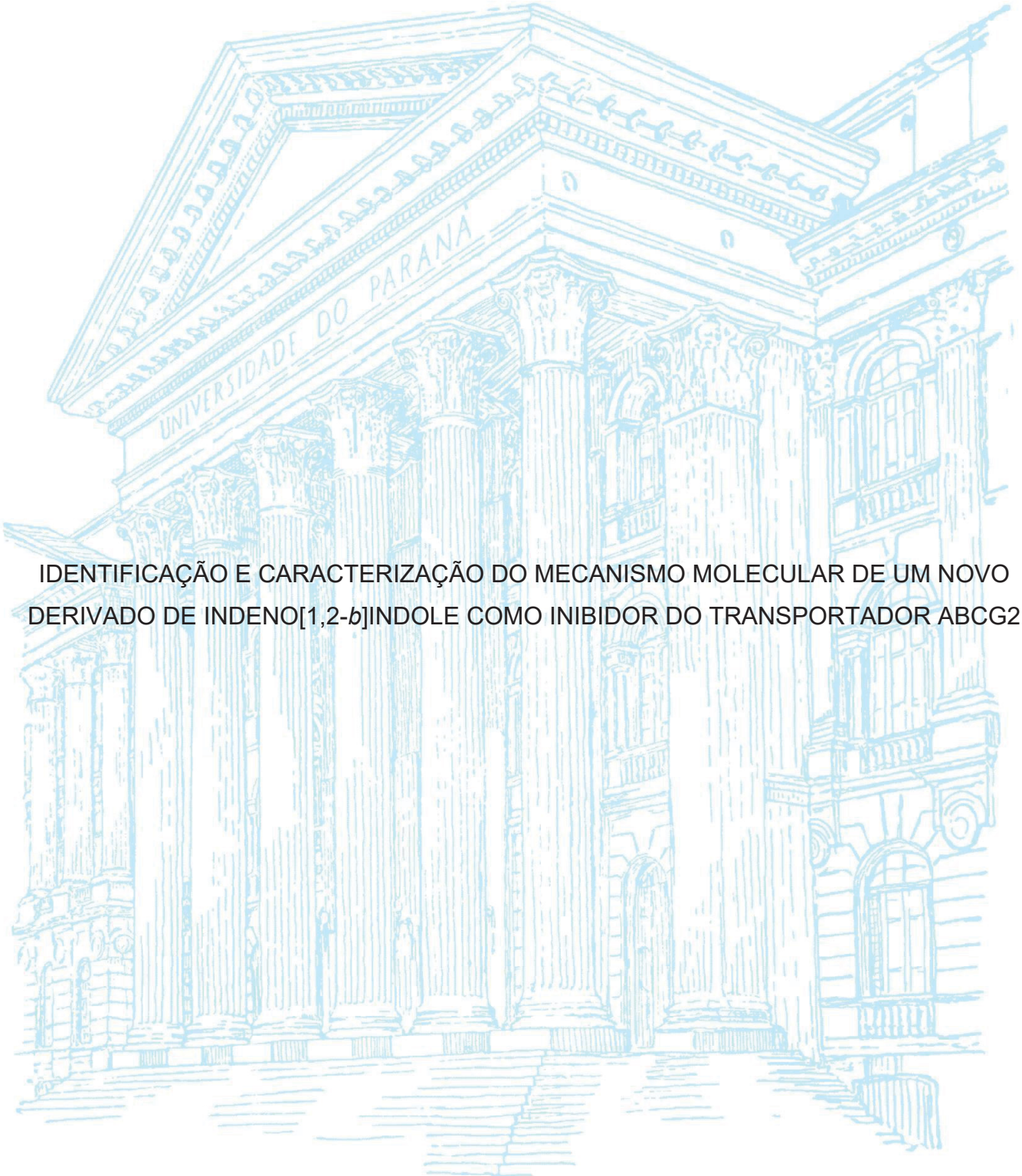
UNIVERSIDADE FEDERAL DO PARANÁ

MARINA HEMBECKER



IDENTIFICAÇÃO E CARACTERIZAÇÃO DO MECANISMO MOLECULAR DE UM NOVO DERIVADO DE INDENO[1,2-*b*]INDOLE COMO INIBIDOR DO TRANSPORTADOR ABCG2

CURITIBA

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas, Setor de Ciências da Saúde, Universidade Federal do Paraná, como requisito para a obtenção do título de Mestre em Ciências Farmacêuticas.

Orientador: Prof. Dr. Glaucio Valdameri
Coorientadora: Prof. Dra. Vivian Rotuno Moure

CURITIBA

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PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO CIÊNCIAS FARMACÊUTICAS - 40001016042P8

ATA Nº485

ATA DE SESSÃO PÚBLICA DE DEFESA DE MESTRADO PARA A OBTENÇÃO DO GRAU DE MESTRA EM CIÊNCIAS FARMACÊUTICAS

No dia quinze de agosto de dois mil e vinte e tres às 13:30 horas, na sala Auditório Mauricio Bissoli, Botânico, foram instaladas as atividades pertinentes ao rito de defesa de dissertação da mestranda **MARINA HEMBECKER**, intitulada: **Identificação e caracterização do mecanismo molecular de um novo derivado de indeno[1,2-b]indole como inibidor do transportador ABCG2**, sob orientação do Prof. Dr. GLAUCIO VALDAMERI. A Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação CIÊNCIAS FARMACÊUTICAS da Universidade Federal do Paraná, foi constituída pelos seguintes Membros: GLAUCIO VALDAMERI (UNIVERSIDADE FEDERAL DO PARANÁ), JAQUELINE PAZINATO (UFPR), GUILHERME FADEL PICHETH (UNIVERSIDADE FEDERAL DO PARANÁ), DIOGO HENRIQUE KITA (INSTITUTO DE PESQUISA PELÉ PEQUENO PRÍNCIPE). A presidência iniciou os ritos definidos pelo Colegiado do Programa e, após exarados os pareceres dos membros do comitê examinador e da respectiva contra argumentação, ocorreu a leitura do parecer final da banca examinadora, que decidiu pela APROVAÇÃO. Este resultado deverá ser homologado pelo Colegiado do programa, mediante o atendimento de todas as indicações e correções solicitadas pela banca dentro dos prazos regimentais definidos pelo programa. A outorga de título de mestra está condicionada ao atendimento de todos os requisitos e prazos determinados no regimento do Programa de Pós-Graduação. Nada mais havendo a tratar a presidência deu por encerrada a sessão, da qual eu, GLAUCIO VALDAMERI, lavrei a presente ata, que vai assinada por mim e pelos demais membros da Comissão Examinadora.

CURITIBA, 15 de Agosto de 2023.

GLAUCIO VALDAMERI
Presidente da Banca Examinadora

JAQUELINE PAZINATO
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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação CIÊNCIAS FARMACÊUTICAS da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **MARINA HEMBECKER** intitulada: **Identificação e caracterização do mecanismo molecular de um novo derivado de indeno[1,2-b]indole como inibidor do transportador ABCG2**, sob orientação do Prof. Dr. GLAUCIO VALDAMERI, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestra está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 15 de Agosto de 2023.

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Aos professores Glaucio Valdameri e Vivian Rotuno Moure, pelo apoio e por compartilharem o conhecimento para a realização deste trabalho.

A todos os meus colegas do LCDR (*Laboratory of Cancer Drug Resistance*), em especial: Arthur, Bruna, Júlia e Isadora, pela troca de conhecimentos e auxílio (e também pelos docinhos pós almoço).

Aos meus pais e irmãs pelo suporte e carinho durante toda minha vida.

Ao meu namorado, Johnatan, por sempre me encorajar e me apoiar em todas as minhas conquistas.

À UFPR e ao Programa de Pós-Graduação em Ciências Farmacêuticas, por todo o ensino e aprendizado e pela oportunidade de pesquisa.

RESUMO

O câncer consiste em uma das principais causas de mortalidade precoce, e o surgimento de resistência à quimioterapia convencional, torna esse problema ainda mais preocupante. Existem vários mecanismos conhecidos relacionados à resistência a múltiplas drogas (MDR) em pacientes com câncer, sendo a superexpressão de transportadores ABC considerada a principal causa dessa condição clínica. Dentre os 48 transportadores ABC codificados pelo genoma humano, três se destacam por seu envolvimento no fenótipo de MDR: a glicoproteína-P (P-gp ou MDR1), a proteína associada à resistência a múltiplas drogas (MRP1) e a proteína de resistência ao câncer de mama (BCRP ou ABCG2). Esses três transportadores são capazes de reconhecer e eliminar uma ampla variedade de agentes antineoplásicos, quimicamente não relacionáveis. Dessa forma, estão sendo realizados estudos para identificar novos compostos capazes de inibir a atividade dessas proteínas. Embora o ABCG2 seja um dos principais transportadores ABC associados à MDR, ainda não existem inibidores direcionados para essa proteína em estágios clínicos de desenvolvimento de novos fármacos. Portanto, é de extrema relevância a identificação e caracterização de novas moléculas que possam atuar como inibidores seletivos dessa proteína. Tendo isso em vista, quatro novos derivados de indeno[1,2-*b*]indoles foram testados quanto à capacidade de inibição de ABCG2. Somente um dos compostos, o NB4, apresentou seletividade para ABCG2 quando testados frente aos transportadores P-gp e MRP1. Além disso, constatou-se que a capacidade inibitória e não dependente de substrato, apresentando valores de IC_{50} de 208 nM para Hoechst 33342 e de 162 nM para Mitoxantrona. O composto, ainda, não apresentou citotoxicidade significativa, com um valor de IG_{50} >10 μM e razão terapêutica (RT) > 62 para Hoechst e >48 para Mitoxantrona. Foi também demonstrada a capacidade de reversão do fenótipo de resistência do NB4, mesmo este sendo um inibidor parcial, e de promover o aumento do reconhecimento do anticorpo conformacional 5D3, bem como a ausência de interações agonistas e antagonistas com outros inibidores. Por fim, ao incorporar o NB4 em uma formulação farmacêutica baseada em vesículas extracelulares (VEs), ambas as VEs de células sanguíneas e de plasma foram capazes de inibir o transportador ABCG2, demonstrando seu potencial em ensaios *in vivo*.

Palavras-chave: câncer; resistência à múltiplas drogas; transportadores ABC; ABCG2; inibidores; derivados de indeno[1,2-*b*]indole.

ABSTRACT

Cancer is one of the leading causes of premature mortality, with the added complexity of resistance to conventional chemotherapy intensifying this challenge. Several known mechanisms related to multidrug resistance (MDR) in cancer, including the overexpression of ABC transporters, are considered the primary cause of the MDR. Among the 48 ABC proteins encoded by the human genome, three stand out for their involvement in the MDR phenotype: P-glycoprotein (P-gp or MDR1), multidrug resistance-associated protein 1 (MRP1), and breast cancer resistance protein (BCRP or ABCG2). These three ABC transporters can actively transport a wide variety of chemically unrelated antineoplasic agents. To overcome the MDR mediated by ABC transporters, studies are being conducted to identify new compounds that can inhibit the activity of these proteins. Although ABCG2 is one of the main ABC transporters associated with MDR, there are currently no specific inhibitors for this protein in clinical trials. Therefore, identifying and characterizing new molecules that can act as selective ABCG2 inhibitors is of utmost importance. Hence, four new indeno[1,2-*b*]indole derivatives were tested for their ability to inhibit ABCG2 activity. Only one of the compounds, NB4, showed selectivity towards ABCG2 when tested against P-gp and MRP1. Furthermore, it was found that the inhibitory capacity was substrate-independent, showing IC_{50} values of 208 and 162 nM for hoechst 33342 and mitoxantrone, respectively. NB4 also did not exhibit significant cytotoxicity, with an IG_{50} value higher than 10 μM and a therapeutic ratio (TR) > 62 for hoechst 33342 and > 48 for mitoxantrone. A cell viability assay also demonstrated the ability of NB4 to reverse the resistance phenotype. In addition, this inhibitor increased the binding of the conformational antibody 5D3 and did not cause additive or antagonistic inhibition effects when associated with other ABCG2 inhibitors. Finally, human extracellular vesicles (EVs) were tested as a pharmaceutical formulation for NB4. Both blood cell-derived EVs and plasma-circulating EVs loaded with NB4 were able to deliver the inhibitor on target cells, demonstrating their potential for *in vivo* studies.

