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Newly synthetized xanthones as potential P-glycoprotein and BCRP modulators at the intestinal barrier: in vitro and ex vivo studies

Vera Lúcia Santos Silva

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FACULDADE DE FARMÁCIA

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Newly synthetized xanthones as potential P-glycoprotein and BCRP modulators at the intestinal barrier: *in vitro* and *ex vivo* studies

Dissertation of the Master's Degree in Analytical, Clinical and Forensic Toxicology

Elaborated under the supervision of:

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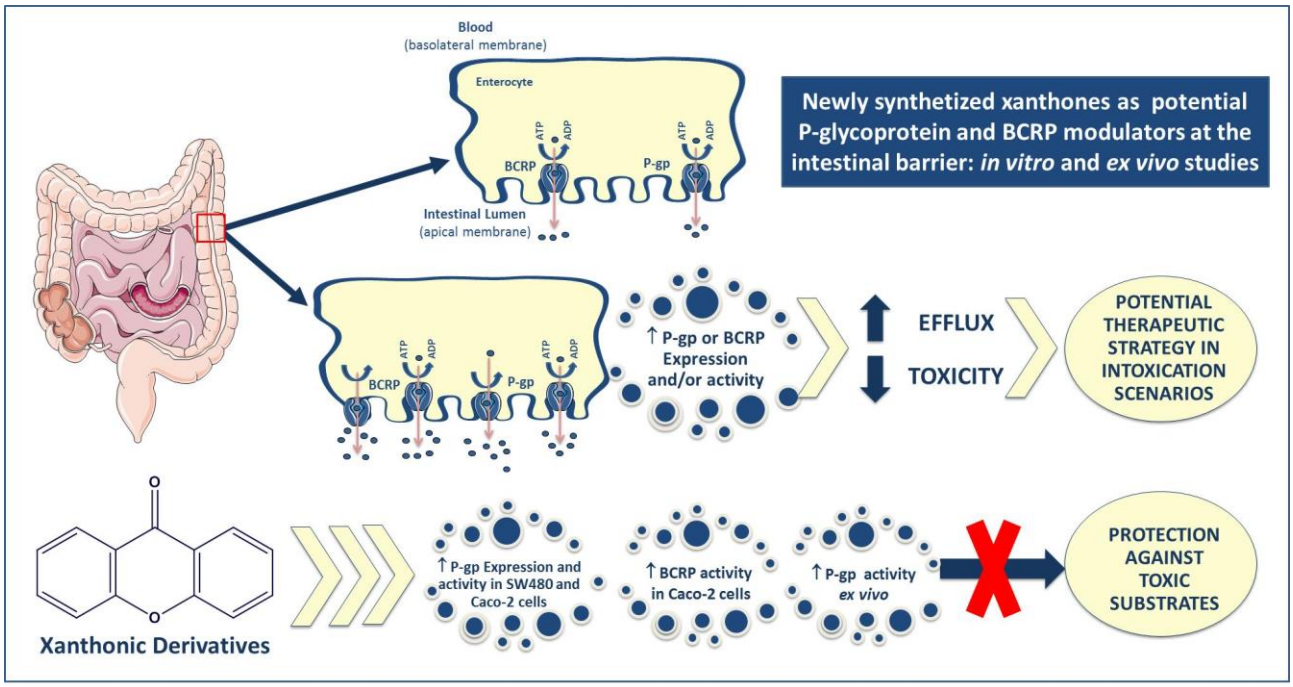
Graphical Abstract



Abstract

ABC (ATP-binding cassette) transporters are a superfamily of carrier proteins that play a crucial role in cell physiology and homeostasis, promoting the efflux of substrates by using the energy resultant from ATP hydrolysis. **P-glycoprotein** (P-gp) and **breast cancer resistance protein** (BCRP) are important members of the ABC superfamily, having an ubiquitous and constitutive distribution throughout the body, with major relevance in normal human epithelial tissues, being also overexpressed in tumor cells, fact that justifies their involvement in the development of multidrug resistance (MDR) in anticancer therapy. Due to their wide distribution, with a polarized expression in barrier and excretory tissues, to their wide range of substrates and to their large efflux capacity, these ABC transporters have a large impact in the pharmaco/toxicokinetics of xenobiotics. This defense mechanism is particularly important at the intestinal level, significantly reducing the intestinal absorption of xenobiotics, limiting their access to the target organs, thus resulting in a decrease in their toxicity. For this reason, P-gp and BCRP induction and/or activation may be a promising new therapeutic strategy in intoxication scenarios, playing a crucial role in the protection of susceptible organs.

