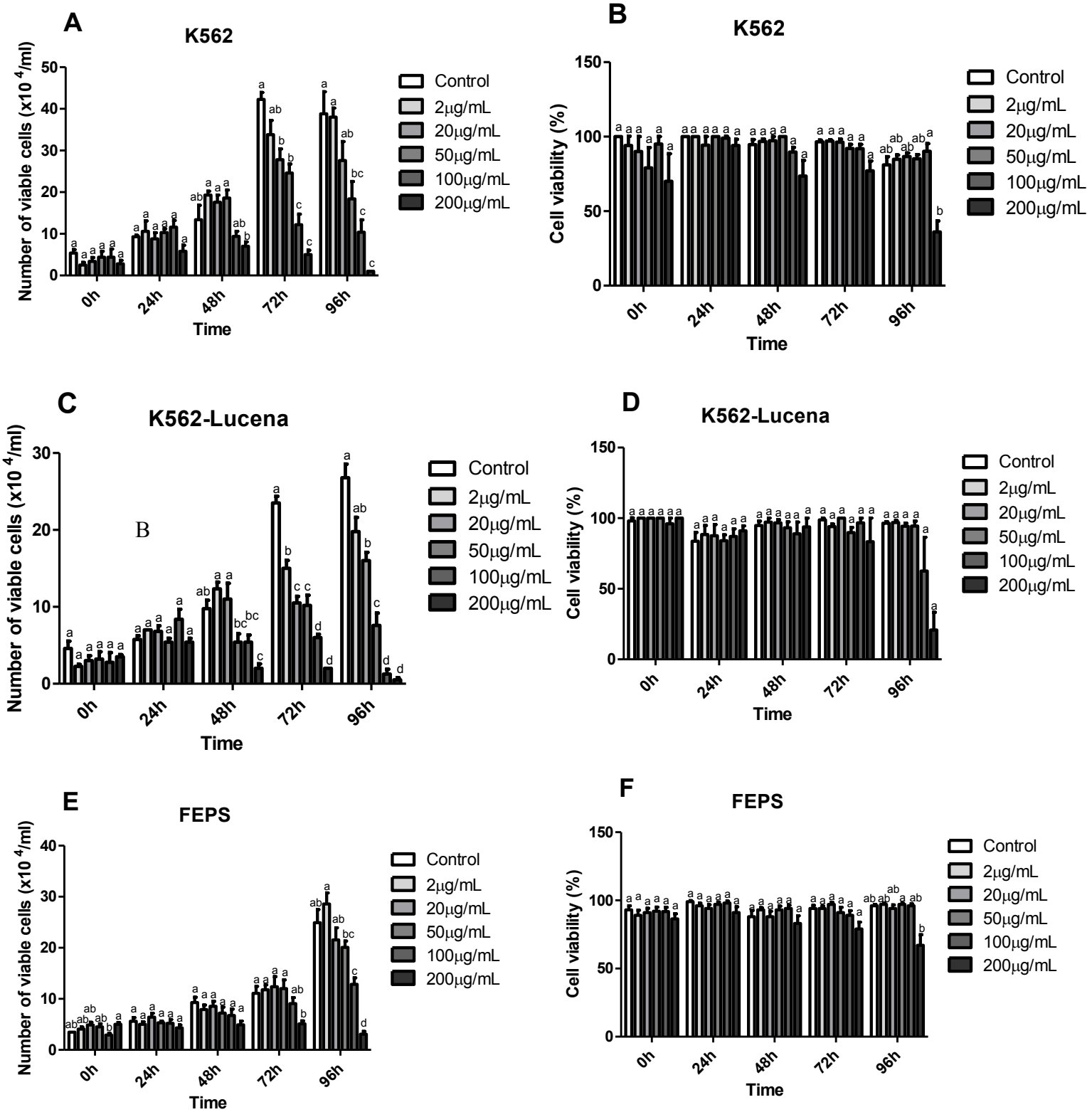

Figure 2 – Sensitivity of human erythroleukemia cell lines to C-PC by Trypan Blue exclusion test: Number of viable cells ($\times 10^4$) and Cell viability (%) of K562 (A, B), K562-Lucena (C, D) and FEPS (E, F)exposed to different concentrations C-PC for until 96 h. Results are expressed as means , ± S.E.M, different letters indicate statistical difference by the ANOVA test followed by *Tukey* (parametric data) or Kruskal-Wallis test (non-parametric data) in each time separately ($p<0.05$).

Supplementary data 1: Interface Residues Contacts between full structure of C-PC and ABCB1 (⇔: amino acid residue linkage on amino acid residue)

C-PC amino acid residue (Chain F)		ABCB1 amino acid residue (Chain A)
ASN 143	⇔	LEU 215
ALA 140	⇔	LEU 215
SER 154	⇔	LEU 223
SER 154	⇔	PRO 219
ALA 140	⇔	LEU 212
ALA 137	⇔	LEU 212
ASN 143	⇔	PRO 219
LYS 133	⇔	LYS 209
ASN 143	⇔	SER 218
PRO 145	⇔	PHE 196
ALA 157	⇔	PRO 219
ALA 61	⇔	GLU 321
GLU 62	⇔	SER 319
ILE 141	⇔	LEU 212
GLU 136	⇔	VAL 213
GLU 136	⇔	LEU 212
SER 58	⇔	GLU 321
GLU 62	⇔	LEU 316
LEU 139	⇔	LEU 215
LEU 139	⇔	ALA 216

Abbreviations: Asparaginr (ASN); Alanine (ALA); Serine (SER); Lysine (LYS); Proline (PRO); Glutamate (GLU); Isoleucina (ILE); Leucine (LEU); Phenylalanine (PHE); Valine (VAL).

Supplementary data 2: Interface Residues Contacts between chain F of C-PC and ABCB1 (⇔: amino acid residue linkage on amino acid residue)

Chain F of C-PC amino acid residue		ABCB1 amino acid residue (Chain A)
LEU 19	⇔	TRP 208
ALA 32	⇔	PHE 197
PHE 98	⇔	LEU 223
ILE 24	⇔	LEU 212
ASP 25	⇔	PHE 204
PHE 5	⇔	LEU 212
VAL 31	⇔	PHE 196
ALA 146	⇔	VAL 33
ALA 146	⇔	ALA 32
ASP 39	⇔	ILE 348
SER 34	⇔	PHE 196
THR 149	⇔	PHE 190
ALA 146	⇔	ALA 352
ILE 148	⇔	ILE 348
ASP 144	⇔	ALA 32
ASP 3	⇔	PRO 219
ASN 35	⇔	PHE 189
VAL 31	⇔	PHE 200
LEU 19	⇔	LEU 212
GLY 147	⇔	PHE 351
GLY 147	⇔	ALA 352
PHE 98	⇔	PRO 219

ALA 146	⇔	ARG 355
GLY 147	⇔	ARG 355
ILE 24	⇔	PHE 204
ILE 24	⇔	TRP 208
PHE 5	⇔	ALA 216
THR 149	⇔	PHE 189
ILE 148	⇔	PHE 351
LEU 27	⇔	LEU 212
LEU 38	⇔	VAL 341
VAL 31	⇔	LEU 215
GLY 147	⇔	ILE 348

Abbreviations: Leucine (LEU); Alanine (ALA); Phenylalanine (PHE); Isoleucine (ILE); Aspartate (ASP); Valine (VAL); Serine (SER); Threonine (THR); Asparagine (ASN); Glycine (GLY); Tryptophan (TRP); Proline (PRO); Arginine (ARG).

Supplementary data 3: Interface Residues Contacts between chain F of C-PC and ABCC1 (⇔: amino acid residue linkage on amino acid residue)

Chain F of C-PC amino acid residue		ABCC1 amino acid residue (Chain A)
ILE 24	⇔	PHE 979
VAL 31	⇔	LEU 982
ASN 42	⇔	PRO 1119
LEU 38	⇔	LEU 1108
VAL 31	⇔	PHE 1098
ASN 35	⇔	ILE 1101
ASP 39	⇔	PRO 1119
GLY 147	⇔	LEU 1123

ASN 42	⇔	ALA 1115
ASN 42	⇔	VAL 1116
SER 34	⇔	ILE 1101
SER 46	⇔	VAL 1116
THR 21	⇔	PHE 979
ILE 148	⇔	PRO 1119
SER 28	⇔	LEU 982
LEU 27	⇔	LEU 982
ILE 24	⇔	LEU 982
PRO 150	⇔	PHE 1126
PHE 98	⇔	LEU 1108
ASP 25	⇔	PHE 975
ASP 25	⇔	PHE 979
ILE 148	⇔	LEU 1123
SER 28	⇔	ILE 978
THR 149	⇔	LEU 1123
THR 149	⇔	GLY 1122

Abbreviations: Isoleucine (ILE); Valine (VAL); Asparagine (ASN); Leucine (LEU); Aspartate (ASP); Glycine (GLY); Serine (SER); Threonine (THR); Proline (PRO); Phenylalanine (PHE); Alanine (ALA).

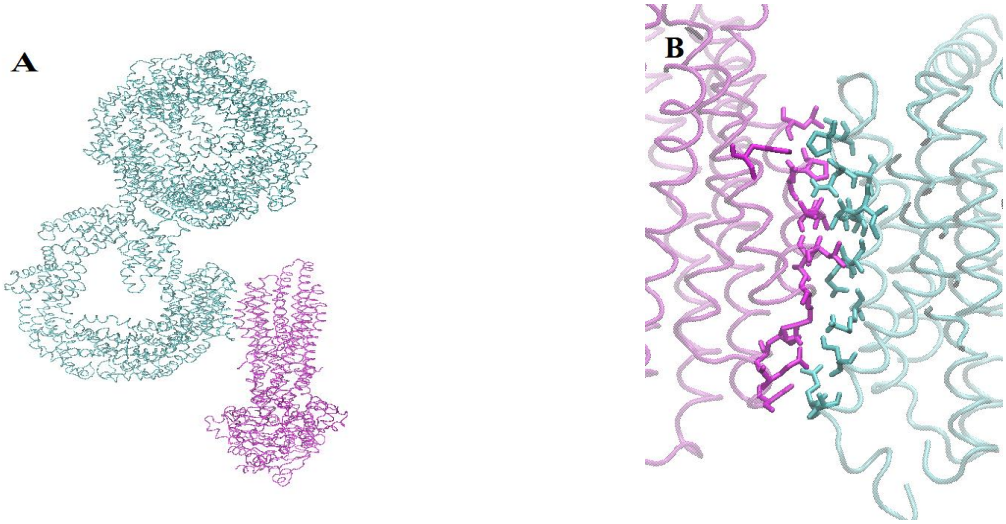

Figure 3 – Protein-protein docking: (A) Full structure of C-PC (blue) and ABCB1 (purple) interaction (B) Amino acids of C-PC (blue) and ABCB1 (purple) in contact.