Key words: cancer; multidrug resistance (MDR); ABC transporters; ABCG2; inhibitors; indeno[1,2-*b*]indole derivatives.

LISTA DE FIGURAS

REVISÃO DE LITERATURA

CHAPTER 1

LISTA DE ABREVIATURAS E SIGLAS

ABC	*ATP binding cassette*
ATP	*Adenosine Triphosphate*
BCPR	*Breast Cancer Resistance Protein*
BSA	*Bovine serum albumine*
CK2	*Casein Kinase II*
C4a	*Chromone 4a*
DMEM	*Dulbecco's Modified Eagle's Medium*
DMSO	*Dimethyl sulfoxide*
EGTA	*Ethylene-bis(oxyethylenenitrilo)tetraacetic acid*
EVs	*Extracellular Vesicles*
FBS	*Fetal bovine serum*
FDA	*Food and Drug Administration*
INCA	Instituto Nacional de Câncer
IC_{50}	*Concentration that inhibits 50% of maximum inhibition*
IG_{50}	*Concentration that reduces 50% of cell viability*
MDR	*Multidrug Resistance*
MRP1	*Multidrug Resistance Protein 1*
MTT	*3-(4,5-dimethylthiazol-2-yl)-2,5-iphenyltetrazolium bromide*
MTX	*Mitoxantrone*
MXR	*Mitoxantrone Resistance*
PBS	*Phosphate Buffer Solution*
P-gp	*P-glycoprotein*
TR	*Therapeutic Ratio*
WT	*Wild-type*

SUMÁRIO

OBJETIVOS

OBJETIVO GERAL

- Estudo de derivados de indeno[1,2-*b*]indoles como inibidores seletivos do transportador ABCG2.

OBJETIVOS ESPECÍFICOS

- *Screening* de quatro derivados de indeno[1,2-*b*]indoles como inibidores do transportador ABCG2;
- Verificar a seletividade de inibição frente aos transportadores P-gp e MRP1;
- Determinar o valor de EC_{50} da inibição do transportador ABCG2 do composto mais promissor usando diferentes substratos;
- Determinar o valor de IG_{50} do composto mais promissor por ensaio de citotoxicidade;
- Determinar a razão terapêutica (TR) do composto mais promissor;
- Verificar as mudanças conformacionais no transportador ABCG2 causada pelo inibidor por meio do ensaio de ligação de anticorpo conformacional (5D3);
- Propor uma formulação farmacêutica baseada em vesículas extracelulares para o inibidor mais promissor.

1 RESUMO DA DISSERTAÇÃO

Os resultados descritos nessa dissertação trazem uma nova compreensão a respeito da identificação e caracterização do mecanismo de inibição de derivados de indeno[1,2-*b*]indoles sobre o transportador ABG2. Assim, o documento foi escrito da seguinte forma:

- Uma introdução para contextualização do tema abordando câncer e resistência à múltiplas drogas, transportadores ABC - com foco no transportador ABCG2, uso de inibidores, derivados de indeno[1,2-*b*]indoles e um tópico sobre sistemas de liberação de drogas baseadas em vesículas extracelulares;
- O capítulo 1 mostra a identificação e a caracterização do NB4, um derivado do indeno[1,2-*b*]indole como um novo inibidor seletivo e não citotóxico do transportador ABCG2.

2 REVISÃO DE LITERATURA

2.1 TUMORES MULTIRRESISTENTES E O FENÔMENO MDR

O câncer compreende um conjunto de mais de 100 doenças que apresenta maior índice de mortalidade precoce (antes dos 70 anos de idade) (INCA, 2022). No Brasil, dados apresentados pelo INCA em 2022 apontam uma estimativa de mais de 704 mil casos novos para o triênio de 2023 a 2025, sendo o câncer de mama e de próstata os mais incidentes (Fig. 1)(INCA, 2022). Na população masculina, por exemplo, o câncer mais frequente - excetuando o câncer de pele não melanoma - serão o de próstata (30,0%), cólon e reto (9,2%), traqueia, brônquio e pulmão (7,5%) e estômago (5,6%). Por outro lado, nas mulheres - também excetuando o câncer de pele não melanoma - os cânceres de mama (30,1%), cólon e reto (9,7%), colo do útero (7,0%), traqueia, brônquio e pulmão (6,0%) e glândula tireoide (5,8%) serão os mais prevalentes (INCA, 2022).

FIGURA 1 - DISTRIBUIÇÃO PROPORCIONAL DOS DEZ TIPOS DE CÂNCER MAIS INCIDENTES ESTIMADOS PARA 2023-2025 POR SEXO, EXCETO DE PELE NÃO MELANOMA

Distribuição proporcional dos dez tipos de câncer mais incidentes estimados para 2023 por sexo, exceto pele não melanoma*

Localização Primária	Casos	%			Localização Primária	Casos	%
Próstata	71.730	30,0%	Homens	Mulheres	Mama feminina	73.610	30,1%
Cólon e reto	21.970	9,2%			Cólon e reto	23.660	9,7%
Traqueia, brônquio e pulmão	18.020	7,5%			Colo do útero	17.010	7,0%
Estômago	13.340	5,6%			Traqueia, brônquio e pulmão	14.540	6,0%
Cavidade oral	10.900	4,6%			Glândula tireoide	14.160	5,8%
Esôfago	8.200	3,4%			Estômago	8.140	3,3%
Bexiga	7.870	3,3%			Corpo do útero	7.840	3,2%
Laringe	6.570	2,7%			Ovário	7.310	3,0%
Linfoma não Hodgkin	6.420	2,7%			Pâncreas	5.690	2,3%
Fígado	6.390	2,7%			Linfoma não Hodgkin	5.620	2,3%

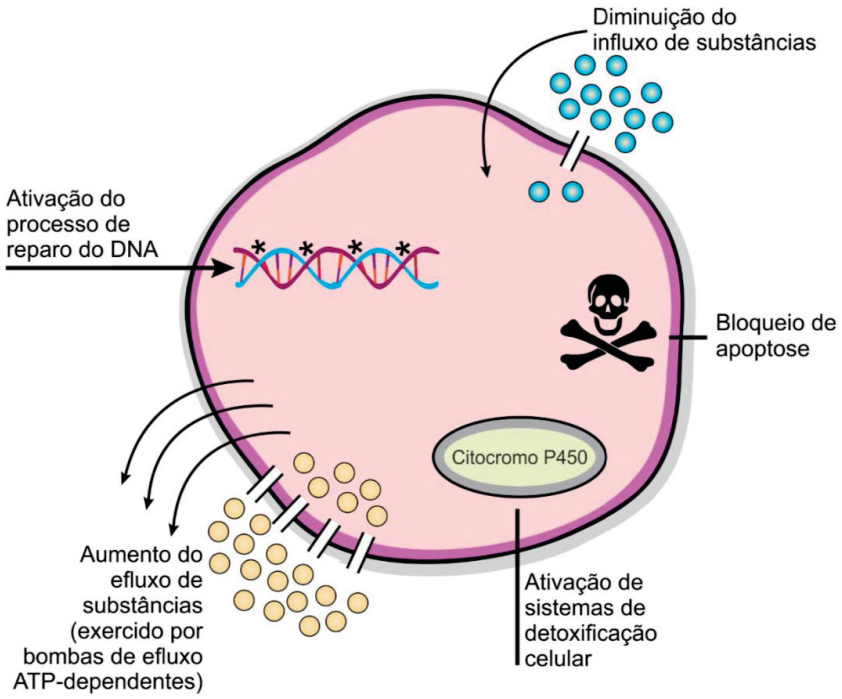
Fonte: INCA, 2022.
Nota: números arredondados para múltiplos de 10.

Apesar do grande progresso com relação à prevenção, detecção e tratamento do câncer, o desenvolvimento de resistência à quimioterapia (também conhecido como *multidrug resistance* - MDR) é um dos maiores desafios encontrados na terapêutica atual, sendo responsável pelo insucesso no tratamento de mais de 90% de cânceres metastáticos (BUKOWSKI; KCIUK; KONTEK, 2020; SAMPSON *et al.*, 2019; SUN *et al.*, 2012). Esse quadro clínico deve-se a inúmeras condições, a citar:

aumento da capacidade de reparo do DNA, diminuição do influxo de substâncias e falha dos mecanismos de morte celular programada, como a apoptose. Contudo, o principal mecanismo de MDR é o efluxo ativo de quimioterápicos mediados pelos transportadores ABC (ATP *binding cassete*) (GOTTESMAN; FOJO; BATES, 2002; SZAKÁCS *et al.*, 2006).

Ainda, a MDR mediada pelos transportadores ABC está relacionada ao desenvolvimento de resistência à inúmeros fármacos não relacionados quimicamente, haja vista que esses transportadores são descritos como promíscuos (FALASCA; LINTON, 2012; ROBEY *et al.*, 2018; SZAKÁCS *et al.*, 2006). Portanto, pode-se dizer que a expressão dos transportadores ABC em pacientes oncológicos com quadros clínicos de MDR possui um impacto significativo, sendo considerado o principal mecanismo de resistência ao tratamento, uma vez que o quimioterápico não permanece dentro da célula na concentração correta e nem pelo tempo suficiente para exercer seu efeito biológico (Fig. 2) (FALASCA; LINTON, 2012; GOTTESMAN; FOJO; BATES, 2002; ROBEY *et al.*, 2018).

FIGURA 2 - Representação dos mecanismos celulares capazes de causar resistência à múltiplas drogas



Fonte: ZANZARINI, 2019.

Nota: As células cancerosas podem se tornar resistentes a drogas por diversos mecanismos. Um deles é efluxo destas por meio do aumento da atividade de bombas de efluxo dependente de ATP, como transportadores ABC; outro é a redução do influxo de drogas. Nos casos em que o acúmulo de drogas não é alterado, a ativação de proteínas de detoxificação celular - como o citocromo P450 - pode promover a resistência. As células podem, também, ativar mecanismos que reparam danos no DNA

induzidos por drogas. Por fim, alterações nas vias de sinalização da apoptose faz com que as células se tornem resistentes à morte celular induzida por drogas.

2.2 TRANSPORTADORES ABC

Os transportadores ABC fazem parte de uma superfamília de proteínas que está presente em todos os reinos biológicos. São responsáveis pelo transporte de substratos pelas membranas celulares, utilizando energia proveniente da ligação e hidrólise de ATP (FORD; BEIS, 2019; KATHAWALA *et al.*, 2015). Em bactérias, esses transportadores realizam a captação de substâncias do meio extracelular para o intracelular, enquanto, em mamíferos, são responsáveis quase que exclusivamente pelo efluxo de substâncias (KATHAWALA *et al.*, 2015). De maneira geral, os transportadores ABC regulam os níveis celulares de hormônios, lipídeos, íons, xenobióticos e outras moléculas. Além disso, participam do controle intracelular de organelas, a exemplo da mitocôndria, lisossomos, retículo endoplasmático e Complexo de Golgi (ROBEY *et al*., 2018).

Em humanos foram identificados 48 genes que codificam proteínas da família ABC, as quais são classificadas de acordo com a similaridade filogenética e organização dos domínios em sete subfamílias, de ABCA a ABCG (FORD; BEIS, 2019; KATHAWALA *et al.*, 2015), todas com função de transporte, exceto as famílias ABCE e ABCF, as quais não apresentam função clara de efluxo de substâncias (FORD; BEIS, 2019).

Essas proteínas são geralmente expressas em tecidos como intestinos, cérebro e placenta, com o objetivo de prevenir o acúmulo de xenobióticos. Consequentemente, este papel protetivo leva os transportadores a afetar parâmetros farmacocinéticos de drogas, como absorção, distribuição, metabolismo e excreção (ROBEY *et al.*, 2018; SZAKÁCS *et al*., 2006).