Therefore, the main aim of the present work was to investigate the potential modulatory effect of six newly synthetized **xanthonic derivatives** on P-gp and BCRP expression and/or activity, in **SW480** and **Caco-2** cells, human colorectal adenocarcinoma cells widely used to mimic the intestinal barrier. Additionally, the most promising compound found *in vitro* was tested for its ability to increase P-gp activity *ex vivo*, using rat everted intestinal sacs and rhodamine 123 (RHO 123) as a P-gp fluorescent substrate. The protective effect of the tested xanthones against the cytotoxicity induced by mitoxantrone (MTX), a toxic P-gp and BCRP substrate, was also explored in both cell lines.

To select a non-cytotoxic working concentration, SW480 cells were initially exposed to the newly synthetized xanthonic derivatives (0 - 50 μM) and their cytotoxicity assessed by the NR uptake and MTT reduction assays, 24 h after exposure. According to the obtained results, and given the lack of significant effects, the 20 μM concentration was selected as the non-cytotoxic working concentration to be used in the subsequent studies. Previous studies performed in the Laboratory of Toxicology also demonstrated the lack of toxicity of the tested xanthones (0 - 50 μM) towards Caco-2 cells.

In SW480 cells, using the concentration of xanthonic derivatives that did not cause significant toxicity (20 μM), and after 24 h of exposure, several compounds demonstrated the ability to positively modulate P-gp expression (**X1**, **X2**, **X6** and **X12**), as evaluated by flow cytometry using the anti-P-gp UIC2 monoclonal antibody conjugated with phycoerythrin. However, a lack of correlation between P-gp expression and activity was

observed 24 h after exposure to some of the tested compounds (e.g. X2 and X5), highlighting the need for simultaneous evaluation of P-gp expression and activity in the screening for P-gp inducers and/or activators.

Additionally, the synthetic xanthones **X1**, **X2**, **X5**, **X6** and **X12**, after a short incubation period of 90 minutes with the P-gp fluorescent substrate, revealed the ability to efficiently and immediately increase P-gp activity in SW480 cells, without altering its protein expression, suggesting their **potential** as **activators** of this efflux pump. On the other hand, **X16** caused a small but significant decrease in P-gp activity.

In addition, **X12**, the most promising compound according to the *in vitro* activation potential, also demonstrated the ability to significantly increase the efflux of RHO 123 *ex vivo*, in rat intestinal everted sacs, confirming the *in vitro* results and presenting itself as a P-gp activator.

In vitro experimental studies concerning BCRP were performed on both cell lines (SW480 and Caco-2 cells), aiming to assess the potential of the tested xanthones for BCRP activation. In SW480 cells, none of the tested compounds demonstrated the ability to positively and immediately modulate BCRP activity, thus not behaving as BCRP activators. However, **X16** demonstrated to slightly but significantly decrease the protein activity. Similar studies were performed in the Caco-2 cell line, which highlighted the ability of **X1**, **X5** and **X6** to efficiently and immediately increase BCRP activity, without altering its expression levels, thus behaving as BCRP activators. In contrast, **X16**, and as observed in the SW480 cell line, decreased the efflux of MTX, suggesting its potential BCRP inhibitory effect. However, despite the P-gp and BCRP activation potential of the tested xanthones, they failed to afford protection against the cytotoxicity induced by the harmful substrate, MTX.

In conclusion, the *in vitro* and *ex vivo* results included in this dissertation suggest the P-gp and BCRP activation potential of some of the tested xanthones, a mechanism of ABC transporters modulation that has a particular therapeutic interest in cases of intoxication by toxic substrates of these efflux pumps, since there is a decrease in the access of their substrates to the target tissues. Therefore, these newly synthetized compounds represent a potential new source of P-gp and BCRP modulators, disclosing new perspectives in the therapeutics of intoxications by ABC transporters substrates.