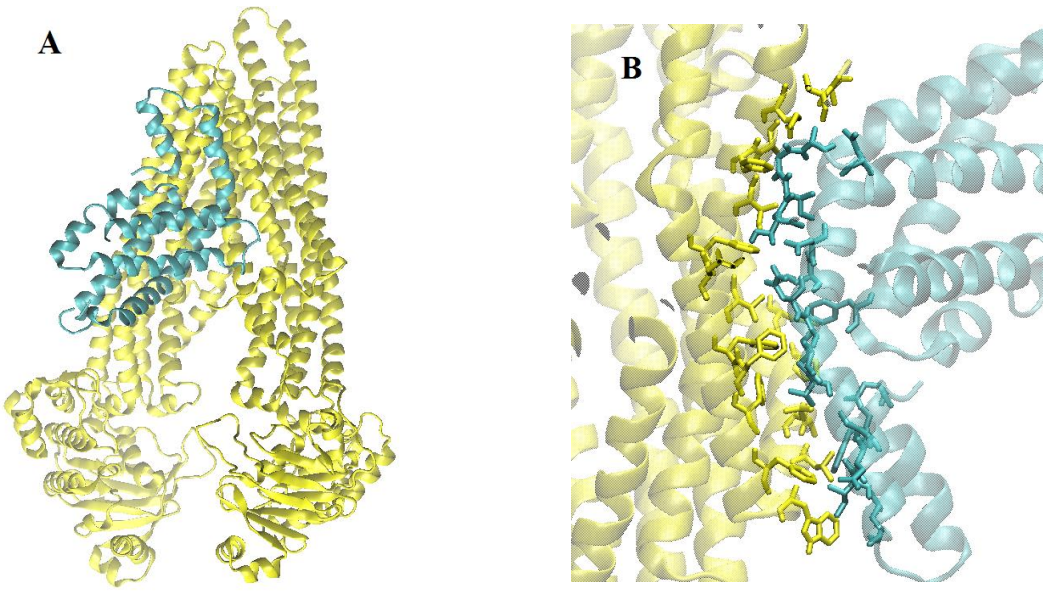

Figure 4 – Protein-protein docking: (A) Chain F of C-PC (blue) and ABCB1 (yellow) interaction (B) Amino acids chain F of C-PC (blue) and ABCB1 (yellow) in contact.

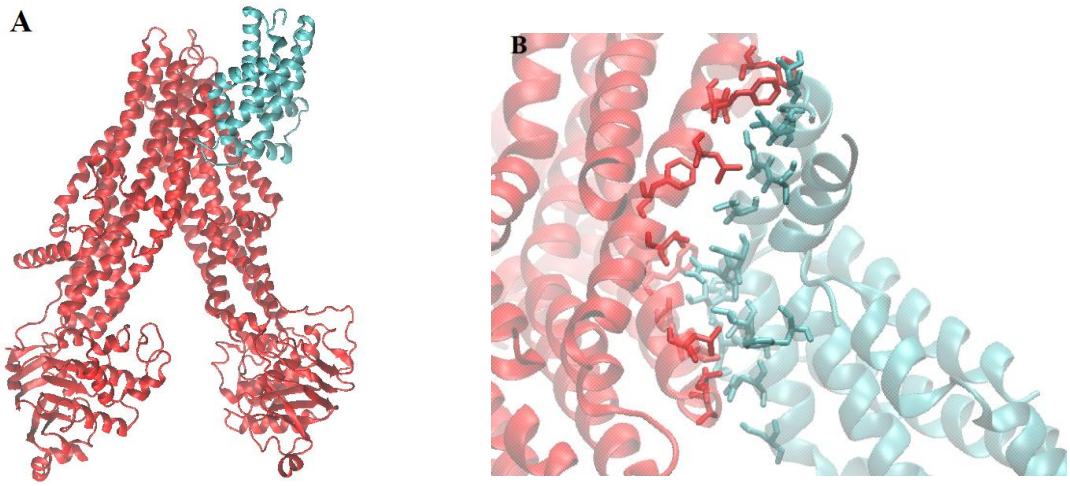

Figure 5 – Protein-protein docking: Chain F of C-PC (red) and ABCC1 (blue) interaction (B) Amino acids chain F of C-PC (blue) and ABCC1 (red) in contact.

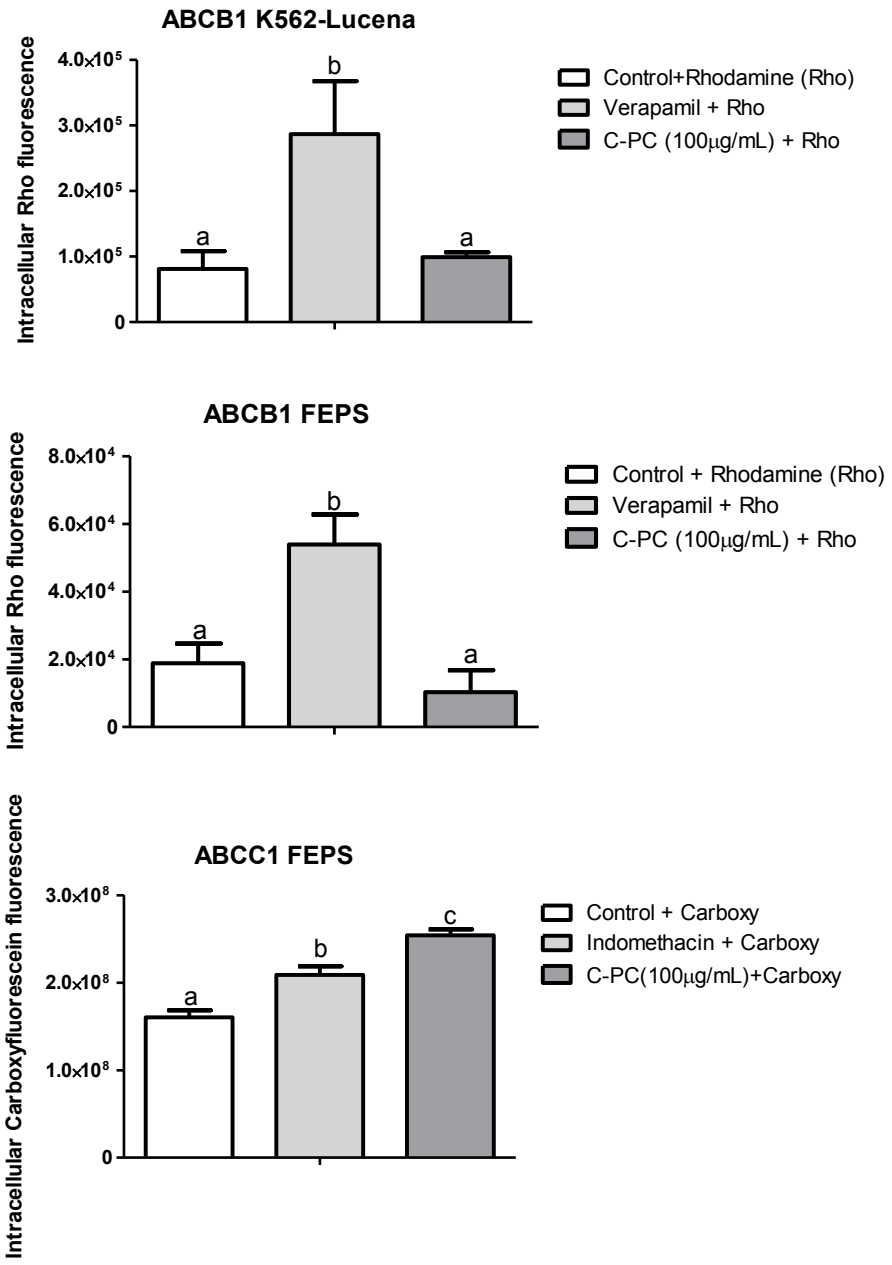

Figure 6 – Intracellular rhodamine 123 (Rho) and carboxyfluorescein diacetate (CF-DA) fluorescence: K562-Lucena and FEPS treated with 100μg/mL of C-PC after 48h. Results are expressed as means , ± S.E.M, different letters indicate statistical difference by the ANOVA test followed by *Tukey* ($p<0.05$).

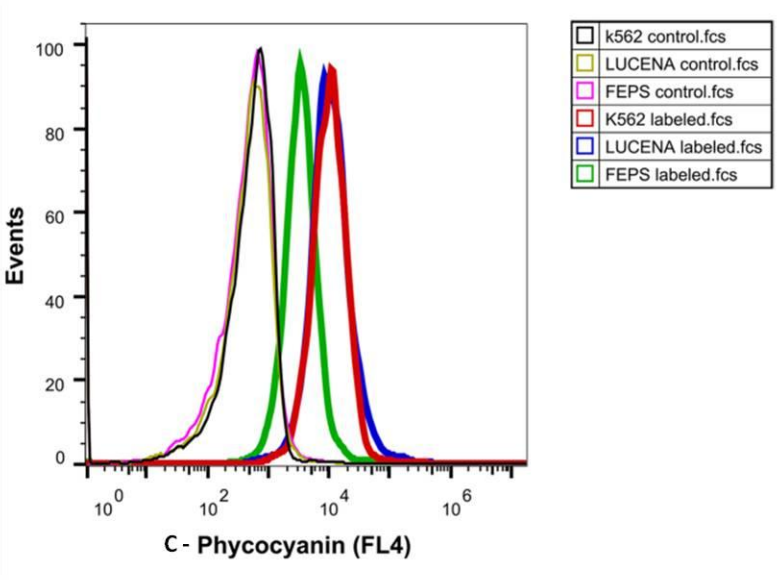

Figure 7 – Overlay graphic showing fluorescence of K562, FEPS and K562-**Lucena labeled or not with C-PC.** 200µg/mL of C-PC, incubation for 1h and cytometric reading after PBS lavage.