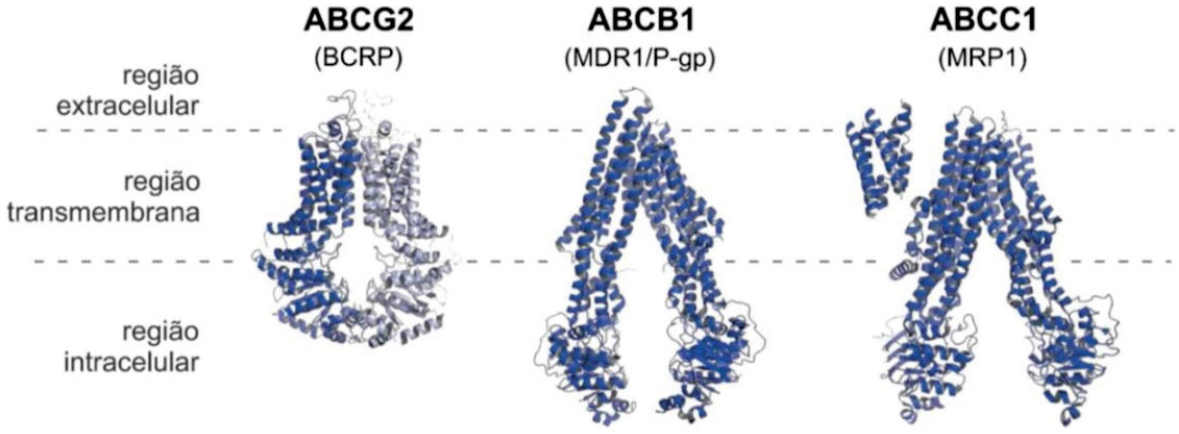
Importante ressaltar que os principais transportadores ABC envolvidos no fenômeno de MDR são a glicoproteína P (também conhecida como MDR1 ou P-gp), a proteína 1 associada à resistência a múltiplas drogas (MRP1) e a proteína de resistência ao câncer de mama (conhecida por BCRP- *breast cancer resistance protein*). Estes são codificados pelos genes *ABCB1*, *ABCC1* e *ABCG2*, respectivamente (ECKENSTALER; BENNDORF, 2020; SZAKÁCS *et al.*, 2006).

Os transportadores ABC são essencialmente compostos por dois domínios transmembrana (TMDs - *Transmembrane Domains*) e dois domínios ligantes de

nucleotídeo (NBDs - *Nucleotide Binding Domain*). Os TMDs são constituídos por 6 α-hélices transmembrana, os quais são responsáveis por reconhecer e transportar os substratos; enquanto os últimos encontram-se no citoplasma e são responsáveis pela ligação e hidrólise do ATP necessário para o transporte (ECKENSTALER; BENNDORF, 2020; FORD; BEIS, 2019; ROBEY *et al.*, 2018).

Quando esses quatro domínios estão presentes como uma única cadeia polipeptídica, esses transportadores são chamados de completos (*full transporters*), como observado na estrutura do transportador ABCB1 (P-gp). Em contrapartida, os transportadores incompletos (*half transporters*), como o ABCG2, possuem somente um domínio TMD e um domínio NBD - também expressos em uma cadeia polipeptídica - e precisam dimerizar-se para apresentar funcionalidade (ECKENSTALER; BENNDORF, 2020; ROBEY *et al.*, 2009). Ainda, há a variante estrutural presente no subtipo ABCC, a exemplo do transportador ABCC1 (MRP1), composta por um domínio transmembrana adicional (designado TMD0), o qual possui 5 α- hélices que não faz parte da cadeia principal (ROBEY *et al.*, 2018; SZAKÁCS *et al.*, 2006). Os três modelos estruturais citados estão representados na figura a seguir (Fig. 3).

FIGURA 3 - Estruturas tridimensionais em alta resolução dos transportadores ABCG2, P-gp e MRP1

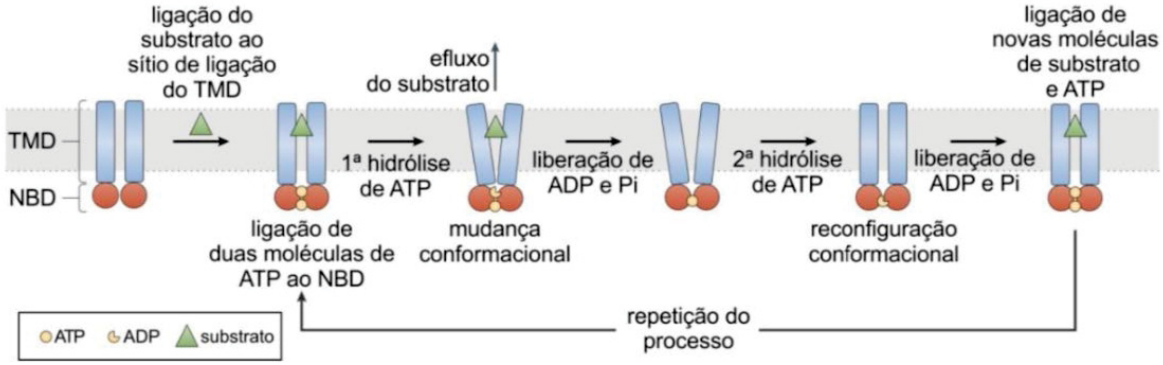


Fonte: Adaptado (ROBEY *et al.*, 2018).

Os domínios TMDs, presentes na membrana plasmática, desempenham um papel crucial no reconhecimento e transporte de substratos. A ligação e liberação desses substratos no meio extracelular requer mudanças conformacionais nos TMDs. Essas alterações são induzidas pela ligação e hidrólise de duas moléculas de ATP nos NBDs, que são partes conservadas da proteína. Dessa forma, após o substrato

se ligar ao sítio de ligação nos TMDs, duas moléculas de ATP se ligam aos NBDs. A hidrólise da primeira molécula de ATP direciona as mudanças conformacionais nos TMDs, resultando na liberação do substrato, devido à abertura da proteína em direção ao meio extracelular. A segunda molécula de ATP é então hidrolisada para que o transportador retorne à sua conformação inicial, permitindo a ligação e saída de novos substratos. Esse processo completa um ciclo catalítico (Fig. 4) (CHEN *et al.*, 2016; ECKENSTALER; BENNDORF, 2020; ROBEY *et al.*, 2018).

FIGURA 4 - Representação esquemática do processo completo de efluxo de substratos



Fonte: Adaptado (ROBEY *et al.*, 2018).

É importante destacar que, embora a estrutura e a função dos NBDs sejam semelhantes entre os transportadores ABC, os TMDs apresentam uma composição altamente heterogênea, o que permite o reconhecimento de uma ampla variedade de substratos. Além disso, a energia derivada da ligação e hidrólise das moléculas de ATP permite o transporte de substâncias através da membrana independentemente do gradiente de concentração predominante na célula (ROBEY *et al.*, 2018).

2.2.1 Transportador ABCG2

O transportador ABCG2 foi descoberto praticamente em concomitância por três laboratórios diferentes, sendo denominado de BCRP (*breast cancer resistance protein)* devido a conferir resistência em células de câncer de mama (MCF-7) (DOYLE *et al.*, 1998), ABCP, nome relacionado à sua expressão abundante na placenta (ALLIKMETS *et al.*, 1998), e MXR, relacionado à resistência a mitoxantrona (MYIAKE *et al*., 1999).

Este transportador, codificado pelo gene *ABCG2*, ao contrário dos demais já citados, é classificado como um transportador incompleto, uma vez que possui apenas

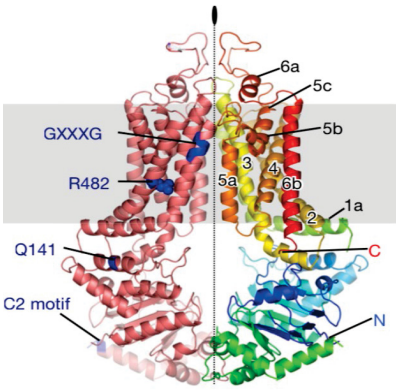
um domínio TMD - constituído por 6 α-hélices - e um domínio NBD, unidos por uma única cadeia polipeptídica. Assim, sua conformação funcional é a de homodímero estabilizado por ligações dissulfeto (ROBEY *et al.*, 2009; SCHINKEL; JONKER, 2003), como representado na Figura 5.

Essa proteína, de 72kDa, encontra-se expressa regularmente em células-tronco, nas barreiras hematoencefálica (BBB), hematotesticular (BTB) e hematoplacentária (BPB), além de células hepáticas e renais. Como ABCG2 é expresso na membrana apical das células e, portanto, na superfície de órgãos que realizam a excreção, também possui papel fundamental na proteção e detoxificação dos tecidos contra xenobióticos, bem como apresenta a capacidade de alterar características farmacocinéticas de certas drogas, o que reforça a sua função protetiva (ROBEY *et al*., 2007; SCHINKEL; JONKER, 2003; TAYLOR *et al*., 2017).

O ABCG2 é responsável pelo transporte de uma ampla variedade de compostos, incluindo agentes citotóxicos como mitoxantrona, topotecan e metotrexato, corantes fluorescentes como o Hoechst 33342, e certos compostos tóxicos encontrados em alimentos (SARKADI *et al*., 2004).

Diversos estudos demonstraram que a superexpressão desse transportador leva a uma diminuição significativa na eficácia dos regimes quimioterápicos, resultando em um fenótipo resistente a várias drogas. Essa superexpressão também foi observada em células hematopoiéticas e linfoides malignas no ambiente tumoral, sugerindo uma possível função do ABCG2 como marcador de células-tronco imaturas e leucêmicas. Além disso, o referido transportador também está presente na "*side population*" (SP), uma subpopulação de células encontradas em certos tumores que exibem características semelhantes às células-tronco, como resistência a múltiplas drogas e alta capacidade de formação de tumores (ROBEY *et al.*, 2007; SCHINKEL; JONKER, 2003; TAYLOR *et al.*, 2017).

FIGURA 5 - Estrutura tridimensional do transportador ABCG2

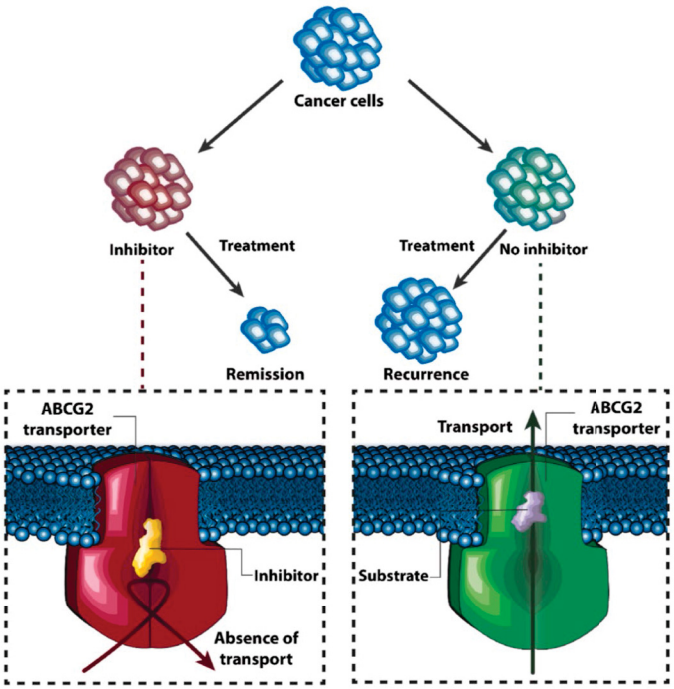


Fonte: Taylor *et al.*, 2017
Nota: Estrutura tridimensional do transportador ABCG2, resolvida por crio-microscopia eletrônica de única partícula com resolução de 3.8 å.