Keywords: P-glycoprotein; Breast cancer resistance protein; Induction; Activation; Xanthones; Intestinal Barrier; Intoxication scenarios.

Resumo

Os transportadores ABC (*ATP-binding cassette*) são uma superfamília de proteínas transportadoras que desempenham um papel crucial na fisiologia e homeostasia celular, promovendo o efluxo de substratos mediante a utilização da energia proveniente da hidrólise do ATP. **A glicoproteína-P** (P-gp) e a **proteína de resistência do cancro da mama** (BCRP) são importantes membros da superfamília ABC, tendo uma distribuição ubíqua e constitutiva em todo o organismo, com grande relevância nos tecidos epiteliais humanos, estando também sobre-expressas em células tumorais, o que justifica o seu envolvimento no desenvolvimento de resistência a múltiplos fármacos (MDR) na terapia anticancerígena. Devido à sua ampla distribuição, com uma expressão polarizada em tecidos de barreira e excretores, à sua ampla gama de substratos e à sua grande capacidade de efluxo, estes transportadores ABC têm um grande impacto na fármaco/toxicocinética dos xenobióticos. Esse mecanismo de defesa é particularmente importante a nível intestinal, reduzindo significativamente a absorção intestinal de xenobióticos, limitando o seu acesso aos órgãos-alvo, resultando na diminuição da sua toxicidade. Por essa razão, a indução e/ou ativação da P-gp e BCRP podem ser uma estratégia terapêutica promissora em cenários de intoxicação, desempenhando um papel crucial na proteção de órgãos suscetíveis.

Assim, o principal objetivo do presente trabalho foi investigar o potencial efeito modulador de seis novos **derivados xantónicos** na expressão e/ou atividade da P-gp e da BCRP, em células **SW480** e **Caco-2**, células de adenocarcinoma colorretal humano amplamente utilizadas para mimetizar a barreira intestinal. Adicionalmente, o composto mais promissor encontrado *in vitro* foi testado quanto à sua capacidade de aumentar a atividade da P-gp *ex vivo*, usando sacos intestinais invertidos de rato e rodamina 123 (RHO 123) como substrato fluorescente da P-gp. O efeito protetor das xantonas testadas contra a citotoxicidade induzida pela mitoxantrona (MTX), um substrato tóxico da P-gp e da BCRP, foi também explorado em ambas as linhas celulares.

Para selecionar uma concentração não citotóxica, as células SW480 foram inicialmente expostas aos derivados xantónicos (0 - 50 μM) e a sua citotoxicidade avaliada pelos ensaios de incorporação do vermelho neutro (NR) e de redução do MTT, 24 h após a exposição. De acordo com os resultados obtidos, e dada a falta de efeitos significativos, a concentração de 20 μM foi selecionada como a concentração não citotóxica a ser utilizada nos estudos subsequentes. Estudos anteriores realizados no Laboratório de Toxicologia também demonstraram a falta de toxicidade das xantonas testadas (0 - 50 μM) para as células Caco-2.

Nas células SW480, usando a concentração de xantonas que não causou toxicidade significativa (20 μM), e após 24 h de exposição, vários compostos demonstraram a

capacidade de modular positivamente a expressão da P-gp (**X1**, **X2**, **X6** e **X12**), tal como avaliado por citometria de fluxo utilizando o anticorpo monoclonal anti-P-gp, UIC2, conjugado com ficoeritrina. No entanto, foi observada a falta de correlação entre a expressão da P-gp e a atividade 24 h após a exposição a alguns dos compostos testados (por exemplo, X2 e X5), destacando assim a necessidade da avaliação simultânea da expressão e atividade da P-gp na pesquisa de indutores e/ou ativadores.

Adicionalmente, as xantonas **X1**, **X2**, **X5**, **X6** e **X12**, após um curto período de incubação de 90 minutos com o substrato fluorescente da P-gp, aumentaram de forma eficiente e imediata a atividade da P-gp nas células Sw480, sem alterar a sua expressão proteica, o que sugere o seu **potencial ativador** desta bomba de efluxo. Por outro lado, a **X16** causou uma pequena, mas significativa, diminuição da atividade da P-gp.