Table 1 – Free binding energies (FEB) estimated for C-PC and chemotherapeutic (VCR or DNR) complex

Drug	FEB
VCR	-6.9Kcal/mol
DNR	-7.2Kcal/mol

Table 2 – C-PC amino acid residues involved in binding to chemotherapeutic agents (VCR and DNR)

Drug	Amino acid residues of C-PC
VCR	Gln, Ala (two residues), Ile, Asp (two residues), Asn, Glu, Phe, Tyr, Gly
DNR	Ala, Asp, Ans, Glu, Arg, Thr, Leu, Val, Ser
VCR and DNR	Ala, Asp, Asn, Glu

Abbreviations: Vincristine (VCR); Daunorubicin (DNR); Glutamine (Gln); Alanine (Ala); Isoleucine (Ile); Aspartate (Asp); Asparagine (Asn); Glutamate (Glu); Phenylalanine (Phe); Tyrosine (Tyr); Glycine (Gly); Arginine (Arg); Threonine (Thr); Leucine (Leu); Valine (Val); Serine (Ser);

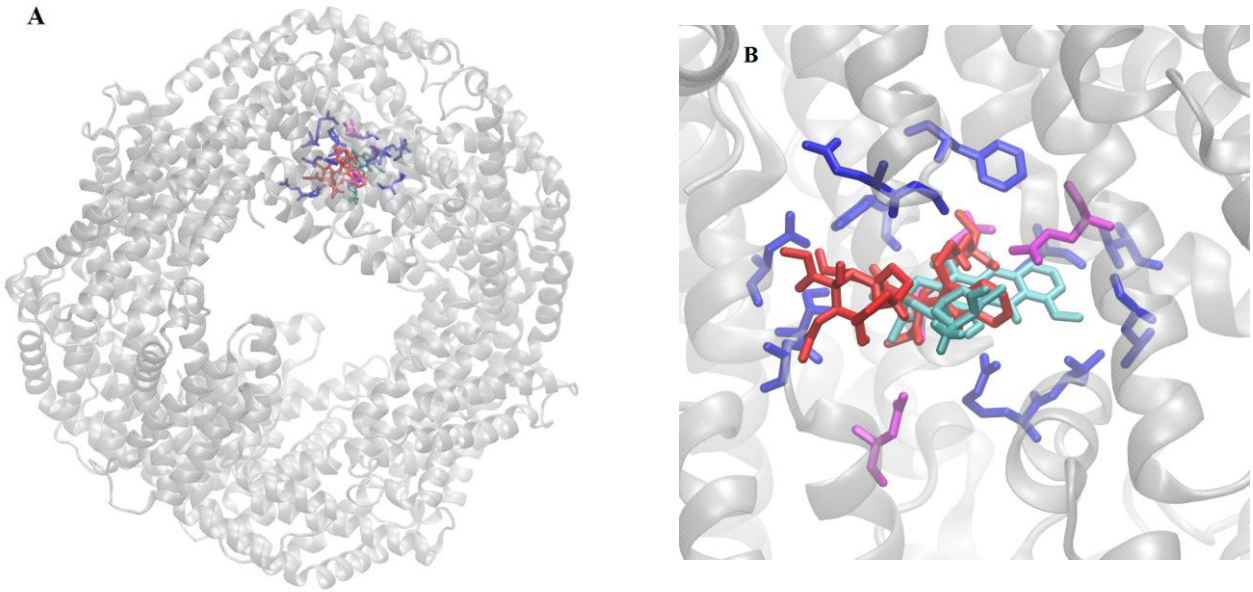

Figure 8 – C-PC + chemotherapeutic (VCR or DNR) complex Gray: (A) C-PC; Red: VCR; Cyano: DNR; (B) Blue: Amino acids of C-PC in contact with each drug; Pink: Amino acids of C-PC in common for VCR and DNR.

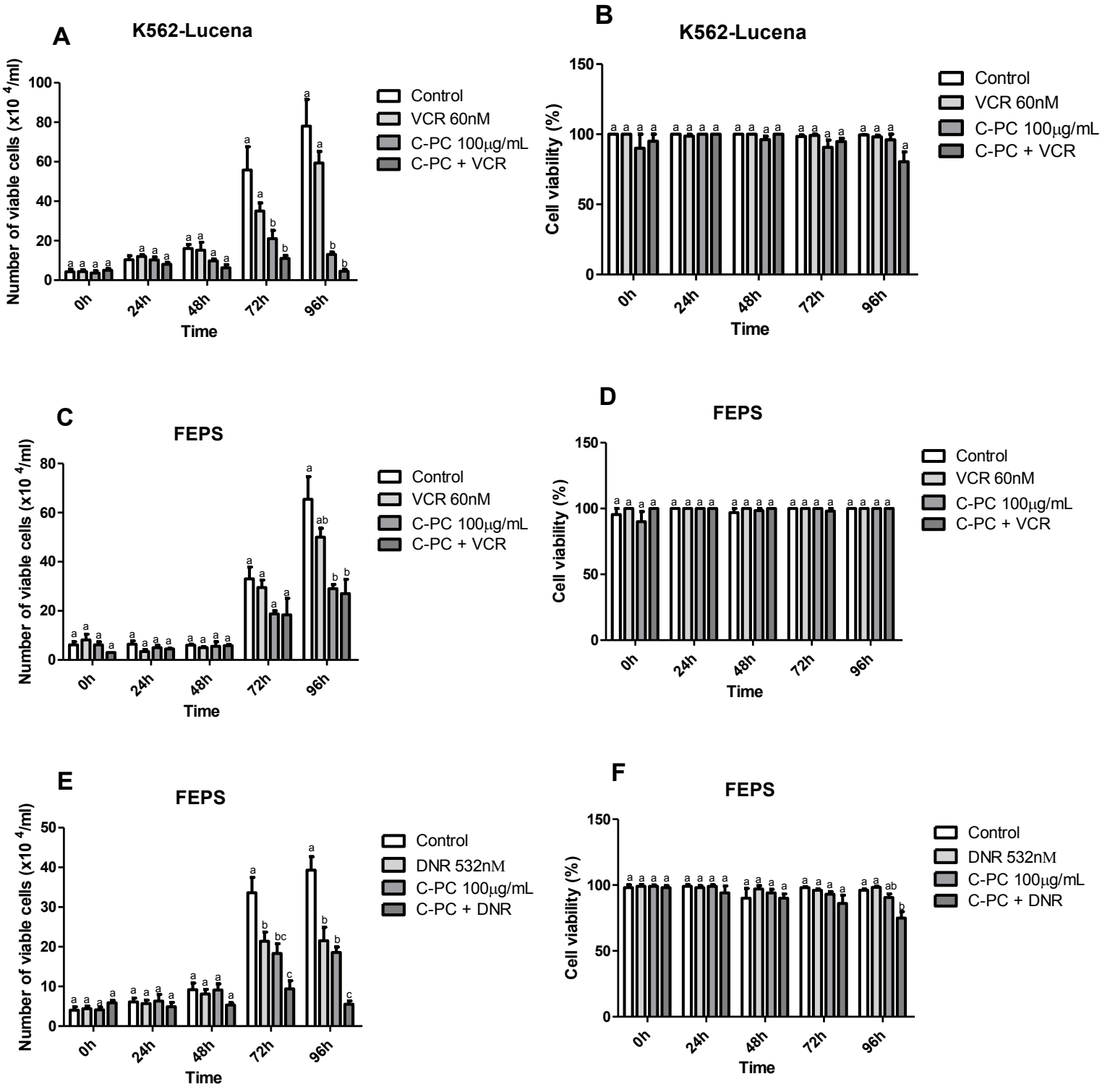

Figure 9 – Sensitivity of human erythroleukemia cell lines to C-PC plus chemotherapeutic drug by Trypan Blue exclusion: Number of viable cells ($\times 10^4$) and Cell viability (%) of K562-Lucena (A, B) and FEPS (C, D, E and F). Results are expressed as means ± S.E.M.; different letters indicate statistical difference by the ANOVA test followed by *Tukey* (parametric data) or Kruskal-Wallis test (non-parametric data) ($p<0.05$) in each time separately.

Manuscrito II

Modulation of the reactive oxygen levels and gene expression in sensitive and resistant tumoral cells by C-phyocyanin

(A ser submetido para a revista Investigational New Drugs como Short Report)

Modulation of the reactive oxygen levels and gene expression in sensitive and resistant tumoral cells by C-phyocyanin

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Abstract

In this work we investigated the possible cellular changes induced by C-phycocyanin (C-PC) in erythroleukemic cells with and without the multidrug resistance (MDR) phenotype. The levels of reactive oxygen species (ROS) (evaluated by fluorimetry) were increased in relation to the control for the K562 and K562-Lucena treated with 100 μg/mL, without increase of the ROS levels for the FEPS. The expression of genes related to resistance was evaluated by real-time PCR: *PTGS2*; ALOX5; *ABCB1* and *ABCC1*, being that with 100 μg/mL of C-PC increased *PTGS2* and *ABCB1* expression for the K562-Lucena and reduced the expression of *ALOX5* for K562-Lucena and FEPS. ROS levels appears to be involved in the biological responses of C-PC for the K562 and K562-Lucena, although the genes studied here have their expression modified by the C-PC only for K562-Lucena cell line. Thus, it is possible to suggest that C-PC modulates the expression of *PTGS2* and *ABCB1* in a ROS-dependent manner for the K562-Lucena and modulates the expression of *ALOX5* in a ROS-independent manner for the FEPS, however more studies needed to elucidate these mechanisms.

Keywords: *PTGS2*; *ALOX5; ABCB1*; *ABCC1*.