2.3 USO DE INIBIDORES DE TRANSPORTADORES ABC E DESENVOLVIMENTO DE NOVAS DROGAS

A estratégia mais promissora para contornar o fenótipo de MDR é o uso de inibidores dos transportadores ABC. Estes, ao bloquearem o efluxo das drogas anticâncer, melhoram a resposta ao tratamento farmacológico, resultando idealmente na remissão do tumor, como representado a seguir (Fig. 6) (ZATTONI *et al.*, 2022).

FIGURA 6 - Representação do uso de inibidores como estratégia para reverter o fenótipo de MDR



Fonte: ZATTONI *et al.*, 2022

Essa busca por novos inibidores compreende várias abordagens, como o reposicionamento de drogas, a triagem de diferentes classes de compostos e o desenho racional de drogas (ZATTONI *et al.*, 2022b).

A primeira delas possui um benefício de que, ao utilizar-se de drogas já testadas em humanos e que já são utilizadas no tratamento de outras doenças, reduziria drasticamente o tempo e o custo do desenvolvimento de uma nova droga (SHIM; LIU, 2014). Por sua vez, a triagem de estruturas químicas de diferentes classes moleculares continua sendo a alternativa mais utilizada, uma vez que compostos naturais - como flavonoides e polifenóis - são grande fonte de esqueletos químicos e, consequentemente, uma fonte abundante de inibidores (GANDHI; MORRIS, 2009). Por fim, estudos de relação estrutura-atividade permitem a síntese racional de drogas, pois, ao analisar a influência de certos substituintes ou grupos carbônicos, pode-se chegar a uma estrutura química otimizada (MATTER *et al.*, 2001).

Porém, existe dúvida quanto a utilização de inibidores dos transportadores ABC na clínica, devido às funções que essa família de proteínas possui no organismo. Dessa forma, é imprescindível a pesquisa e desenvolvimento de compostos específicos para cada transportador, os quais inibiriam suas atividades de maneira seletiva. Importante ressaltar que o bloqueio seletivo de um transportador ABC envolvido na resistência a múltiplas drogas não afetaria a atividade fisiológica dos demais - visto que esses transportadores apresentam similaridades estruturais - e garantiria o processo de detoxificação celular, realizado por estes de maneira regular (ROBEY *et al.*, 2018; THEILE; WIZGALL, 2021).

2.3.1 Uso de inibidores no transportador ABCG2

O primeiro inibidor seletivo para o transportador ABCG2 reportado foi a toxina fúngica *Fumitremorgin C* (FTC), porém, a elevada neurotoxicidade em ensaios *in vivo* levou à impossibilidade do seu uso na clínica (NISHIYAMA; KUGA, 1989; RABINDRAN *et al.*, 1998). A fim de obter inibidores mais potentes e com menor toxicidade, foram desenvolvidos análogos sintéticos do FTC, dentre os quais se destaca o Ko143, que se mostrou promissor *in vitro*, apesar de citotoxicidade residual (ALLEN *et al*., 2002).

Posteriormente, foram desenvolvidos outros inibidores específicos de ABCG2 a partir da modificação estrutural de inibidores já conhecidos, como o elacridar e o

tariquidar. Estas substâncias consistem em séries de compostos não relacionáveis quimicamente, a exemplo de cromonas (HONORAT *et al.*, 2015; PIRES *et al*., 2016; VALDAMERI *et al.*, 2012c), chalconas (VALDAMERI *et al.*, 2012b), estilbenos(VALDAMERI *et al*., 2012a), porfirinas(ZATTONI *et al.*, 2022a) e indeno[1,2-*b*]indoles (GOZZI *et al*., 2015a, 2015b; KITA *et al.*, 2021), as quais apresentaram resultados satisfatórios nos ensaios *in vitro*. Porém, ainda não há inibidores seletivos para ABCG2 que seguiram para a realização de testes clínicos, o que ressalta a relevância e urgência da descoberta de um inibidor seletivo desse transportador.

Algumas características relevantes e que devem ser levadas em consideração no desenvolvimento de um novo inibidor de ABCG2 é a alta potência de inibição - com concentrações de inibição em escala nanomolar, baixa ou nenhuma citotoxicidade e alta seletividade com relação aos principais transportadores ABC envolvidos com MDR (P-gp e MRP1) (ZATTONI *et al.*, 2022b).

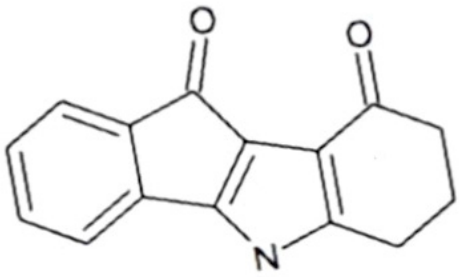
2.4 INDENO[1,2-b]INDOLES

A porção estrutural indenoindólica ocorre em vários produtos naturais, bem como o núcleo indólico (KAUSHIK *et al*., 2013; PRAKASH; RAJA, 2012; RONGVED *et al.*, 2013) e apresentam vasta gama de funções biológicas, como atividades antioxidante e anticancer (KASHYAP *et al.*, 2013; RONGVED *et al.*, 2013). Além disso, os núcleos heterocíclicos permitem modificações e a adição de vários grupos substituintes em sua estrutura (Fig. 7) (LOBO *et al*., 2015; RONGVED *et al*., 2013). Nesse âmbito, derivados de indeno[1,2-*b*]indoles foram desenvolvidos, inicialmente como inibidores da proteína quinase II (CK2)(GOZZI *et al*., 2015a, 2015b).

A CK2 é uma proteína heterotetramérica, composta por duas subunidades catalíticas (α) e duas subunidades regulatórias (β), e pode utilizar-se de ATP ou de GTP como cofator. A superexpressão e a elevação da atividade de CK2 está intimamente relacionada a vários tipos de câncer, como próstata, mama, pulmão e pâncreas, impactando de forma significativa na sobrevida do paciente (BORGO *et al.*, 2021; GOZZI *et al.*, 2015a, 2015b; HUNDSDÖRFER *et al.*, 2012). É também evidenciado que a CK2 está envolvida na potencialização do fenótipo de MDR, uma vez que aumenta a expressão e a atividade de efluxo dos três principais transportadores ABC (P-gp, MRP1 e ABCG2) (BORGO *et al*., 2021).

Devido a essa interação entre CK2 e os transportadores ABC, evidenciou-se que inibidores dessa quinase poderiam agir como inibidores do transportador ABCG2, a depender dos substituintes presentes no núcleo indeno[1,2-*b*]indólico. Dessa forma, novos derivados desses compostos vêm sendo sintetizados como um desenho racional objetivando uma molécula específica e potente como inibidores seletivos desse transportador (BORGO *et al.*, 2021; GOZZI *et al.*, 2015a, 2015b; KITA *et al.*, 2021).

FIGURA 7 - Representação da estrutura base de um Indeno[1,2-*b*]Indole



Fonte: Adaptado (GOZZI *et al.*, 2015a).

2.5 SISTEMA DE LIBERAÇÃO DE DROGAS BASEADAS EM VESÍCULAS EXTRACELULARES

Recentemente, sistemas de liberação de drogas (*drug delivery*) vêm ganhando importância, uma vez que podem solucionar problemas de entrega de medicamentos com baixa solubilidade em água (PATRA *et al*., 2018), como é o caso dos inibidores do transportador ABCG2 (SHUKLA; WU; AMBUDKAR, 2008). Dessa forma, a aplicação de nanoestruturas, como lipossomas (LI *et al.*, 2017), microemulsões (QU *et al.*, 2017) micelas (QU *et al*., 2019) e vesículas extracelulares (ELSHARKASY *et al*., 2020; N'DIAYE *et al*., 2022; PATRA *et al.*, 2018) pode impactar significativamente na solubilidade, absorção e biodisponibilidade, bem como na redução dos efeitos adversos dessas drogas (ELSHARKASY *et al*., 2020; PATRA *et al*., 2018; SERCOMBE *et al.*, 2015).

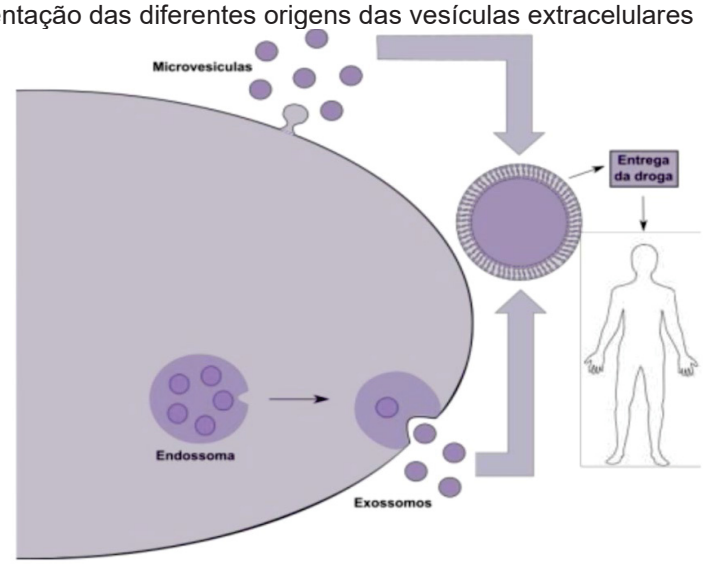
As vesículas extracelulares (VEs), mais que eliminar compostos não necessários para as células, têm papel fundamental na troca de componentes entre células - ácidos nucleicos, lipídeos e proteínas - bem como na sinalização em processos homeostáticos celulares normais ou como consequência de patologias (COLOMBO; RAPOSO; THÉRY, 2014; LO CICERO; STAHL; RAPOSO, 2015).

Os lipossomos - nanocarreadores mais comuns - são compostos por uma bicamada lipídica, a qual possibilita acomodar drogas hidrofóbicas, e uma fase interna aquosa, capaz de acondicionar drogas hidrofílicas (ELSHARKASY *et al.*, 2020; SERCOMBE *et al*., 2015). Esse sistema permitiu com que fossem desenvolvidas formulações farmacêuticas que viabilizassem a administração de diversas drogas. A primeira delas, Doxil®, foi a primeira formulação lipossomal disponível no mercado, sendo aprovada no ano de 1995 pelo *Food and Drug Administration* (FDA) (BARENHOLZ, 2012).

Por sua vez, as VEs - também com composição de bicamada lipídica - são secretadas por todos os tipos celulares, variando em número, composição e características físico-químicas (JONG *et al.*, 2019). Importante ressaltar que as VEs possuem vantagem significativa frente aos sistemas sintéticos uma vez que, por terem origem biológica, seu uso não resulta em reações de hipersensibilidade (ELSHARKASY *et al.*, 2020; PATRA *et al.*, 2018).

As VEs fazem parte de uma população heterogênea de partículas, as quais podem ser classificadas em duas categorias: exossomas e microvesículas. A primeira delas - exossomas - possuem de 30 a 100 nm de diâmetro e são vesículas intraluminais originadas do brotamento interno da membrana endossomal durante o processo de maturação dos endossomos multivesiculares (MVEs). Esses MVEs atuam como intermediários no sistema endossomal e são liberados após fusão com a superfície celular. Por sua vez, as microvesículas são geradas pelo brotamento externo e fissão da membrana plasmática e subsequente liberação no espaço extracelular, e possuem tamanho de 50 a 1000 nm de diâmetro (Figura 7) (ALBERRO *et al*., 2021; ELSHARKASY *et al*., 2020; JONG *et al*., 2019; VAN NIEL; D'ANGELO; RAPOSO, 2018).

FIGURA 8 - Representação das diferentes origens das vesículas extracelulares



Fonte: Dutra, 2022
Nota: Representação das origens de vesículas extracelulares secretadas por células viáveis: exossomos e microvesículas.