Além disso, a **X12**, o composto mais promissor de acordo com o potencial de ativação da P-gp *in vitro*, demonstrou igualmente a capacidade de aumentar significativamente o efluxo da RHO 123 *ex vivo*, em sacos intestinais invertidos de rato, confirmando os resultados *in vitro* obtidos e apresentando-se como um ativador da P-gp.

Estudos experimentais *in vitro* sobre a BCRP foram realizados em ambas as linhas celulares (células SW480 e Caco-2), com o objetivo de avaliar o potencial das xantonas testadas para a ativação da BCRP. Nas células SW480, nenhum dos compostos testados demonstrou capacidade de modular positiva e imediatamente a atividade da BCRP, não se comportando, assim, como ativadores. No entanto, a **X16** demonstrou diminuir ligeiramente, mas de forma significativa, a atividade da proteína. Estudos semelhantes foram realizados em células Caco-2, e revelaram a capacidade da **X1**, **X5** e **X6** para aumentar, de forma eficiente e imediata, a atividade da BCRP, sem alterar os níveis de expressão, comportando-se como ativadores. Em contraste, a **X16**, e tal como observado nas células SW480, diminuiu o efluxo da MTX, sugerindo o seu potencial efeito inibitório da BCRP. No entanto, apesar do potencial ativador da P-gp e BCRP das xantonas testadas, elas falharam na proteção contra a citotoxicidade induzida pelo substrato tóxico, a MTX.

Em conclusão, os resultados *in vitro* e *ex vivo* incluídos nesta dissertação sugerem o potencial ativador da P-gp e BCRP de algumas das xantonas testadas, tendo este mecanismo de modulação um particular interesse terapêutico em casos de intoxicação por substratos tóxicos destas bombas de efluxo, uma vez que há uma diminuição do acesso dos seus substratos aos tecidos-alvo. Portanto, esses novos compostos sintetizados representam uma potencial nova fonte de moduladores da P-gp e BCRP, revelando novas perspetivas na terapêutica de intoxicações por substratos dos transportadores ABC.

Palavras-chave: Glicoproteína-P; Proteína de resistência do cancro da mama; Indução; Ativação; Xantonas; Barreira Intestinal; Cenários de intoxicação.

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Abbreviations List

2-AAF - 2-Acetylaminofluorene
AAAF - N-Acetoxy-2-acetylaminofluorene
ABC - ATP-binding cassette
ADME - Absorption, distribution, metabolism and excretion
ADMET- Absorption, distribution, metabolism, elimination and transport
ADP - Adenosine diphosphate
AhR - Aryl hydrocarbon receptor
AhRE - Aryl hydrocarbon response element
AP-1 - Activator protein 1
ARE - Antioxidant response element
ATCC - American Type Culture Collection
ATP - Adenosine triphosphate
ATXs - Aminated thioxanthones
AZT - Zidovudine
BA - Bioavailability
BBB - Blood-brain barrier
Bcl-2 - B-cell lymphoma 2 protein
BCRP - Breast cancer resistance protein
BCSFB - Blood-cerebrospinal fluid barrier
cAMP - Cyclic adenosine monophosphate
CAR - Constitutive androstane receptor
cGMP - Cyclic guanine monophosphate
CITCO - (E)-1-[6-(4-Chlorophenyl) imidazol[2,1-b][1,3]thiazol-5-yl]-N-[(3,4 dichlorophenyl)methoxy]methanimine
CNS - Central nervous system
Co^{2+}- Cobalt ion (II)
CYP 450 - Cytochrome P450
CYP 3A4 - Cytochrome P450 3A4
DEX - Dexamethasone
DHEA - Dehydroepiandrosterone
DMEM - Dulbecco's modified Eagle's medium
DMSO - Dimethyl sulfoxide
DNA - Deoxyribonucleic acid
DOX - Doxorubicin
ECACC - European Collection of Authenticated Cell Cultures