1. Introduction

The resistance to chemotherapy is one of the major challenges for the treatment of cancer, a disease that generates high mortality rates worldwide [2,3,56]. Besides the alterations in the process of cell division, caused by genetic mutations [57,58], cancer cells can exhibiting the phenotype of multiple drug resistance (MDR). This phenotype provides for the cells additional and/or enhanced features such as, the overexpression of ABC family drug efflux proteins (ATP binding cassete) [6,56], high concentration and/or activity of antioxidant (enzymatic and non-enzymatic) [59], as well as the overexpression of proteins related to the inflammatory process [60,61].

There are a model with three human erythroleukemia cell lines (K562, K562-Lucena and FEPS) which present distinct characteristics in relation to the MDR phenotype and therefore present an alternative for understanding the mechanisms of resistance. K562 cell line was obtained by the selective culturing of positive Philadelphia chromosome (Ph^+) retaining myeloblastic cells from a chronic myeloid leukemia (CML) patient [26]. The K562 is the parental cell line of this model, it is sensitive to chemotherapy, does not present proteins for drug efflux and has low antioxidant activity and expression of *PTGS2* gene (encoding for COX-2 which converts arachidonic acid into prostaglandins) in relation to the K562-Lucena cell line [59,60]. The K562-Lucena cells were derived from the K562 cell line by selection in progressively higher concentrations of vincristine (VCR) [27,28]. The FEPS cell line also was derived from the K562 cell line but by selection in progressively higher concentrations of daunorubicin (DNR). The FEPS cells differ from the K562 and K562-Lucena cells due to the expression of cellular death receptor CD95, overexpression of ABCB1 (greater than the K562-Lucena cells) and ABCC1 and overexpression of the *Alox-5* gene encoding for arachidonate 5-lipoxygenase (5-LOX) [37,62].

The C-phycocyanin (C-PC) a protein that acts as a photosynthetic pigment in cyanobacteria /microalgae has antitumoral capacity reported in several studies [21,63,64]. However, investigations in chemotherapy-resistant cells are poorly explored and the antitumoral and anti-MDR mechanisms of C-PC remain superficially understood [11,65]. Thus, the objective of this study was to verify the mechanisms of action of C-PC on sensitive and resistant tumor cells with respect to: cell death; production of reactive oxygen species and

expression of genes related to the MDR phenotype (genes involved with the inflammatory process: *PTGS2* and *ALOX5*; genes for efflux proteins: *ABCB1* and *ABCC1*).

2. Materials and Methods

2.1. Human erythroleukemic cells culture

K562, K562-Lucena, and FEPS cells were obtained from Laboratório de Imunologia Tumoral of Instituto de Bioquímica Médica Leopoldo de Meis (Universidade Federal do Rio de Janeiro, Brazil). K562 parental cells were grown in RPMI 1640 medium (Sigma), supplemented with sodium bicarbonate (0.2 g/l) (Synth), L-glutamine (0.3 g/l) (Vetec), with 10 % fetal bovine serum (FBS) (Gibco) and 1% antibiotic and antimycotic (penicillin [100 U/mL], streptomycin [100 μg/mL], and amphotericin B [0.25 μg/mL]) (Gibco), in disposable plastic flasks at 37 °C and 5% of CO_2. K562-Lucena and FEPS cells were grown under the same conditions above, but with 60 nM vincristine (VCR) (Sigma) or 532 nM daunorubicin (DNR), respectively, in order to preserving MDR phenotype. During the experiments, no VCR or DNR was added to K562-Lucena and FEPS cell cultures.

2.2. C-phycocyanin extraction and purification

Spirulina platensis LEB 52 was cultivated [38] and supplied by Laboratório de Engenharia Bioquímica (Universidade Federal do Rio Grande – FURG, Brazil).

C-phycocyanin extraction process occurred according to Moraes et al. [39]. Briefly, crude broth containing cells was centrifuged (1890Xg, 20 min) and pH was adjusted at 6.5. Purification process was by ultrafiltration performed in dead-end ultrafiltration cell with 50 kDa polyethersulfone membrane (UH050 P, Nadir, Germany) at 25 ºC and 1.0 kgf/cm² in diafiltration/ultrafiltration mode [40]. Purity of C-PC extract was calculated according to Abalde et al., [41], using absorbance ratio OD_{620}/OD_{280}. Treatments were constructed using purified extract with purity degree of 1.00, considered as food grade [42]. C-PC concentration (mg/mL) was calculated according to Bennet and Bogorad [43] by following equation, with changes in the wavelength:

$$\text{C-PC} = \frac{(OD620 - 4.74 \times (OD652))}{5.34}$$

2.3. Quantitative analysis of apoptosis and necrosis

Cells were centrifuged (197X*g* for 2 minutes), suspended (2.10^4 cells/mL) in RPMI 1640 medium, plated in 96-well culture plates, and treated with 100.0 and 200.0 μg/mL of C-PC (concentrations based on a previous study from our research group / unpublished data) and incubated at 37 °C. A control group received the same volume of sterile water. After 72h of exposure the cells were centrifuged (461X*g* for 10 minutes), washed with phosphate buffer saline (PBS), centrifuged again (461X*g* for 10 minutes) for PBS removal and suspended in 100μl RPMI 1640 medium. The evaluation occurred according to Kosmider et al., [66] modified, with the addition of 2 μL of work solution (composed 100 μg/mL of acridine orange and 100 μg/mL of ethidium bromide in PBS) in each well containing RPMI 1640 medium. Apoptosis and necrosis were quantified by areas captured from the well plate (40X) with epifluorescence microscope (Olympus IX81) and the data were expressed in percentage related to total cells number visualized on the captured area. The cells were classified according Kosmider et al. modified [66], as following: living cells: green nucleus; apoptosis: nuclei orange, while fragmentation or condensation of chromatin is still observed and necrosis: uniformly red-orange stained cell nuclei.

2.4. Intracellular ROS levels

Cells were centrifuged (197x*g* for 2 minutes), suspended (1.10^5 cells/mL) in RPMI 1640 medium, plated in 24-well culture plates, and treated with 100.0 μg/mL of C-PC and a control group (receiving the same volume of sterile water), incubated at 37 °C. After 72h, cells were washed twice with PBS and incubated for 30 min at 37°C with the fluorigenic compound H_2DCF-DA (2',7'- dichlorofluorescein diacetate; 40 μM; Sigma) oxidized by ROS into a fluorescent compound [DCF (2',7'- dichlorofluorescein)] according to Myhre and Fonnum [67]. After incubated for 30 min cell lines were washed twice with PBS and suspended in fresh PBS. Each treatment was performed in quadruple. Aliquots from 160 μl of each sample (three replicates) were placed into an ELISA plate and the fluorescence intensity determined during 90 min at 37◦C, using a fluorimeter (Victor 2, PerkinElmer) with λex and λem of 485 and 520 nm respectively. ROS levels were expressed in terms of fluorescence area, after fitting fluorescence data to a second order polynomial and the estimated functions integrated between 0 and 90 min in order to obtain its area.

2.5. Gene expression

The cells were treated with 100.0 μg/mL of C-PC for 72 h for gene expression analysis of *PTGS2*, *ALOX5*, *ABCB1* and *ABCC1*. Total RNA was extracted from five samples of each cell line (2.10^5 cells per sample) according to the manufacturer's protocol for TRIzol Reagent (Invitrogen, Brazil), and quantified by BioDrop μLite spectrophotometer (BioDrop, England). The RNA integrity was determined by electrophoresis in a 1.5% agarose gel stained with ethidium bromide (0.5μg/mL). The cDNA synthesis was performed by reverse transcription of 2 μg RNA using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Brazil). Gene expression analysis was performed by real-time quantitative PCR (qPCR). Gene-specific primers (Table 1) were designed based on sequences available in GenBank using the Primer-Blast tool (http://www.ncbi.nlm.nih.gov). Previously, the PCR amplification efficiency of each primer pair was evaluated by serial dilution reactions where the efficiency of reactions showed the appropriate parameters (Table 1). *EF1α* and *B2M* were chosen as reference genes after stability was confirmed with the geNorm applet [68].The gene expression analysis was realized using the ABI 7300 platform Real Time Systems (Applied Biosystems, Brazil) and the detection system Gotaq qPCR Master Mix (Promega Corporation, Brazil). The normalization factor was calculated as the geometric mean of the expression values of the reference genes tested by the geNorm applet. Gene expression levels of the target genes were calculated by dividing the expression value of the target gene by the normalization factor.

2.6. Statistical analysis

All experiments occurred with at least three replicates of the samples. Normality of the data was verified by the Shapiro-Wilk test. Parametric data were compared by Student's t-tests or analysis of variance ANOVA (one or two way) followed by Tukey post-test. Non-parametric data were compared by the Kruskal-Wallis test. The data were expressed as mean and standard error of the mean, being $p < 0.05$ utilized.

3. Results

3.1. Apoptosis and necrosis

Cell death by apoptosis was detected in the three cell lines analyzed, although these levels of cell death did not differ from the control for any of the analyzed cells (Figure 1) (P-value for K562: viables=0.1512; apoptosis=0.1512; necrosis=nonexistent; P-value for K562-Lucena: viables=0.0865; apoptosis=0.0803; necrosis=0.406; P-value for FEPS: viables=0.4750; apoptosis=0.4750; necrosis= nonexistent).

3.2. Intracellular ROS levels

The basal level of ROS (control) differs among the three cell lines, being the lowest for the FEPS and higher for K562 and K562-Lucena (P-value K562 x K562-Lucena: 0.0891; P-value K562 x FEPS: 0.0004; P-value K562-Lucena x FEPS: 0.0001). The treatment with C-PC generated increased levels of ROS for the K562 (P-value: 0.0003) and K562-Lucena (P-value: 0.0004) cell lines (Figure 2).

3.3. Gene Expression

The basal level of expression (control) to *PTG2* and *ALOX5* has a similar profile for K562-Lucena and FEPS, being higher than K562 for *PTG2* expression (P-value K562 x K562-Lucena: 0.0001; P-value K562 x FEPS:0.0003; P-value K562-Lucena x FEPS: 0.9193) and lower than K562 for *ALOX5* expression (P-value K562 x K562-Lucena: 0.0001; P-value K562 x FEPS:0.0109; P-value K562-Lucena x FEPS: 0.0821) (Figure 3A, B). For *ABCB1* expression, each cell line has a basal level of expression, being low for K562, intermediate for K562-Lucena and high for FEPS cells (P-value K562 x K562-Lucena: 0.0006; P-value K562 x FEPS: 0.0001; P-value K562-Lucena x FEPS: 0.0001) (Figure 3C). Already for the basal level of *ABCC1* expression the K562 and FEPS cell lines are similar having a higher expression in relation to K562-Lucena cell line (P-value K562 x K562-Lucena: 0.0001; P-value K562 x FEPS:0.0144; P-value K562-Lucena x FEPS: 0.0008) (Figure 3D).