As VEs podem ser encontradas em diversos fluidos biológicos, como saliva, leite materno, urina, líquido amniótico e saliva; porém uma das fontes mais estudadas e aplicadas é o sangue (CABY *et al*., 2005; ELSHARKASY *et al.*, 2020; KELLER *et al.*, 2007; KIM; LEE; GHO, 2017). Neste caso, tem-se as VEs secretadas pelas próprias células sanguíneas e aquelas circulantes, as quais podem possuir diversas origens e são facilmente obtidas por meio de amostras minimamente invasivas (ALBERRO *et al.*, 2021). Vale ressaltar que o processo de vesiculação também pode ser induzido por meio do aumento da concentração de cálcio citoplasmático aliado à alteração na simetria dos fosfolipídios da membrana plasmática, embora o processo ainda não seja completamente esclarecido (RAPOSO; STOORVOGEL, 2013). Sugere-se, então, que a elevação dos íons Ca^{2+} realiza a ativação de algumas enzimas - escramblase, calpaína e gelsolina - que desempenham papel no transporte de fosfolipídios e nas alterações no citoesqueleto (COCUCCI; RACCHETTI; MELDOLESI, 2009). Assim, leva-se à formação de microvesículas constituídas por uma bicamada lipídica composta por fosfolipídios intercalados com proteínas de membrana e de adesão, além do colesterol (RAPOSO; STOORVOGEL, 2013).

Dessa forma, pode-se evidenciar que as VEs produzidas por células sanguíneas podem ser utilizadas como um sistema de liberação de drogas, uma vez que há na literatura exemplos bem-sucedidos de estudos *in vitro*, a exemplo da entrega da Cromona 4a como inibidor do transportador ABCG2 (VALDAMERI *et al*., 2023), bem como *in vivo* com a utilização dessas VEs para entrega de dopamina (QU

*et a*l., 2018) e de um peptídeo terapêutico (BAY55-9837), que tem como alvo células pancreáticas objetivando o aumento da secreção de insulina (ZHUANG *et al*., 2019).

Portanto, devido à sua alta capacidade de incorporação de drogas - principalmente de compostos hidrofóbicos - e sua baixa toxicidade e imunogenicidade, as vesículas extracelulares surgem como uma alternativa promissora para sistemas de liberação de drogas.

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CHAPTER 1

Title: Identification of a new indeno[1,2-*b*]indole as a potent, selective and non-cytotoxic ABCG2 inhibitor

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1 INTRODUCTION

The emergence of multidrug resistance (MDR) poses a significant challenge in cancer treatment. MDR is marked by the resistance to unrelated drugs and can result from various factors, such as alterations in drug metabolism, disruptions in programmed cell death regulatory mechanisms, diminished drug uptake, and more [1]. However, the predominant mechanism behind MDR is associated with the active expulsion of anticancer drugs facilitated by ATP-binding cassette (ABC) transporters. The overexpression of these transporters profoundly impacts intracellular drug concentrations, thereby affecting treatment effectiveness [2,3].

The human genome encodes 48 ABC proteins, with three of them playing a pivotal role in the MDR phenotype: P-glycoprotein (P-gp or MDR1 - encoded by the *ABCB1*), Multidrug Resistance Protein 1 (MRP1 - encoded by the *ABCC1*), and Breast Cancer Resistance Protein (BCRP - encoded by the *ABCG2*) [3–5].

Overcoming MDR mediated by ABC transporters is commonly achieved through the use of functional inhibitors [6]. These inhibitors, when combined with anticancer drugs, can elevate intracellular drug concentrations, thereby sensitizing tumors to their cytotoxic effects [6].

Despite recent advancements, potent and non-toxic ABCG2 inhibitors suitable for clinical trials remain in short supply, given the overexpression of these transporters in various cancer types. In recent years, several classes of compounds have shown promise as potent ABCG2 inhibitors, including chromones [7–9], chalcones [10], stilbenes [11], porphyrins (ZATTONI *et al*., 2022a), and indeno[1,2-*b*] indoles [12–14]. While these compounds have shown promising results in vitro, there is still a lack of selective inhibitors for ABCG2 that have progressed to clinical trials, underscoring the importance and urgency of discovering a selective inhibitor for this transporter.

Initially developed as inhibitors of human protein kinase casein kinase II (CK2), indeno[1,2-*b*]indoles were found to interact with ABC transporters associated with the MDR phenotype, such as ABCG2, albeit with distinct structure-activity relationships [12]. This discovery suggests that with appropriate modifications, CK2 inhibitors can be converted into ABCG2 inhibitors and vice versa [12].

Based on these insights, a rational drug design approach was initiated to identify the optimal structure-activity relationship for an ABCG2 transporter inhibitor. In this study, new derivatives of indeno[1,2-*b*]indole were tested as ABCG2 inhibitors, and the inhibitory mechanism of the most promising compound was characterized using cell-based assays to assess its potential.

2 MATERIAL AND METHODS

2.1 Chemistry

The indeno[1,2-*b*]indoles derivatives (NB4, NA16-1, AF95a and AF84a) were synthesized by Prof. Dr. Marc Le Borgne's research group from *Université Claude Bernard Lyon 1*, Institute of Pharmaceutical and Biological Sciences, Lyon - France. All the compounds were prepared in DMSO at 10 mM and maintained at -20ºC and thawed when necessary.

2.2 Cell lines and culture

HEK293 cells, as well the stably transfected HEK293-*ABCG2*, NIH3T3-*ABCB1* and BHK21-*ABCC1* cells were kindly provided by Dr. Attilio Di Pietro (IBCP, Lyon, France). All cells were maintained in High Glucose Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% of fetal bovine serum (FBS), 1% penicillin/streptomycin, and 0,25 mg/mL amphotericin at 37ºC and 5% CO_2, under controlled humidity.

2.3 Transport Assay

The transfected cells (HEK293-*ABCG2*, NIH3T3-*ABCB1* and BHK21-*ABCC1*) were seeded in a 24-well plate with a density of $2.0x10^5$ cells/well and incubated for 48 hours at 37ºC and 5% CO_2. When the cell confluence reached 90%, the medium was removed, and the cells were treated with different concentrations of the tested compounds (or with the reference inhibitors) and the substrates for 30 minutes at 37ºC and 5% CO_2. After incubation, the media was removed, and the cells were washed with saline solution (PBS) at 37ºC, trypsinized, and resuspended in cold PBS. For bimodulation assays, cells were treated with one or two inhibitors simultaneously under conditions as described before.

The intracellular fluorescence was measured by flow cytometry with acquisition of at least 10,000 events. The maximum fluorescence, taken as 100%, was determined by the median of fluorescence of the transfected cells incubated with the reference inhibitors (Verapamil 30 µM for MRP1, GF120918 1 µM for P-gp and Ko143 1 µM for ABCG2).

The percentage of inhibition was calculated according to the formula:

$$\%\, Inibition = \frac{(C - S)}{(I - S)} \times 100$$

in which "*C*" represents the intracellular fluorescence of the substrate plus the tested compounds, "*S*" represents the intracellular fluorescence in the presence of the substrate alone and "*I*" represents the intracellular fluorescence of the substrate plus the reference inhibitor.

2.4 Cytotoxicity assay

Cell viability was evaluated by a colorimetric assay using diphenyltetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-bromide (MTT) [15]. First, parental cell line (HEK293) and transfected cell line (HEK293-*ABCG2*) were seeded in 96-well plates at a density of $1.5x10^4$ cell per well. After overnight incubation, the cells were treated with the compounds at various concentrations (0.020 - 10 µM) for 72 hours at 37ºC and 5% CO_2. For chemosensitization assay (MDR phenotype reversion), cells were simultaneously treated with NB4 at 1 µM (saturating concentration) and SN-38 at 0.1 µM for 72 hours. Afterward, 20 µL of MTT (5 mg/mL) was added, and the cells were incubated for 4 hours at 37ºC and 5% CO_2. Then, the formazan crystals were dissolved with a solution 1:1 of DMSO and ethanol, and the absorbance was measured using an iMark microplate reader (Bio-Rad) at 595 nm wavelength. Results express the percentage of viable cells compared to the control cells (1% DMSO), taken as 100%.

2.5 Conformational antibody binding (5D3) assay

The conformational antibody binding (5D3) assay was performed with HEK293-*ABCG2* cells. Cells were detached with trypsin and centrifuged at 1,000 x g for 3 minutes. The supernatant was removed and the cells were resuspended in 100 µL of (PBS). Then, 4 µL of BSA solution (1 mg/mL) and NB4 at 10 µM or reference inhibitor (Ko143) at 2 µM were added and incubated for 10 minutes at 37ºC. After that, 1 µL of the primary antibody (5D3) was added (Purified mouse anti-human CD338, BD Pharmigen - 1:100 dilution) and incubated for 30 minutes at 37ºC. Another centrifugation was made at 1,000 x g for 3 minutes, the supernatant was removed, and the cells were resuspended in 100 µL of PBS, and then 0.5 µL of the secondary antibody was added (PE Goat anti-mouse IgG, Abcam- 1:200 dilution) and incubated for 30 minutes at 37ºC. After this step, the cells were centrifuged at 1,000 x g for 3 minutes and resuspended in 300 µL of PBS. Then, an analysis by flow cytometry (FACS Celesta - Becton Dickinson) at B-575 channel was performed. The results were expressed as a ratio related to the control.

2.6 Preparation of human extracellular vesicles (EVs) containing NB4

To obtain the human EVs, 9 mL of blood was collected and 8 mM of EGTA was added. Then, the material was centrifuged at 425 x g for 10 minutes to allow the separation of the blood cells from the plasm. Plasma was stored in a 15 mL falcon tube (approximately 4 mL) while the cells were further processed. RPMI medium (same amount of volume, approximately 5 mL) was added to the cells and the material was centrifuged again at 425 x g for 10 minutes. The supernatant was discarded and the cell pellet resuspended with 5 mL of RPMI medium containing 2 mM $CaCl_2$ and incubated for one hour at 37ºC to stimulate the vesiculation.

After this time, the plasm and the cells were centrifugated at 425 x g for 10 minutes at 10ºC, and the supernatant was transferred to microcentrifuge tubes. This material was centrifuged at 4,000 x g for 30 minutes at 10ºC, transferring the supernatant from this step again to new 1.5 mL tubes that were subjected to the last centrifugation step at 11,000 x g for 120 minutes at 10ºC. In this last step, the supernatant was discarded and the pellet containing the EVs was solubilized in 60 µL of PBS.

The EVs were then transferred to new microcentrifuge tubes (10 µL) and diluted in PBS (190 µL) and treated with the reference inhibitor Ko143 (1 µM) and the tested inhibitor (NB4) at a concentration of 10 µM and incubated at room temperature for 30 minutes to enable the incorporation of the compounds on the EVs. After this time, the tubes were centrifuged at 11,000 x g for 120 minutes and 10ºC to remove the solvent DMSO. The supernatant was transferred to other microcentrifuge tubes, and the EVs from cells and plasm pellets were resuspended with 50 µL of PBS.

The inhibition was evaluated as described in the subsection 2.3 - Transport Assay. Cells were treated with EVs (from cells and plasm) and the supernatant (from cells and plasm) for three and a half hours at 37ºC and 5% CO_2.

2.7 Statistics

Data was analyzed by the software GraphPad Prism version 6.01. Normality of the data was verified by Shapiro-Wilk's test. In the case of the Chemosensitization assay, one-way ANOVA test was applied and presented P- value of 0.0026. For Bimodulation assay, Kruskal-Wallis test was applied and P- value was <0.0001.

3 RESULTS

3.1 Identification of NB4 as a selective ABCG2 inhibitor

The four indeno[1,2-*b*]indoles derivatives (Fig. 1) tested in this work were obtained by substitution reactions on the indeno[1,2-*b*]indole core structure. These compounds were evaluated as potential ABCG2 inhibitors by assessing their effect on ABCG2-mediated efflux using the substrate Hoechst 33342. Cells stably transfected to overexpress the ABCG2 transporter (HEK293-*ABCG2*) were incubated with 10 and 50 μM of the indeno[1,2-*b*]indoles derivatives. The intracellular fluorescence of Hoechst 33342 was measured by flow cytometry, and the inhibition capacity caused by these compounds was calculated based on the effect caused by the reference inhibitor (Ko143).