ECs - Endothelial cells
EDTA - Ethylenediamine tetraacetic acid
ERα - Estrogen receptor α
ERE - Estrogen response element
FBS - Fetal bovine serum
FTC - Fumitremorgin C
FXR - Farnesoid X receptor
GSH - Glutathione
HBSS - Hank's balanced salt solution
HDACI - Histone deacetylase inhibitor
HIF-1α - Hypoxia-inducible factor 1α
HMG-CoA - 3-hydroxy-3-methylglutaryl-coenzyme A
HRE - Hypoxia response element
HSE - Heat shock element
HSP90 - Heat shock protein 90
HYP – Hypericin
IA - Inhibited activity
ICD1 - Intracellular domain 1
invMED1 - Inverted mediator-1
KH - Krebs-Henseleit buffer
Km - Affinity constant (transport kinetic parameter)
LPO - Lipid peroxidation
LXR - Liver X receptor
MDR - Multidrug resistance
MDR1 - Multidrug resistance protein 1
MDR3 - Multidrug resistance protein 3
MFI - Mean of fluorescence intensity
Mg^{2+} - Magnesium ion
MPO - Myeloperoxidase
mRNA - Messenger RNA
MRPs - Multidrug resistance-associated proteins
MRP1 - Multidrug resistance-associated protein 1
MRP2 - Multidrug resistance-associated protein 2
MRP3 - Multidrug resistance-associated protein 3
MRP4 - Multidrug resistance-associated protein 4
MRP5 - Multidrug resistance-associated protein 5
MRP6 - Multidrug resistance-associated protein 6

MRP7 - Multidrug resistance-associated protein 7

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

MTX - Mitoxantrone

NA - Normal activity

NBDs - Nucleotide binding domains

NEAA - Nonessential amino acids

NF-κB - Nuclear factor κB

NF-γ - Nuclear factor-γ

NR – Neutral Red

Nrf2 - Nuclear factor (erythroid-derived 2)-like-2

NSAIDs - Nonsteroidal anti-inflammatory drugs

ORBEA - *Organismo Responsável pelo Bem-Estar Animal*

p53 - p53 tumor suppressor protein

PAF - Platelet-activating factor

PBS - Phosphate-buffered saline

PE - Phycoerythrin

P-gp - P-glycoprotein

PhIP - 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine

P_i - Inorganic phosphate

PKC - Protein kinase C

PMA - Phorbol 12-myristate 13-acetatePCN – Pregnenolone 16α-carbonitrile

PPARα - Peroxisome proliferator-activated receptor α

PPARγ- Peroxisome proliferator-activated receptor γ

PQ - Paraquat

PRB - Progesterone receptor B

PRE - Progesterone response element

PXR - Pregnane X receptor

QSAR - Quantitative structure-activity relationship

RedRif - Reduced rifampicin

RHO 123 - Rhodamine 123

RNA - Ribonucleic acid

RXR – Retinoid X receptor

SLC - Solute carrier

SP - Side population

SP1 - Specificity protein 1

SXR - Steroid xenobiotic receptor

TBHQ - Tert-Butylhydroquinone

TBP - TATA binding protein
TCDD - Tetrachlorodibenzo-p-dioxin
TMDs - Transmembrane domains
TMHs - Transmembrane α-helices
TPA - *12-O*-Tetradecanoylphorbol-*13*-acetate
UV - Ultraviolet
vcMsbA - *Vibrio cholera* MsbA
VER - Verapamil
YB-1 - Y-box-binding protein 1
ZOS - Zosuquidar

I. INTRODUCTION

1. MEMBRANE TRANSPORTERS – ABC TRANSPORTERS

In order to reach the target organs and exert their pharmacodynamic or toxicodynamic effects, the compounds need to cross tissue barriers, *i.e.*, membranes. To pass through the cell membrane, the compound needs to have a physical structure compatible with the specific characteristics of the biological barrier, namely weight, size, distribution capacity and hydrophobicity, or needs to have the ability to interact with specific carriers expressed at the membranes and which mediate its transport through them (1, 2).