The C-PC treatment did not alter expression of *ABCC1* for any of the cell lines (Figure 3D). However, C-PC treatment generated an increased in the expression of *PTGS2* and *ABCB1* for K562-Lucena (Figure 3C, E) (P-value for *PTGS2*: 0.0052; P-value for *ABCB1*: 0.0204), besides decreasing the expression of *ALOX5* for K562-Lucena (P-value: 0.0109) and FEPS (P-value: 0.0001) (Figure 3B).

4. Discussion

In an earlier study of our laboratory it was found that concentrations from 20 μg/mL of C-PC possessed the capacity of proliferation inhibition for K562 (non-MDR) e K562-Lucena (MDR) and that only the concentration of 200μg/mL of C-PC was able to inhibit the proliferation of the FEPS line in 72h. For the present study, the concentration of 100 μg/mL of C-PC was chosen to compare if the final biological response (proliferation inhibition only for K562; K562-Lucena) was due to a different pattern of cellular changes induced by C-PC for the three cell lines. The possible cellular alterations evaluated in this study were: cell death, ROS levels and expression of genes related to the MDR phenotype. The exploration of the anti-MDR role of the C-PC is restricted, as well as the knowledge about antitumoral and anti-MDR mechanisms are poorly understood, which demonstrates the need for research in the area.

No cell death by necrosis was detected and although different levels of apoptosis were observed in the three cell lines evaluated, these were not significant in relation to the control. These results indicate that C-PC does not generate cytotoxicity for the human erythroleukemia lines evaluated, which is in agreement with data previously verified in our research group (unpublished data), since C-PC showed an antiproliferative character (observed for K562 and K562-Lucena in 72h and for FEPS in 96h) by reducing the number of cells and possible inhibition of the mitosis process.

It is known that C-PC has a diversity of cellular targets and mechanisms of action [11], however, the reduction of reactive oxygen species (ROS) levels seems to be a recurrent mechanism for activation of pathways related to the biological effects of C-PC [25,69,70]. For erythroleukemic cell lines (except FEPS) C-PC generated increased ROS levels, contrary to literature data (which demonstrate a reduction of ROS levels) [29-31]. For the FEPS line, the increase in ROS levels by C-PC was not verified and this cell line presented the lowest basal level of ROS production indicating a possible increased antioxidant capacity in relation to the K562 and K562-Lucena cell lines, which may have neutralized the ROS generated by the C-PC.

The involvement of ROS in the modulation of gene expression previously verified in some studies [25,69,70] may have occurred in our study, however, by increasing ROS levels. C-PC may have altered gene expression through ROS for the K562-Lucena because it reduced

the expression of *ALOX5* (gene encoding 5-lipoxygenase/5-LOX protein, which produces leukotrienes) [71] and induced the expression of *ABCB1* and *PTGS2* for this cell line . Studies have shown that *ALOX5* is involved in several physiological and pathological processes such as oxidative stress (ROS increased), inflammation and cancer [72–74]. C-PC also decreased expression of *ALOX5* to the FEPS line, however ROS levels were not increased for this cell line, then there is likely to be involvement of other ROS-independent cell pathways for the modulation of *ALOX5* expression. The signaling pathway involving ROS inhibition by C-PC proposed by Nishant et al., [31] is as follows: Reduction of ROS levels inhibits pathways involving the Akt protein pathway (also stimulated by prostaglandins) and thus NF-κB does not stimulate the activation of the expression of *ABCB1*. It is possible that for the K562-Lucena cell line the increase in ROS levels stimulate a pathway involving the Akt protein and thus increase the expression of *ABCB1*.

Studies reveal the close relationship between inflammatory mediators and drug resistance [75,76]. Our gene expression results demonstrate for the first time for the FEPS cell line the basal expression of *PTGS2*, a gene encoding the cycloxygenase-2/ COX-2 (prostaglandin-generating arachidonic acid). This gene expression was the largest for the K562-Lucena as previously reported by Carrett-Dias et al. [60] and FEPS in relation to K562. The increase in the expression of *PTGS2* induced by the C-PC for the K562-Lucena was great (about 3 times in relation to the control), therefore we performed a statistical analysis only for the K562-Lucena cell line for *PTGS2* gene expression of. Thus, by comparing control and treatment with C-PC alone in the K562-Lucena cell line, a significant increase in *PTGS2* expression can be observed.

Nishanth et al., [25] and Carrett-Dias et al. [60] demonstrated the possible relationship of COX-2 with the MDR phenotype (for cell which overexpressing *ABCB1*): The first demonstrates that inhibition of prostaglandin synthesis by the COX-2 enzyme reduces *ABCB1* gene expression in HepG2 cell line (liver tumor) and the second verifies that the K562-Lucena overexpresses both *ABCB1* and *PTGS2* in relation to the non-MDR K562. In the present study, an increase in *ABCB1* gene expression was observed in the K562-Lucena treated with C-PC (Figura 3C). It is possible that this increase is directly induced by the increase in ROS or that this increase is caused by induction of COX-2, which could be inducing of *ABCB1*expression, due to the relationship between these genes demonstrated by authors mentioned above.

The ABC family proteins are one of the main factors responsible for the MDR phenotype [8]. Daflon-Yunes et al., [37] characterized by immunoblotting the presence of the ABCB1 and ABCC1 proteins for the cell lines of the present study, for K562: without overexpression , for K562-Lucena: presence of ABCB1; absence of ABCC1 and for FEPS: presence of ABCB1 and ABCC1. Our gene expression results are in agreement with what was observed at the ABCB1 protein level, since the basal (control) levels of *ABCB1* gene expression were increasing according to the MDR phenotype (Figure 3C). However, when comparing the gene expression of *ABCC1* with the levels for this protein found Daflon-Yunes et al., [37] an important difference is revealed: the K562 cell line which does not have ABCC1 immunostaining on its membrane has a basal overexpression of the *ABCC1* gene, which equals the overexpression of the FEPS MDR cell line, being both larger than the K562-Lucena MDR cell line. Therefore, it is possible that for the K562 cell line the processes of posttranslational modifications and delivery of the ABCC1 protein to the membrane are not performed.

Our results revealed that for K562-Lucena MDR cell line the C-PC could use a ROS-dependent pathway for generate decreased in *ALOX5* gene expression, increased in *PTGS2* and *ABCB1* gene expression. The *ALOX5* gene appears to be an important target of C-PC in MDR lines with or without ROS involvement, since although the FEPS presenting a smaller level of cell proliferation inhibition by the C-PC (unpublished data) and not presented ROS production in relation to K562-Lucena, the *ALOX5* gene was also inhibited for the FEPS. It is likely that C-PC also modulates gene expression for the non-MDR line K562, however such modulation may occur genes different from those modulated in an MDR cell.

5. Conclusion

The increased of ROS levels appears to be involved in the biological responses of C-PC for the K562 (non-MDR) and K562-Lucena (MDR) cell lines, despite this the genes evaluated here have their expression modified only for the K562-Lucena cell line. C-PC appears to modulate *PTGS2* and *ABCB1* gene expression in a ROS-dependent manner for the K562-Lucena and appears to modulate *ALOX5* expression for FEPS in a ROS-independent manner.

6. Conflict of interest

The author(s) confirm that this article content has no conflict of interest.

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Table 1 - Primers sequences used in gene expression analysis

Gene	Primers sequence 5' – 3'	Primers Efficiency (%)	GenBank accession number
ABCB1	F:TCCTCAGTCAAGTTCAGAGTCTTCA R: TCTCCACTTGATGATGTCTCTCACT	102,01	NM_000927
ABCC1	F: GGATCTCTCCAGCCGAAGTCT R: GTGATGGGAGCCAGAAGCA	99,25	XM_017023237.1
ALOX5	F: GTGGCGCGGTGGATTC R: TGGATCTCGCCCAGTTCCT	94,99	XM_011539564
B2M	F: CTCACGTCATCCAGCAGAGAA R: TCGGATGGATGAAACCCAGAC	98,19	NM_004048.2
EF1α	F: GCCAGTGGAACCACGCTGCT R: ATCCTGGAGAGGCAGGCGCA	103,14	NM_001402
PTGS2	F: ACGCTTTATGCTGAAGCCCT R: GCCGAGGCTTTTCTACCAGA	102,51	NC_000001.11

ABCB1: ATP-binding cassette, sub-family B (MDR/TAP); *ABCC1*: ATP Binding Cassette, subfamily C (MRP1); *ALOX5:* arachidonate 5-lipoxygenase (5-LOX); *PTGS2:* prostaglandin-endoperoxide synthase 2 ; *EF1α*: eukaryotic translation elongation factor 1 alpha 1; *B2M*: Beta-2-Microglobulin