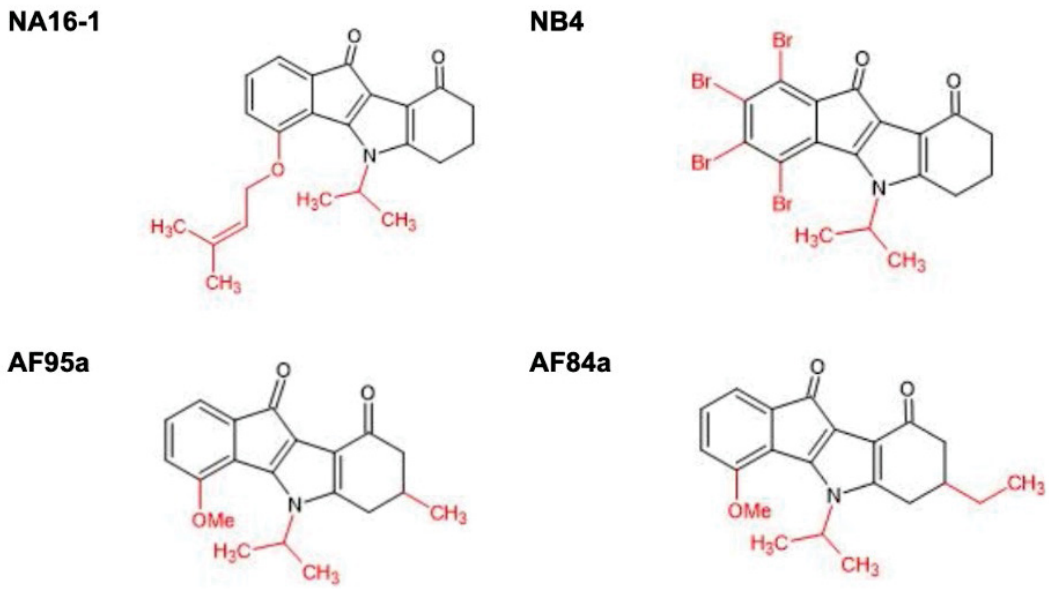

Fig. 1 - The chemical structure of the four indeno[1,2-b]indole derivatives studied in this work. Indeno[1,2-b]indole core appears in black and in red the modifications made in the structure.

All compounds inhibited ABCG2 (Fig. 2A). NA16 inhibited approximately 100% at both concentrations tested, while NB4 presented 55% and 65% at 10 and 50 μM, respectively. On the other hand, AF95 showed 20% at 10 μM and 32% at 50 μM, whereas AF84 inhibited 60% at 10 μM and 93% at 50 μM (Fig. 2A).

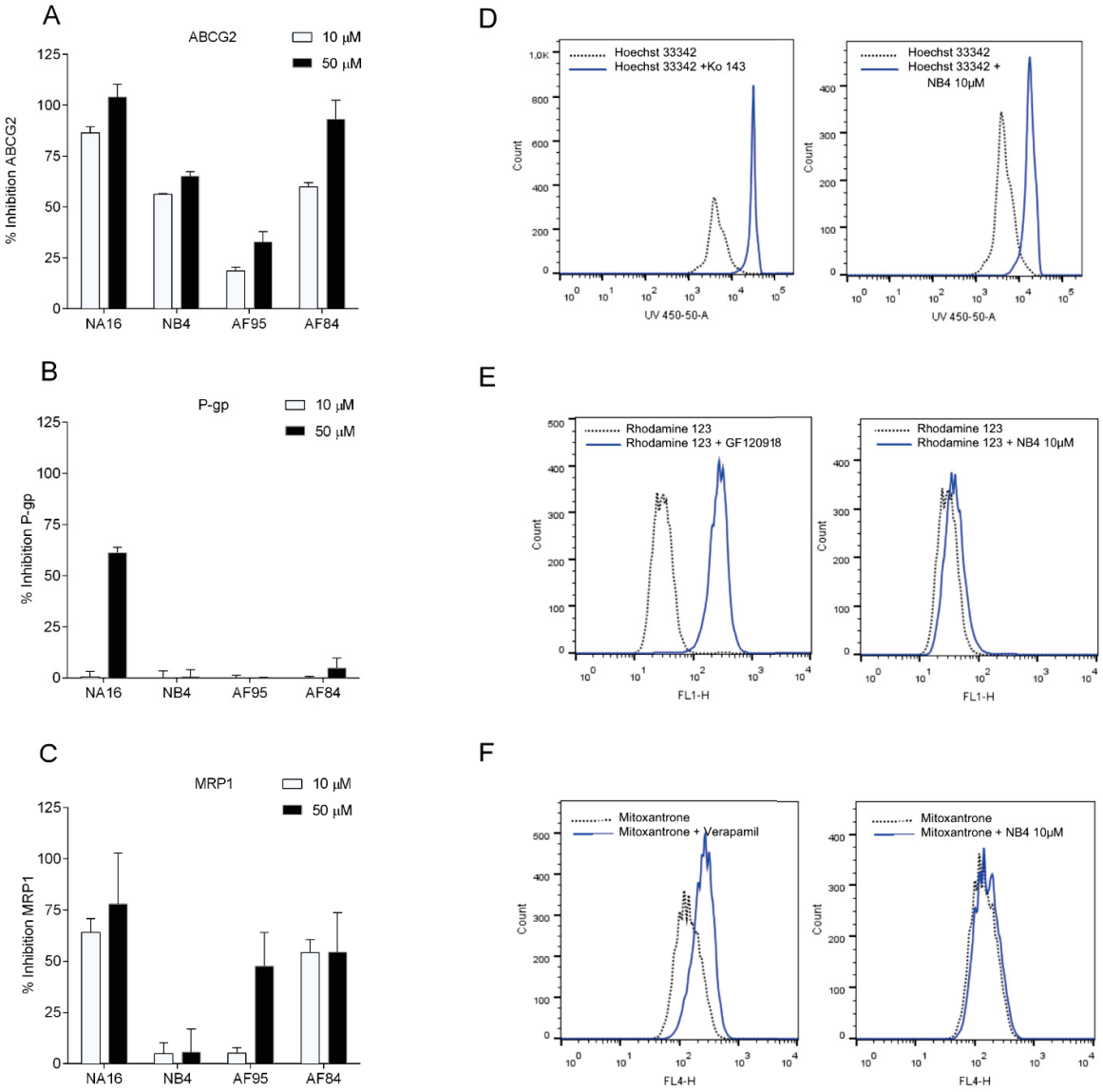

Fig. 2 - Screening of indeno[1,2-*b*]indoles derivatives as inhibitors of ABCG2 (A), P-gp (B) and MRP1(C). HEK293-*ABCG2*, NIH3T3-*ABCB1*, and BHK-*ABCC1* were incubated with the indeno[1,2-*b*]indole derivatives at 10 µM and 50 µM and the substrates Hoechst 33342 (3 µM), Rhodamine 123 (10 µM) and Fluorescein (50 µM), for ABCG2, P-gp and MRP1, respectively. The reference inhibitors (taken as 100% of inhibition) used were Ko143 (1 µM), GF 120918 (1 µM) and Verapamil (50 µM) ABCG2, Pgp and MRP1, respectively. The intracellular accumulation of the fluorescent substrates was monitored by flow cytometry. The data are represented as mean ± SD of at least three independent experiments. (D), (E), and (F): Histograms of ABCG2, P-gp and MRP1 inhibition comparing the reference inhibitors with the compound NB4.

In order to evaluate the selectivity of the indeno[1,2-*b*]indoles derivatives towards the ABCG2 transporter, the same assay was performed using cells stably transfected to overexpress the P-gp and MRP1 transporters (NIH3T3-*ABCB1* and BHK21-*ABCC1*) using Fluorescein and Rhodamine 123 as fluorescent substrates, respectively. For P-gp, only NA16 presented a significant inhibition at 50 µM (Fig. 2B). Moreover, for MRP1, NA16 presented an inhibition of 63% and 78% at 10 µM and 50

μM, respectively; NB4 did not show significant inhibition regarding this transporter, nor AF95 at 10 μM, while AF95 at 50 μM presented 47% of inhibition and AF84 showed nearly 50% of inhibition at both concentrations tested (Fig. 2C).

Among the indeno[1,2-*b*]indoles derivatives tested against the ABC transporters, only NB4 demonstrated specificity towards ABCG2 since there was no significant inhibition regarding the other two transporters (Fig. 2D, E and F). Selectivity is crucial to prevent unintended consequences arising from the inhibition of other ABC transporters, which hold significant roles in physiological functions. Thus, a characterization of the mechanism of ABCG2 inhibition caused by NB4 was performed.

3.2 Identification of the therapeutic ratio (TR) for NB4

In order to determine the EC_{50} value (concentration that causes half of the maximal effect of inhibition) of ABCG2 inhibition for the only selective indeno[1,2-*b*]indole derivative, NB4, HEK293-*ABCG2* cells were treated with increasing concentrations of the compound. The EC_{50} value was initially calculated using the substrate Hoechst 33342. As shown in figure 3A, NB4 showed a high affinity toward ABCG2, showing a EC_{50} value of 208 nM. To further characterize the ABCG2 inhibition caused by NB4, a second ABCG2 substrate was used. NB4 inhibited the mitoxantrone-mediated efflux, showing a EC_{50} value of 162 nM (Fig. 3B), and confirming that the ABCG2 inhibition caused by this compound is not substrate-dependent. As the EC_{50} values were in the same order of magnitude (nM) for both substrates, NB4 appears as a new promising very potent ABCG2 inhibitor.

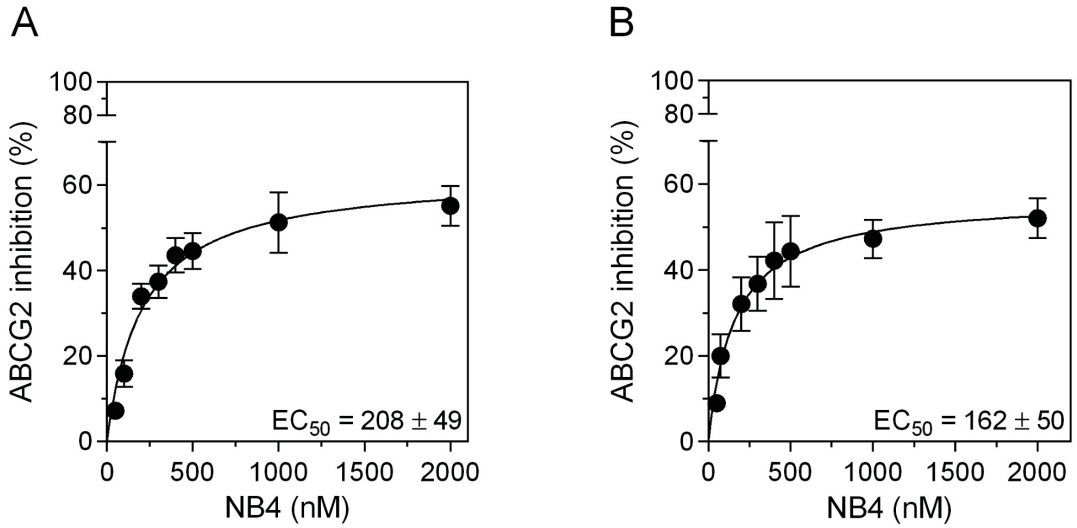

Fig. 3 - EC_{50} values of ABCG2 inhibition. HEK293-*ABCG2* cells were treated with 3 μM of Hoechst 33342 (A) or 10 μM of Mitoxantrone (B), respectively, and the NB4 (50, 100, 200, 300, 400, 500, 1000 e 2000 nM). Intracellular accumulation of the fluorescent substrates was monitored by flow cytometry. Results were expressed as inhibition percentages compared to the reference inhibitor (Ko143 (1 μM)). Data represent mean ± SD of at least three independent experiments.

The cytotoxic effect of NB4 was evaluated in cells overexpressing ABCG2 (HEK293-*ABCG2*) and the parental cell line (HEK293) after 72 hours of exposure. The cytotoxic effect was evaluated in both cell lines to additionally investigate a possible transport mediated by ABCG2. As shown in Figure 4A, NB4 did not show any cytotoxicity even at high concentrations, since it did not significantly reduce the cell viability neither on parental cells nor on cells overexpressing the ABCG2 transporter. In addition, the absence of a cytotoxic effect hampers the transport analysis, however, the similar pattern in both cell lines suggests that NB4 is not recognized as an ABCG2 substrate.