Membrane transporters are proteins located on tissue, cell and cellular organelles membranes capable of promoting the uptake or efflux of compounds. The bioavailability (BA) refers to the fraction of the administered dose that reaches the systemic circulation, being able to trigger a biological effect (3). The BA of compounds, namely endobiotics or xenobiotics that are not able to permeate the membrane by passive diffusion, is determined by the balance between these inflow and outflow carriers, which facilitate their movement through the membranes (4, 5). These carriers are important both for the maintenance of cellular homeostasis and for the detoxification of substances with toxic potential (4). For this reason, membrane carriers are considered an integral part of the pharmacokinetic process, which led to the update of the acronym ADME to ADMET meaning absorption, distribution, metabolism (phase I and phase II), elimination and transport (phase 0 and phase III) (1). In phase 0, multispecific transporters that promote the entry of the compounds into the cells are involved, participating in the absorption process, being called solute carriers (SLC); phase I comprises metabolic processes of oxidation, reduction and hydrolysis; phase II involves the conjugation of metabolites or intact compounds with endogenous substances, such as glutathione (GSH) and glucuronic acid, in order to increase their size and polarity to be more easily excreted; phase III corresponds to the elimination process, where the ATP-binding cassette (ABC) efflux transporters are involved, limiting the cellular uptake of the compounds with high passive permeability, but also facilitating the elimination of compounds with low passive permeability, such as phase II conjugates or toxic compounds (1, 5-7).

The ABC superfamily represents one of the largest families of carrier proteins in living organisms, being universally expressed in different species including bacteria, plants and mammals (4, 8, 9). Currently, 49 human genes coding for ABC transporters are identified, classified into seven subfamilies from ABCA to ABCG, based on the similarity of gene structure, sequence of domains and sequence homology: ABCA (12 members), ABCB (11 members, including P-gp), ABCC (13 members), ABCD (4 members), ABCE (1

member), ABCF (3 members) and ABCG (5 members, including BCRP) (**Table 1**) (8, 10, 11).

Table 1. Human ABC genes subfamilies. Adapted from (8)

Subfamily name	Alias	Number of genes	Number of pseudogenes
ABCA	ABC1	12	5
ABCB	MDR	11	4
ABCC	MRP	13	2
ABCD	ALD	4	4
ABCE	OABP	1	2
ABCF	GGN20	3	2
ABCG	White	5	2
Total		49	21

ABC transporters mediate the efflux of substrates through all cell membranes and cellular organelles, namely mitochondria, endoplasmic reticulum, Golgi apparatus and other vesicles, against the concentration gradient, by using the energy resultant from the hydrolysis of adenosine triphosphate (ATP) (4, 12-14). These transporters have several functions in the different organisms, being involved in nutrient uptake, cell signaling and in the active transport of a wide variety of chemically different endogenous substrates, including bile salts, ions, peptides, steroids, polysaccharides, vitamins, phospholipids, amino acids, metabolites and exogenous compounds (4, 13, 15). In eukaryotes, ABC transporters are exclusively exporters (16).

From the toxicological point of view, the fact that ABC carriers mediate the efflux of toxic substances from the cells is beneficial, and a potential antidotal approach to be used in intoxications, since there is a reduction in their intracellular concentration and, consequently, in their toxicity, thus contributing to cellular homeostasis (1, 17). However, from the pharmacological point of view, carrier-mediated efflux facilitates the excretion of drugs, thus restricting their BA and, consequently, their therapeutic efficacy (17).

The ABC transporters have a basic structure that defines them as members of the same family, although they have a distinctive modular architecture (13). These transporters can be considered full-transporters, typically having two homologous moieties, each containing a nucleotide binding domain (NBD) and a transmembrane domain (TMD) containing six transmembrane α-helices (TMHs), or half-transporters, consisting of a single NBD and a single TMD (11, 13, 15, 18). P-glycoprotein (P-gp - *Permeability - glycoprotein)* and multidrug resistance-associated proteins 4 and 5 (MRP4 and MRP5) are considered full-transporters because they exhibit the typical structure (11,

18). On the other hand, the breast cancer resistance protein (BCRP) is considered a half-transporter because it only exhibits one NBD and one TMD (6, 8, 13). In addition, some multidrug resistance-associated proteins (MRPs), as MRP1, MRP2, MRP3, MRP6 and MRP7, have an extra TMD with 5 TMHs (19).