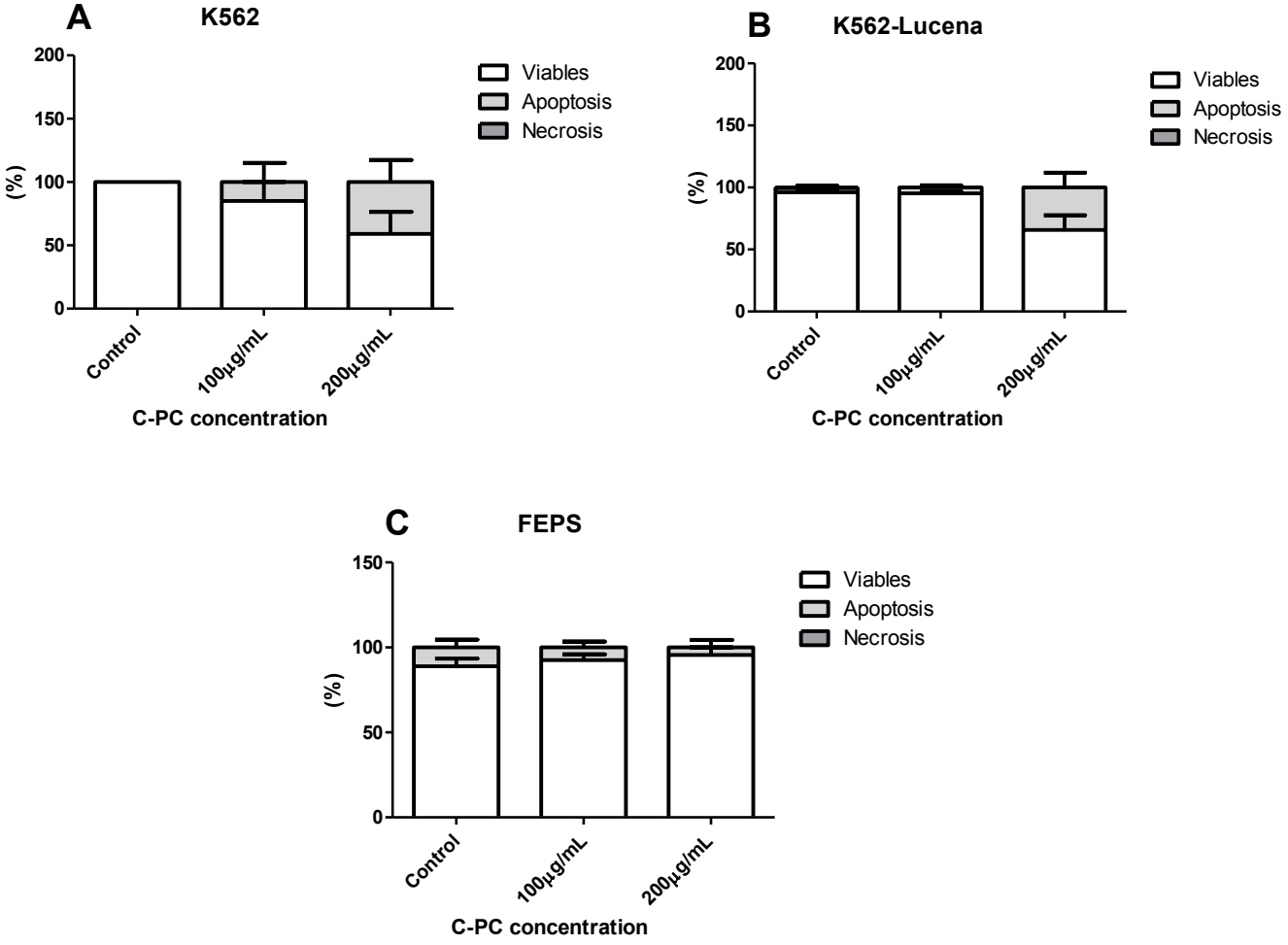

Figure 1: Apoptosis and necrosis for K562 (A), K562-Lucena (B) and FEPS (C) cell lines after 72h of exposure to C-PC. Results are expressed as means ± S.E.M. There was no statistical difference between treatments ($p>0.05$) by the ANOVA test followed by *Tukey* (parametric data) or Kruskal- Wallis test (non-parametric data) ($p<0.05$).

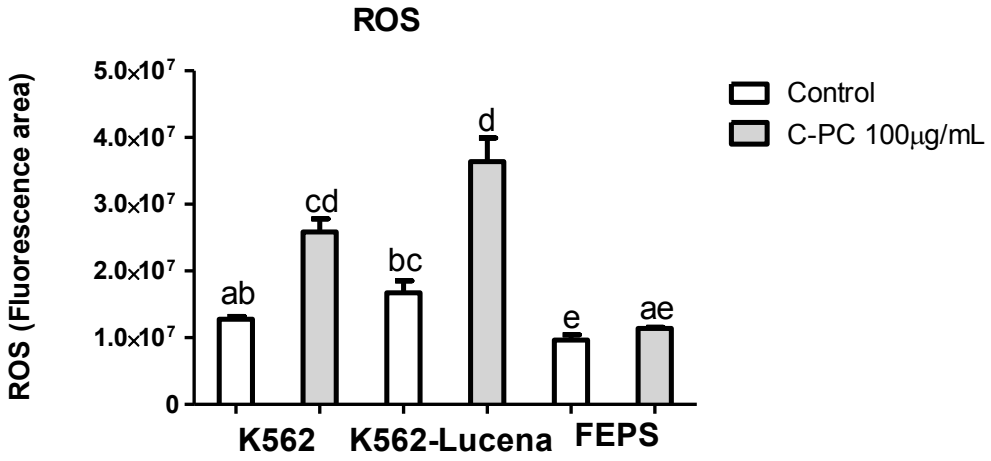

Figure 2: Intracellular ROS levels for K562, K562-Lucena and FEPS cell lines after 72h of exposure to C-PC. Results are expressed as means ± S.E.M. Different letters indicate statistical difference ($p<0.05$) by the two way ANOVA test followed by *Tukey* ($p<0.05$).

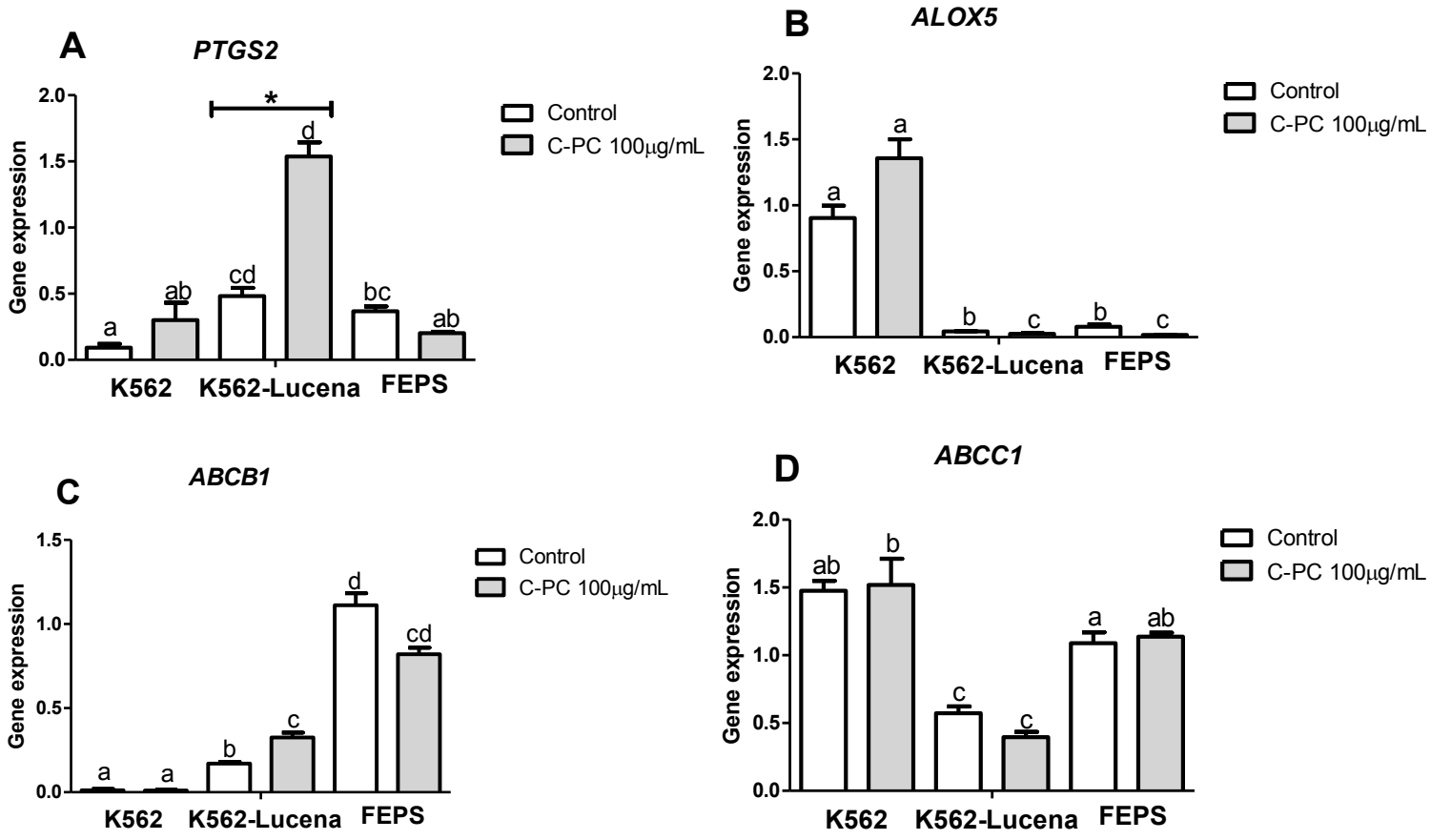

Figure 3: Gene expression (Normalized by *EF1α* and *B2M*) for K562, K562-Lucena and FEPS cell lines after 72h of exposure to C-PC. Results are expressed as means ± S.E.M. Different letters indicate statistical difference ($p<0.05$) by the two way ANOVA test followed by *Tukey*. *Comparison by Student's t-test.

3. Discussão Geral

A descrição do pigmento fotossintético C-ficocianina (C-FC) ocorreu em 1928 (Lemberg, 1928) e os primeiros estudos sobre o efeito antitumoral desta molécula começaram há cerca de 15 anos. Apesar disso, ainda não existe uma completa compreensão sobre os mecanismos antitumorais exercidos pela C-FC (E Silva *et al.*, 2017) e uma exploração abrangente sobre o seu papel em células com fenótipo de resistência a múltiplas drogas (MDR). Os resultados desta tese abordam tanto os mecanismos antitumorais da C-FC, quanto o papel anti-MDR, gerando respostas necessárias para o campo da pesquisa da C-FC no câncer.