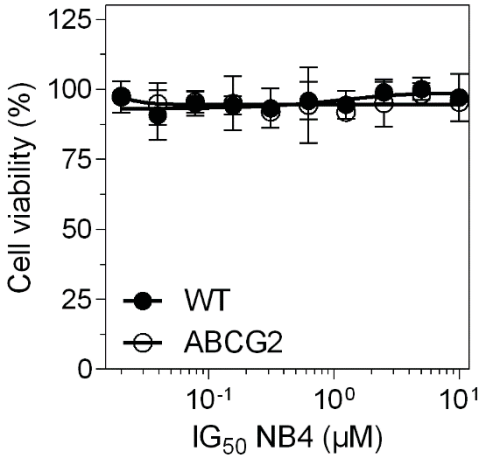

Fig. 4 - Cell viability assay to evaluate the cytotoxic effect of NB4. HEK293-*ABCG2* and HEK293 cells were treated with increasing concentrations of NB4 (0.020 – 10 μM) for 72 hours. The values were obtained by the MTT method, and the results were expressed as a percentage of viable cells compared to the control. Data represent mean ±SD of three independent experiments.

Taking into consideration these two fundamental characteristics of the identification of new ABCG2 inhibitors, the potency of inhibition, given by the EC_{50} value of inhibition, and cytotoxicity, expressed by the IG_{50} value, the therapeutic ratio (TR) was calculated as the ratio between IG_{50} and EC_{50} values. TR values were calculated for both substrates, showing a value > 62 for Hoechst 33342 and > 48 for Mitoxantrone. These high values of TR indicate a promising perspective to use the NB4 in future *in vivo* assays, since the low cytotoxicity is mandatory to follow in animal models.

3.3 NB4 reverse the MDR phenotype mediated by ABCG2

In order to evaluate the capability of the NB4 to reverse the MDR phenotype, a chemosensitization assay was performed with HEK293-*ABCG2* and HEK293 cells after 72 hours of treatment. HEK293-*ABCG2* cells were treated with 0.1 μM of SN-38, the active metabolite of the anticancer drug Irinotecan, used in the treatment of various types of cancer, such as colon and lungs (FUJITA et al., 2015) alone or associated with NB4, while HEK293 cells were treated only with 0.1 μM of SN-38. The cell viability of cells overexpressing the ABCG2 was not affected by treatment with SN-38 (Fig. 5). In contrast, the cell viability of the parental cell line (HEK293) was significantly reduced by SN-38. Interestingly, co-treatment of cells overexpressing ABCG2 with SN-38 and NB4 caused a cytotoxic effect similar to the observed with the effect of SN-38 alone in HEK293 cells. Thus, this result strongly suggests NB4 is able to chemosensitize cells

with MDR phenotype (overexpressing the ABCG2 transporter), demonstrating a phenotype reversion (Fig. 5).

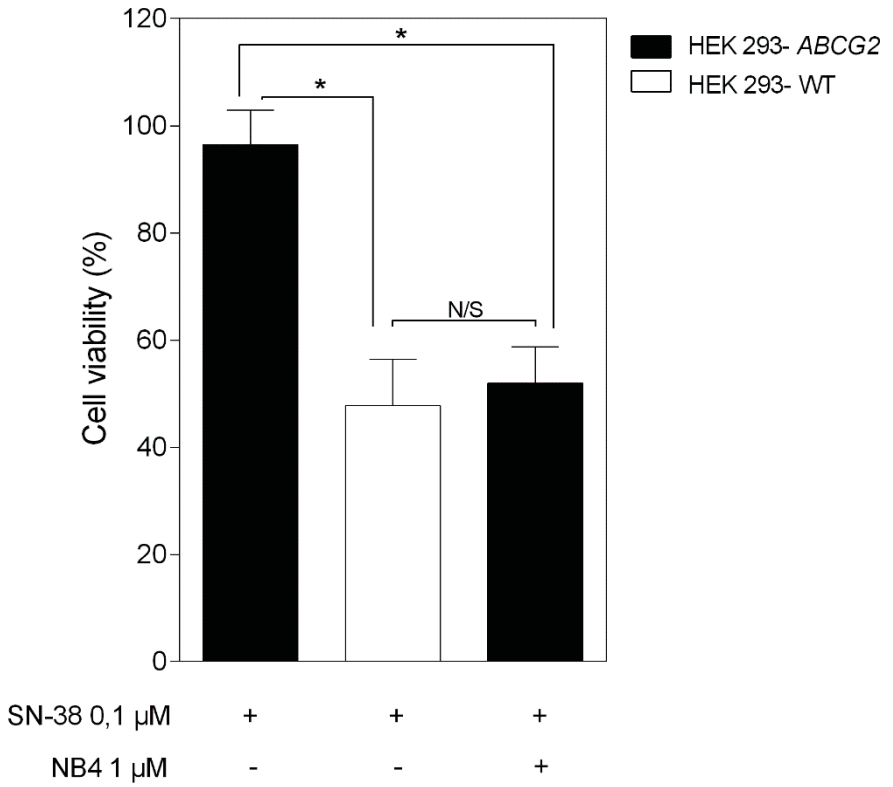

Fig. 5 - The chemosensitization effect was investigated in HEK293-*ABCG2* cells. Cells were treated with SN-38 alone or associated with NB4. The effect on cell viability was compared with the parental cell line HEK293 treated with SN-38. Data represent mean ±SD of three independent experiments. P-value was 0.0026.

3.4 Effect of NB4 on antibody 5D3 binding

The effect of NB4 binding on protein conformation was evaluated by the 5D3 shift assay. This approach involves the use of a conformational antibody called 5D3 that recognize a specific epitope located in the extracellular loop of ABCG2. As shown in figure 5A, the reference inhibitor Ko143 increases the 5D3 binding, confirming the effect that ABCG2 inhibitors produce conformational changes that can be monitored by the 5D3 shift [6]. The indeno[1,2-*b*]indole derivative NB4 caused a similar increase in the 5D3 binding when compared to Ko143 (Fig. 6B). This result suggests that the interaction between NB4 and ABCG2 results in significant conformational changes.

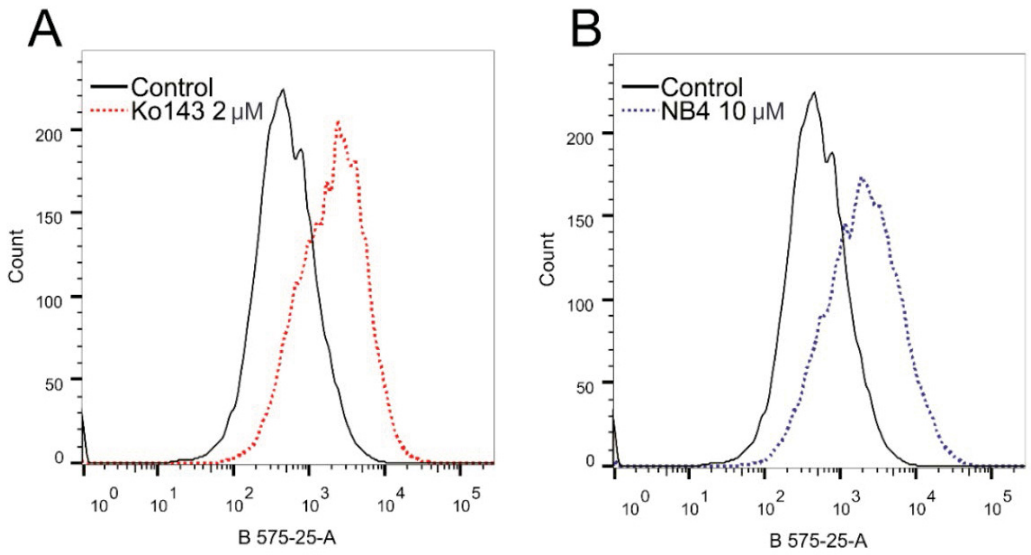

Fig. 6 - 5D3 shift assay. Cells HEK293-*ABCG2* were exposed to a saturating concentration of NB4 (10µM) and incubated with the primary antibody 5D3 and a secondary antibody. Positive and negative controls were performed using the reference inhibitor Ko143 at 2 µM and the fluorescent substrate Hoechst 33342 at 1 µM, respectively. Data represents the overlay of untreated control and cells treated with Ko143 2µM (A) and NB4 10µM (B).

3.5 Study of agonist and antagonist effects of NB4

To get insights about the binding of NB4 on ABCG2, a bimodulation assay was performed. This bimodulation assay is possible to be performed only for the partial inhibitors, such as NB4. Thus, to evaluate its capacity to promote an agonist or an antagonist effect when treated concomitantly with other inhibitors, this experiment was performed associating several others ABCG2 inhibitors, including a chalcone (MBL II 123), a chromone (C4a), Elacridar (GF120918), Ko143 and the indeno[1,2-*b*]indole derivative 5e. Cells overexpressing ABCG2 were treated for 30 minutes with the inhibitor alone - at saturation and EC_{50} concentrations for total inhibitors and only at a saturating concentration in the case of 5e, a partial inhibitor. In all cases, a comparison of the presence and absence of NB4 was evaluated. As showed in figure 7, no statistically significant antagonist or agonist behavior was observed for all conditions and inhibitors evaluated.

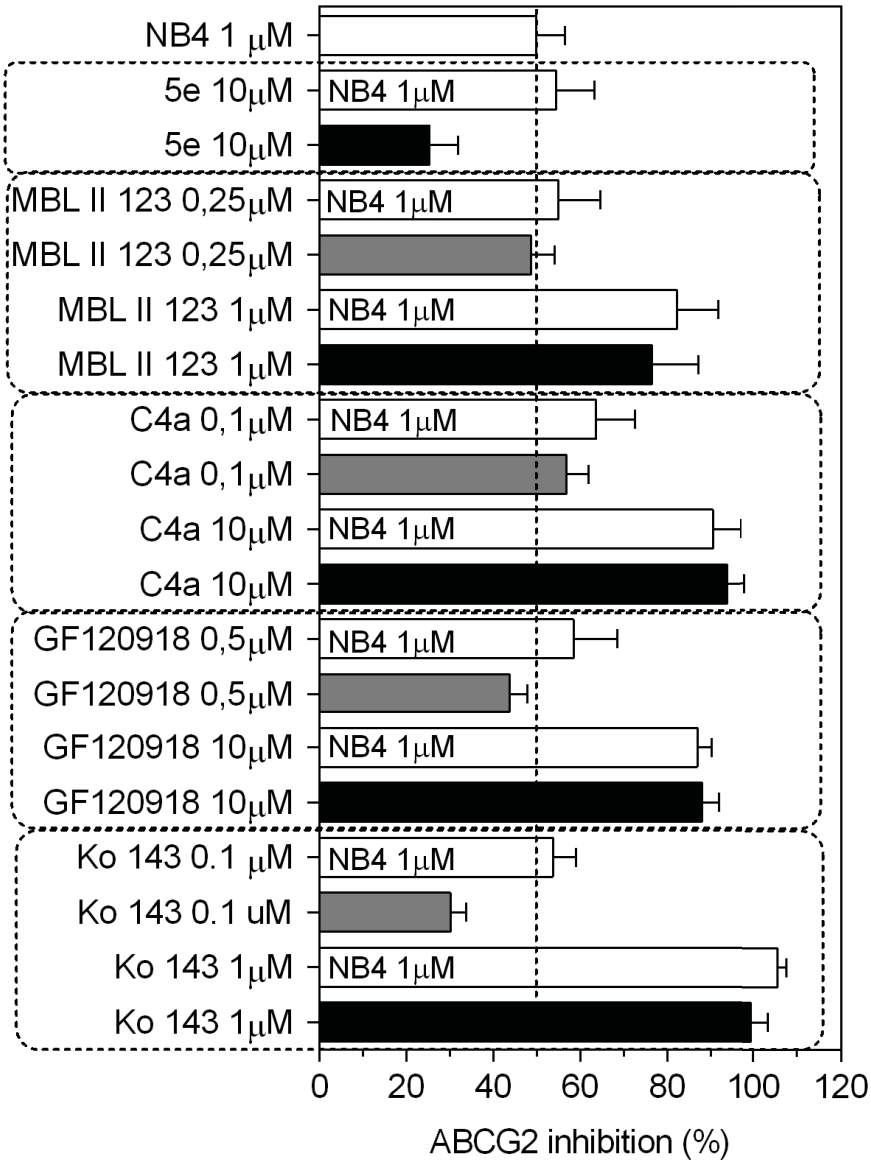

Fig. 7 - Bimodulation assay using the partial inhibitor NB4 at the saturating concentration of 1 μM and in association with different ABCG2 inhibitors (white bars). Four complete inhibitors (Ko143, GF120918, C4a and MBL II 123) at saturating (black bars) and EC_{50} concentrations (grey bars) and one partial inhibitor (5e) at a saturating concentration (black bar) were used. The transport activity was evaluated in HEK293-*ABCG2* cells using Hoechst 33342 as a substrate. Data represent mean ±SD of at least three independent experiments.