O modelo de estudo desta tese possibilita um padrão de comparação muito interessante, pois são utilizadas três linhagens eritroleucêmicas humanas (K562, K562-Lucena e FEPS), porém com diferenças marcantes no que se refere a características relacionadas ao fenótipo MDR: A linhagem parental não-MDR K562 foi estabelecida por Lozzio e Lozzio (1973) através da cultura seletiva de células mieloblásticas apresentando o cromossomo Filadélfia (Ph) (Lozzio e Lozzio, 1973), é sensível à quimioterapia, não possui superexpressão de proteínas da família ABC (ATP binding cassete) para efluxo de drogas e tem baixa atividade antioxidante e expressão do gene *PTGS2* (codificador da enzima COX-2 que converte ácido araquidônico em prostaglandinas) em relação com a linhagem de K562-Lucena (Votto et al., 2010; Carrett-Dias et al., 2011). A linhagem MDR K562-Lucena foi derivada da K562 através da seleção de células resistentes a concentrações progressivamente crescentes de Vincristina (VCR; tem como alvo o citoesqueleto) (Rumjanek *et al.*, 1994; Rumjanek *et al.*, 2001). A linhagem MDR FEPS também foi derivada da K562, mas por seleção em concentrações progressivamente crescentes de Daunorrubicina (DNR; tem como alvo o DNA). As células FEPS diferem das células K562 e K562-Lucena devido à baixa expressão do receptor de morte celular CD95, superexpressão de ABCB1 (maior que as células de K562-Lucena) e ABCC1 (Daflon-Yunes *et al.*, 2013).

Verificou-se que a principal ação antitumoral da C-FC para as linhagens eritroleucêmicas concentra-se no papel citostático e não citotóxico. Além disso, não houve um papel citotóxico da C-FC para as células saudáveis (macrófagos peritoneais de *Mus musculus*), estando este resultado de acordo com outros estudos que apontam a segurança da utilização de C-FC em células saudáveis (Wang *et al.*, 2007; Li *et al.*, 2010).

Até o momento a compreensão do papel da C-FC em células com fenótipo MDR restringia-se a linhagem HepG2 (tumor hepático) (Nishanth *et al.*, 2010), contudo a expressão induzida do gene *ABCB1* por agentes químicos em células saudáveis pode ser inibida pela C-FC através do mesmo mecanismo utilizado em HepG2: Redução dos níveis de espécies reativas de oxigênio (ROS) que inibe certas vias de sinalização responsáveis pela expressão de *ABCB1* (Roy *et al.*, 2007; Roy *et al.*, 2008; Nishanth *et al.*, 2010). Nossos resultados revelam que para as linhagens eritroleucêmicas, exceto a FEPS a C-FC gera um aumento dos níveis de ROS e esse aumento se reflete em uma expressão aumentada de *ABCB1* para a linhagem K562-Lucena, resultados contrários àqueles verificados para a linhagem HepG2. Segundo o mecanismo proposto por de Nishanth et al., (2010) o que interliga ROS e

expressão gênica é uma via envolvendo a proteína *Akt* que induz a produção de NF-κB que por sua vez ativa a expressão de *ABCB1*. A contradição entre nossos resultados e os de Nishanth et al., (2010) com a linhagem HepG2 revela a importância de expandir a pesquisa do papel anti-MDR em mais linhagens celulares resistentes, uma vez que existem diferenças marcantes entre os diferentes tipos tumorais (Cesnik *et al.*, 2016) e o fenótipo MDR pode ser influenciado pelo tipo celular no qual é expresso, gerando diferentes respostas biológicas.

Nesse contexto, diferentes respostas biológicas da C-FC comparando o fenótipo MDR de uma eritroleucemia e de um tumor hepático são menos surpreendentes se compararmos os diferentes resultados obtidos entre K562-Lucena e FEPS, a primeira é mais sensível através de ensaios de viabilidade e tem aumento de ROS que reflete em aumento da expressão de *ABCB1*, ao passo que a segunda é mais resistente nos ensaios de viabilidade, sem aumento dos níveis de ROS e alteração apenas na expressão de *ALOX5*. As linhagens K562-Lucena e FEPS são eritroleucemias humanas derivadas da linhagem parental K562, apesar disso, pelo fato da resistência de cada linhagem dever-se a fármacos distintos foram gerados fenótipos MDR com diferentes origens e características, podendo explicar os diferentes padrões de resposta para a C-FC.

No artigo de revisão que compõe esta tese foi destacada a necessidade de explorar não somente a expressão de *ABCB1*, mas também a atividade desta proteína, bem como a expressão e atividade de outras proteínas da família ABC no que se refere ao papel da C-FC. A necessidade de investigação apontada no artigo de revisão foi sanada através de alguns dos resultados do manuscrito 1: O *docking* proteína-proteína entre C-FC e as proteínas de efluxo (ABCB1 e ABCC1) em conjunto com os ensaios de efluxo para verificar a atividade das proteínas da família ABC contribuem para um campo até agora pouco explorado e que é fundamental para uma compreensão completa da relação entre C-FC e fenótipo MDR. Assim, ao considerar apenas o aumento da expressão de *ABCB1* para a K562-Lucena poder-se-ia gerar uma contradição em relação ao papel anti-MDR da C-FC. Entretanto, ao ser considerado o ensaio de atividade de ABCB1 por fluorimetria verifica-se que a alteração da expressão gênica não reflete em aumento da atividade da proteína, a qual não é capaz de realizar o efluxo de C-FC, tanto para a linhagem K562-Lucena quanto para a FEPS.

Além disso, através da análise de atividade de ABCC1 encontrou-se a principal via de resistência da linhagem FEPS (linhagem menos marcada por C-FC na citometria de fluxo) provavelmente mais resistente que K562 e K562-Lucena por realizar o efluxo de C-FC via ABCC1 (Figura 6). Mais uma vez, se os resultados ficassem restritos à expressão gênica e não fosse verificada alteração na expressão de *ABCC1* (como ocorreu no presente estudo) via C-FC, a proteína ABCC1 não seria considerada um mecanismo de resistência importante. Um fato importante a ser considerado é que possivelmente a C-FC não se encontra em sua forma nativa (Figura 5), pois esta seria maior que o canal para passagem de substrato da ABCC1 (Figura 2 introdução geral; Figura 3 manuscrito 1) para ser substrato para a ABCC1, que deve transportá-la por partes conjugadas a glutationa (Choudhuri e Klaassen, 2006; Szöllősi et al., 2018).

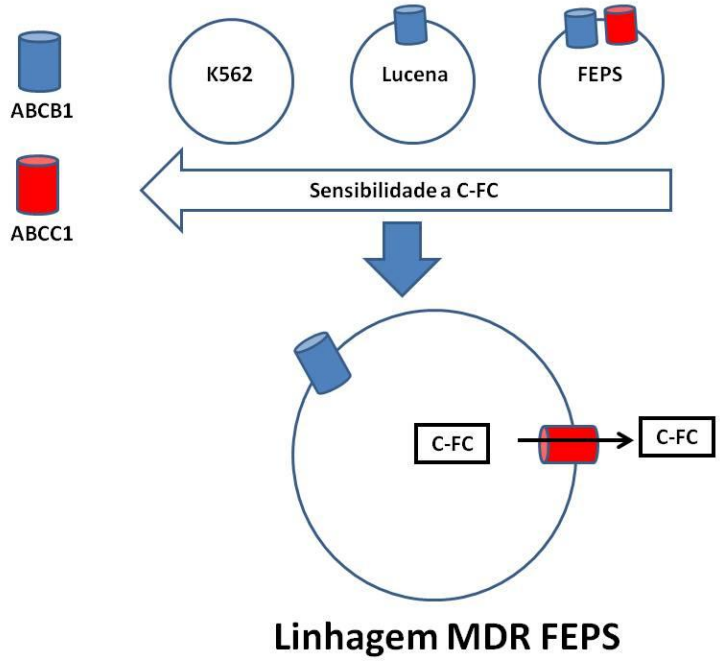

Figura 6: Extrusão de C-FC via ABCC1 para linhagem MDR FEPS como possível mecanismo de resistência.

Outro aspecto novo para a pesquisa da C-FC e fenótipo MDR foi a verificação da capacidade de interação da C-FC com os quimioterápicos Vincristina (VCR) e Daunorrubicina (DNR) através de *docking* proteína-ligante. A formação de complexo com VCR e a incapacidade da ABCB1 realizar o efluxo de C-FC pareciam indicar que a combinação de C-FC e VCR poderiam potencializar os efeitos de inibição de proliferação, contudo não se verificou esse resultado. Entretanto, a combinação de C-FC e DNR potencializou o efeito antiproliferativo em relação às substâncias isoladas para a FEPS que era mais resistente que a K562-Lucena. Isso demonstra uma possível alteração do padrão de interação com ABCC1 do complexo C-FC-DNR em relação às substâncias isoladas que podem sofrer efluxo via ABCC1, sendo este resultado um respaldo para uma das necessidades de estudo apontadas no artigo de revisão que abordava a possibilidade da associação da C-FC com quimioterápicos tradicionais para modificar a relação com proteínas de efluxo e alterar a sensibilidade das células tumorais.

No artigo de revisão da presente tese também foi destacada a possível relação entre fenótipo MDR e superexpressão de *PTGS2* (gene relacionado ao processo inflamatório) que havia sido previamente demonstrada por Carrett-Dias et al., 2011 para a linhagem K562-Lucena. Esta relação foi confirmada através da expressão de *PTGS2* para a linhagem FEPS que teve níveis semelhantes à K562-Lucena e ambas maiores que K562. Ainda, foi destacada no mesmo artigo de revisão a potencial importância da expressão de *PTGS2* como um alvo para a C-FC em linhagens MDR. Esta hipótese confirmou-se para a linhagem K562-Lucena uma vez que a C-FC aumentou os níveis de *PTGS2* e *ABCB1*, sendo que a expressão do primeiro possivelmente influencia a expressão do segundo.

Outro alvo celular para linhagens MDR parece ser o gene *ALOX5* que teve sua expressão reduzida pela C-FC tanto para K562-Lucena quanto para FEPS e semelhantemente ao *PTGS2* esse gene está envolvido com a resposta inflamatória (Reddy *et al.*, 2003). Contudo, o padrão de modulação bem como a resposta biológica gerada pela modulação de *ALOX5* é diferente entre K562-Lucena e FEPS: Para a K562-Lucena a redução da expressão desse gene pode estar envolvida com os níveis aumentados de ROS (que não estão aumentados para a linhagem FEPS) e a resposta biológica final de inibição de proliferação que pode envolver a redução da expressão de *ALOX5* gera efeitos mais pronunciados para a K562-Lucena.

A C-FC foi capaz de aumentar os níveis de espécies reativas de oxigênio (ROS) para as linhagens K562 e K562-Lucena. O fato de não aumentar os níveis de ROS para a FEPS pode indicar uma possível capacidade antioxidante aumentada da FEPS em relação à K562 e K562-Lucena. Este resultado é interessante, uma vez que reforça a ideia de que o fenótipo MDR é complexo e multifatorial (Wu et al., 2014), sendo composto não somente pela superexpressão de proteínas da família ABC, mas também de outras defesas como os antioxidantes (Trindade *et al.,* 1999; Votto *et al.,* 2007; Votto et al., 2010).

É possível que os aumentos dos níveis de ROS tenham modulado a expressão gênica. Contudo, os genes avaliados não foram alterados para a K562, sendo possível que o ROS ative vias diferentes para a K562-Lucena. Apesar dos níveis de ROS não serem aumentados para a linhagem FEPS houve modulação da expressão de *ALOX5* indicando que a modulação da expressão de FEPS deve envolver vias distintas daquelas para K562 e K562-Lucena. A expressão gênica de *ALOX5* foi modulada tanto para K562-Lucena quanto para FEPS, indicando um possível papel do *ALOX5* como alvo MDR-específico da C-FC (Figura 7). Ainda, as principais lacunas no conhecimento são: qual via celular interliga os níveis de ROS à modulação da expressão gênica; quais os genes poderiam ser modulados para a linhagem K562 e qual seria o mecanismo inicial para modular expressão gênica da linhagem FEPS.

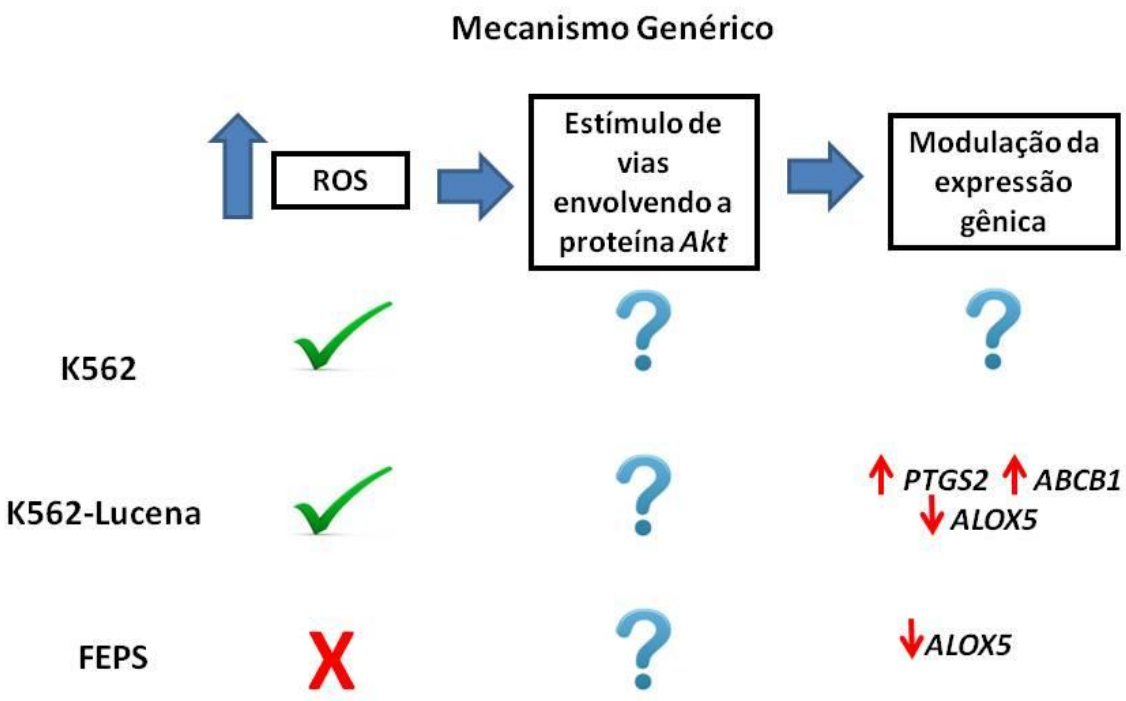

Figura 7: Mecanismo proposto para ação da C-FC sobre o modelo K562, K562-Lucena e FEPS.

Considerando-se o conjunto de dados do artigo de revisão e dos dois manuscritos que compõem esta tese conclui-se que a característica antitumoral predominante da C-FC é a inibição de proliferação e não a citotoxicidade (em menor grau para a FEPS) que pode ser gerada pelo mecanismo genérico de aumento de ROS e modulação da expressão gênica para K562 e K562-Lucena. Além disso, a C-FC é promissora para o tratamento do câncer de células com fenótipo MDR, sendo que a origem do fenótipo MDR poderá determinar uma necessidade de associação da C-FC com fármacos padrão (para linhagens mais resistentes como a FEPS). Finalmente, destaca-se a importância de aprofundamento dos estudos acerca dos mecanismos antitumorais e anti-MDR da C-FC em eritroleucemias e outras linhagens com fenótipo MDR.

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5. Anexos

Documentos de aprovação da Comissão de Ética no Uso de Animais (CEUA), Universidade Federal da Paraíba para uso de animais para obtenção de macrófagos peritoneais:

UNIVERSIDADE FEDERAL DA PARAÍBA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA)

CERTIFICADO

Certificamos que o projeto intitulado **"RELAÇÃO ENTRE RESISTÊNCIA À QUIMIOTERAPIA E O PROCESSO INFLAMATÓRIO: ATUAÇÃO DA C-FICOCIANINA E DA PIPERINA"** protocolo **nº 111/2016** sob a responsabilidade da pesquisadora **Dra. Sandra Rodrigues Mascarenhas** – que envolve a produção, manutenção e/ou a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de controle da Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Federal da Paraíba (CEUA-UFPB).

Vigência do Projeto	2016 - 2020
Espécie/linhagem	*Mus musculus - Swiss*
Número de animais	65
Idade/Peso	6-8 semanas/ 25-30 g
Sexo	Fêmeas
Origem	Biotério Thomas George - UFPB

Prof. Dr. Ricardo Romão Guerra
CEUA-UFPB

UNIVERSIDADE FEDERAL DA PARAÍBA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA)

PARECER CONSUBSTANCIADO DO PROJETO DE PESQUISA

I. DADOS DO PROJETO

1. Título do projeto:
RELAÇÃO ENTRE RESISTÊNCIA À QUIMIOTERAPIA E O PROCESSO INFLAMATÓRIO: ATUAÇÃO DA C-FICOCIANINA E DA PIPERINA

2. Pesquisador responsável:
Sandra Rodrigues Mascarenhas

3. Centro (Sigla):	4. Departamento:
CBiotec	De Biologia Celular e Molecular

5. Número de protocolo:	6. Data de submissão:	7. Data da relatoria:
111/2016	26/10/2016	13/12/2016

8. Apresentação do projeto:
Os fármacos atualmente disponíveis para o tratamento do câncer são incapazes de vencer o fenótipo de resistência a múltiplas drogas (MDR) que gera insucessos na quimioterapia. Os mecanismos que regem este fenótipo são complexos e pouco conhecidos, contudo a inflamação poderia estar envolvida, pois algumas citocinas podem ativar vias de sinalização que tornam uma célula tumoral resistente. Assim, o objetivo deste estudo é verificar a relação resistência tumoral x inflamação na presença tanto de um pigmento, a c-ficocianina (C-FC) extraída da alga *Spirulina platensis,* quanto do composto bioativo da pimenta-do-reino (piperina - PIP) em cultura de células eritroleucêmicas humanas e em cultura de macrófagos peritoneais. As células eritroleucêmicas humanas K562 (sensível a drogas), Lucena-1 e FEPS (MDR) serão expostas a diferentes concentrações das substâncias (isoladamente, sem co-exposição) e a viabilidade será avaliada pelo método de azul de Trypan. Os macrófagos, obtidos do peritônio de camundongos *Swiss* serão expostos às mesmas concentrações de substâncias das células eritroleucêmicas e a citotoxicidade, bem como os níveis de óxido nítrico, IL-1, IL-8 e TNF-α serão verificados. Logo, prende-se determinar os efeitos anti-tumorais e antiinflamatórios da C-FC e da PIP, especialmente em células com fenótipo MDR.

9. Substituição de metodologia: [ ] **SIM** [X] **NÃO**

9.1 Comentários:
(*Se achar necessário, justifique e sugira uma nova metodologia para redução do número de animais)*

II. INFORMAÇÕES RELATIVAS AOS ANIMAIS

1. Espécie:	2. Número amostral:
Mus musculus	65

3. Justificativa do número amostral: [X] **Adequado** [ ] **Inadequado**

3.1 Comentários:

4. Acomodação e manutenção:	[X] Adequado	[ ] Inadequado

4.1 Comentários:

5. Manipulação dos animais:	[X] Adequado	[ ] Inadequado

4.1 Comentários:

6. Analgesia dos animais (se aplicável):	[ ] Adequado	[ ] Inadequado

6.1 Comentários:
Não se aplica.

7. Anestesia dos animais (se aplicável):	[ ] Adequado	[ ] Inadequado

7.1 Comentários:
Não se aplica.

8. Eutanásia dos animais (se aplicável):	[X] Adequado	[ ] Inadequado

8.1 Comentários:

III. SITUAÇÃO DO PROJETO

[X] ***Aprovado*** [ ] ***Com pendência*** [ ] ***Negado***

1. Considerações sobre o parecer:
O projeto apresenta fundamentação teórica, mérito científico, viabilidade técnica e está de acordo com a Resolução Normativa Nº 13, de 20.09.2013, publicada no D.O.U. de 26/09/2013, que trata das Diretrizes da prática de eutanásia do Conselho Nacional de Controle de Experimentação Animal – CONCEA. Sendo assim somos de parecer favorável à aprovação e execução do projeto.

João Pessoa, 13 de dezembro de 2016.
Comissão de Ética no Uso de Animais.