3.6 Delivery of NB4 using extracellular vesicles (EVs)

The very poor solubility of NB4 in water consists in an important challenge for the use of indeno[1,2-*b*]indole derivatives. To overcome this critical feature, we proposed the use of EVs to incorporate and promote the delivery of NB4 in target cells. Here, we have used a system based on EVs derived from stimulated human blood cells and circulating plasm EVs. This approach allows the incorporation of drugs on a

pharmaceutical formulation compatible with the patient, since the EVs comes from the donator.

EVs from stimulated blood cells and circulating plasm EVs were incubated with NB4 at 10 μM for 30 minutes to allow its incorporation. After that, these EVs were centrifuged to remove the solvent DMSO and resuspended in PBS for cell treatment (EVs-NB4). The supernatant containing the non-incorporated NB4 was also tested (SUPT-NB4). HEK293-*ABCG2* cells were incubated for three and a half hours with EVs containing NB4 (EVs-NB4) to allow the release of the drugs inside the cells and with the supernatant containing NB4 (SUPT-NB4). As shown in figure 8, NB4 in DMSO caused a partial inhibition, as observed in figures 2 and 3. Interestingly, both EVs from stimulated blood cells and circulating plasma EVs efficiently incorporated and delivered NB4 for target cells causing ABCG2 inhibition. In addition, the supernatant fraction (SUPT-NB4) also inhibited ABCG2, suggesting that the NB4 was not completely incorporated into EVs. In summary, these results demonstrate that this approach based in the use of human EVs were capable of deliver NB4 inhibiting ABCG2.

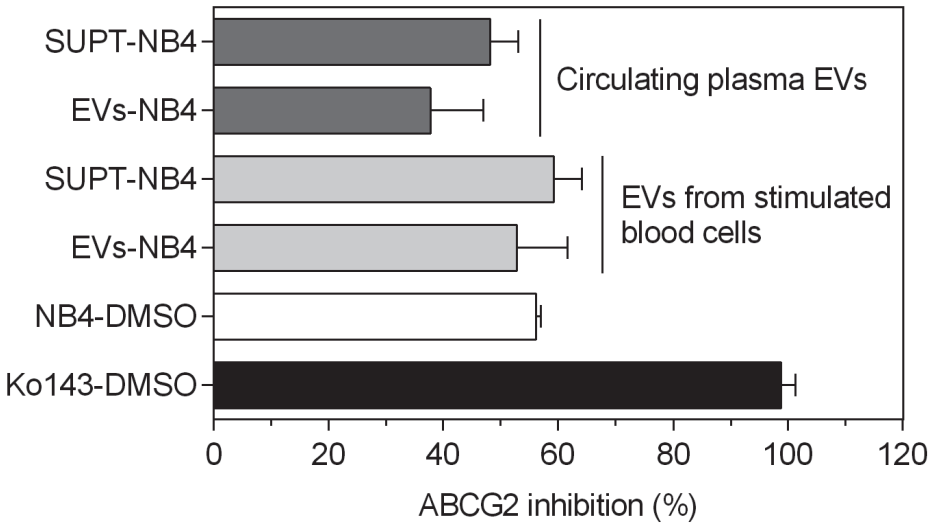

Fig. 8 - Delivery of NB4 using human EVs from stimulated blood cells and circulating plasm EVs. Ko143 at 1 μM was used as control (100 % of inhibition). NB4 in DMSO at 10 μM was also used as a control. Human EVs were incubated with NB4 at 10 μM. Both fractions were tested, EVs containing NB4 (EVs-NB4) and the supernatant containing the non-incorporated NB4 (SUPT-NB4). SUPT-NB4 condition was used as a control for the incorporation of NB4 in the EVs. The transport activity was evaluated in HEK293-*ABCG2* cells using Hoechst 33342 as a substrate. Data represent mean ±SD of three independent experiments.

4 DISCUSSION

The indeno[1,2-*b*]indole derivatives were initially described as human casein kinase II (CK2) inhibitors [16]. After, a series of indeno[1,2-*b*]indole-9,10-dione derivatives were tested against ABCG2, showing promising results and demonstrating that it was possible to convert, through suitable substitutions, indeno[1,2-*b*]indole derivatives designed as CK2 inhibitors into potent ABCG2 inhibitors [12]. In addition to ketonic indeno[1,2-*b*]indole derivatives, it was also demonstrated that phenolic and *p*-quinonic derivatives also inhibited ABCG2 [13]. Recently, structural optimization of the indeno[1,2-*b*]indole was synthesized and tested as ABCG2 inhibitors. The mechanism of ABCG2 inhibition and the differences between compounds that produce a complete versus partial inhibition of mitoxantrone transport were also studied for the best compounds [14].

In this work, four new derivatives (Fig. 1) were initially tested against the three most important transporters related to the MDR phenotype. Only the compound NB4 presented selectivity towards the ABCG2 transporter, showing no effect on P-gp and MRP1 (Fig. 2). Other indeno[1,2-*b*]indole derivatives present similar behavior, such as 6a, that is also selective to ABCG2 [14]. In addition, NA16, AF95 and AF84 showed a dual effect inhibiting ABCG2 and MRP1, without effect against P-gp (Fig. 2). This dual effect was already reported for some ketonic, phenolic and *p*-quinonic derivatives [13,14]. Interestingly, NA16 inhibited the three ABC transporters, consisting in the first indeno[1,2-*b*]indole derivative that inhibit P-gp (Fig. 2) and can be considered a pan-inhibitor.

The selectivity towards ABCG2 is a desirable feature considering the perspective to future clinical trials [6], and this effect observed for NB4 can also be found in other classes of compounds, including methoxy derivatives of stilbenes [11], chalcones and analogs [10], chromones [9], and porphyrin derivatives [17]. Other feature of ABCG2 inhibitors is the saturation effect. Some compounds cause a complete inhibition and others produce a partial effect; thus, these compounds could be divided in complete and partial inhibitors [6]. Kita and collaborators (2021) were the first to characterize this complete and partial effect of inhibition for indeno[1,2-*b*]indole derivatives. The partial inhibitor 6a presented 62% of maximum inhibition of mitoxantrone efflux with EC_{50} of 210 nM, while 5e presented 36% of maximum inhibition and EC_{50} of 150 nM [14]. Here, NB4 showed 55% of maximum inhibition with an EC_{50} of 162 nM (Fig. 3B), demonstrating that NB4 is similar to the best indeno[1,2-*b*]indole derivatives already reported.

Another relevant issue regarding ABCG2 inhibitors is cytotoxicity. There are no compounds yet in clinical trials due to their neurotoxicity, such as FTC[18,19] or residual cytotoxicity, as observed with Ko143 [20]. Indeno[1,2-*b*]indole derivatives appears as a promising class of ABCG2 inhibitors to overcome this challenge, since some compounds presented very low to no cytotoxicity, including the derivatives 5e and 6a, that showed IG_{50} values of 66 and > 100 μM, respectively [14]. NB4 presented a non-cytotoxic effect even in a long-term assay with the use of high concentrations (about 10-fold greater than the IC_{50} value), with an IG_{50} > 10 μM (the higher concentration tested). This feature of NB4 makes this compound very promising to follow in preclinical studies.

The indeno[1,2-*b*]indole derivatives have been previously described as compounds not transported by ABCG2 [14]. In this study, we examined the hypothesis of transport mediated by ABCG2 through a cell viability assay using both the parental and cells that overexpress ABCG2. Since NB4 was not cytotoxic at the highest concentration tested (10 μM), additional techniques need to be applied to investigate a possible transport mediated by ABCG2, since the use of this approach is based in the comparison of the cytotoxic effect in different cells. However, the absence of cytotoxicity is a positive feature for the development of a new inhibitor, that impacts directly in the TR, that for NB4 was estimated to be > 62 for Hoechst 33342 and > 48 for Mitoxantrone. These values are promising, since they are higher than other ABCG2 inhibitors, such as porphyrin derivatives (TR around 10) [17].

In order to evaluate the compound's capacity to sensitize cells overexpressing ABCG2 to an anticancer drug, a cell viability assay was made with the co-treatment of the cells with NB4 at a saturating concentration (10 μM) and SN-38, the active metabolite of Irinotecan. This result (Fig. 5) confirmed that NB4 chemosensitized the cells overexpressing ABCG2, supporting the hypothesis previously identified that a partial inhibitor is sufficient to completely abrogate a chemoresistant phenotype [14].

Generally, ABCG2 inhibitors increases the binding of the conformational-sensitive antibody 5D3 to the extracellular loop of ABCG2, while substrates have little either no effect, or even decreases the binding [21–23]. NB4 showed a similar effect that other functional inhibitors, such as Ko143 and chromone 4a [24], thus suggesting the occurrence of conformational changes on protein structure after the binding of NB4. However, this finding opposes to other indeno[1,2-*b*]indole derivatives (e.g. 5e and 6a), which did not had any effect on 5D3 binding [14].

An interesting way to study the binding site of ABCG2 inhibitors is to perform bimodulation assays, firstly used for study the interaction of stilbenes with ABCG2 [11]. This assay consists in the association of two ABCG2 inhibitors, however, it is mandatory that one of them to be a partial inhibitor. This approach possibilities the investigation of either additive or antagonist effects when two inhibitors are combined. Previously, the association of two partial indeno[1,2-*b*]indole derivatives, 5e and 6a, showed an antagonist effect, decreasing the inhibition effect when compared to the use of these compounds alone [14]. Here, a bimodulation assay was performed using different classes of compounds. Interestingly, the association of NB4 with other inhibitors did not cause significant effect on ABCG2 inhibition, even when NB4 was used with other indeno[1,2-*b*]indole derivative that partially inhibit ABCG2 (Fig. 5).

Finally, most ABCG2 inhibitors present low water solubility imposing difficulties for preclinical tests [24,25], and restricting their delivery and passage through physiological barriers, such as the solid tumor barrier [26]. Thus, to overcome these challenges related with hydrophobic ABC transporter inhibitors, an innovative drug delivery approach based on EVs has been recently described [24]. For the first time was described that human EVs can incorporate and deliver ABCG2 and P-gp inhibitors for target cells [24]. Here, the same approach was successfully applied with the indeno[1,2-*b*]indole derivative NB4, demonstrating that human EVs are promising vehicles for delivering inhibitors targeting membrane proteins. Particularly noteworthy are plasma-circulating EVs, which offer the distinct advantage of being readily and rapidly obtainable [24].

5 CONCLUSION

The indeno[1,2-*b*]indole derivative named as NB4 is a promising selective and non-cytotoxic ABCG2 inhibitor. Also, the characterization of the mechanism of inhibition increased the current knowledge about the drug design and development of ABCG2 inhibitors based on indeno[1,2-*b*]indole core structure. Finally, the incorporation and delivery of NB4 using human EVs makes this compound very attractive for further preclinical studies.

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