Due to their physiological role in the transport of a wide range of endobiotics, as well as xenobiotics, several diseases were already associated with changes in the ABC genes coding for the ABC transporters expressed in humans. Presently, there are 13 known genetic diseases associated with mutations in 14 ABC carriers, namely cystic fibrosis, Stargardt's disease, age-related macular degeneration, adrenoleukodystrophy, Tangier's disease, Dubin-Johnson syndrome and progressive familial intrahepatic cholestasis (**Table 2**) (20).

Table 2. Human ABC transporters, their functions and diseases caused by mutations in ABC genes. Adapted from (21).

Human ABC transporter	Function	Disease
ABCA1	Cholesterol and phospholipids transport	Tangier disease, familial hypoapoproteinemia
ABCA4	Rod photoreceptor retinoid transport	Stargardt/fundus flavimaculatis, Retinitis pigmentosa, Cone-rod dystrophy, Age-related macular degeneration
ABCB2	Peptide transport	Immune deficiency
ABCB3	Peptide transport	Immune deficiency
ABCB4	Bile-acid transport	Progressive familial intrahepatic cholestasis-3
ABCB7	Iron transport	X-linked sideroblastosis and anemia
ABCB11	Bile-acid transport	Progressive familial intrahepatic cholestasis-2
ABCC2	Bile-acid transport	Dubin-Johnson Syndrome
ABCC6	Unknown	Pseudoxanthoma elasticum
ABCC7	Chloride ion channel	Cystic fibrosis
ABCD1	Very long chain fatty acids transport	Adrenoleukodystrophy
ABCG5	Sterol transport	Sitosterolemia
ABCG8	Sterol transport	Sitosterolemia

Due to their high expression in several tissues, their implications in the pharmaco-toxicokinetics process and their involvement in therapeutic strategies, the main carriers of this superfamily, and those more studied and described in the literature, are ABCB1/P-gp, ABCG2/BCRP and the MRPs, particularly ABCC1/MRP1 to ABCC5/MRP5 (4, 10, 13, 22).

1.1. P-glycoprotein

P-gp belongs to the ABCB subfamily of ABC transporters, being also recognized as ABCB1/MDR1 (4). The name multidrug resistance protein 1 (MDR1) is related with the role of this protein in the multidrug resistance (MDR) phenomenon, due to the export of several xenobiotics from the intracellular space. In humans, P-gp is encoded by two MDR genes located, adjacent to each other, on the chromosome 7 (7q21): *ABCB1*/*MDR1*, coding to a protein responsible for the transport of xenobiotics, and *ABCB4*/*MDR3*, which codes to a protein responsible for the efflux of phosphatidylcholine into the bile (23). In the following sections of this dissertation, the term P-gp will be used to indicate the human *ABCB1*/*MDR1* gene product (or *abcb1a*/*abcb1b* gene products in rodents). The designation of this protein results from the study performed by Juliano and Ling (1976), where the isolation of chinese hamster ovary cells was carried out and the existence of colchicine-resistant mutants was observed (24). While studying the surface of the cell membranes, the researchers observed the presence of a glycoprotein, which appeared to be exclusive to the mutant cells, and that had altered the permeability to the drug. For this reason, this protein was called P (permeability) glycoprotein (24).

1.1.1. Structure of P-gp

P-gp is a high molecular weight (170 kDa) transmembrane protein whose synthesis occurs in the endoplasmic reticulum. P-gp is formed by 1280 amino acids arranged in two homologous portions of 610 amino acids each (4, 23, 25). The two homologous halves, which arose from a gene duplication event, present twelve highly hydrophobic TMHs equally distributed by the two hydrophobic TMDs, thus containing six TMHs each. P-gp also presents two hydrophilic NBDs where the ATP hydrolysis takes place, and which are located in the cytoplasmic side of the membrane (25, 26). Given its typical structure, P-gp is considered a full-transporter (**Figure 1**) (13, 26).

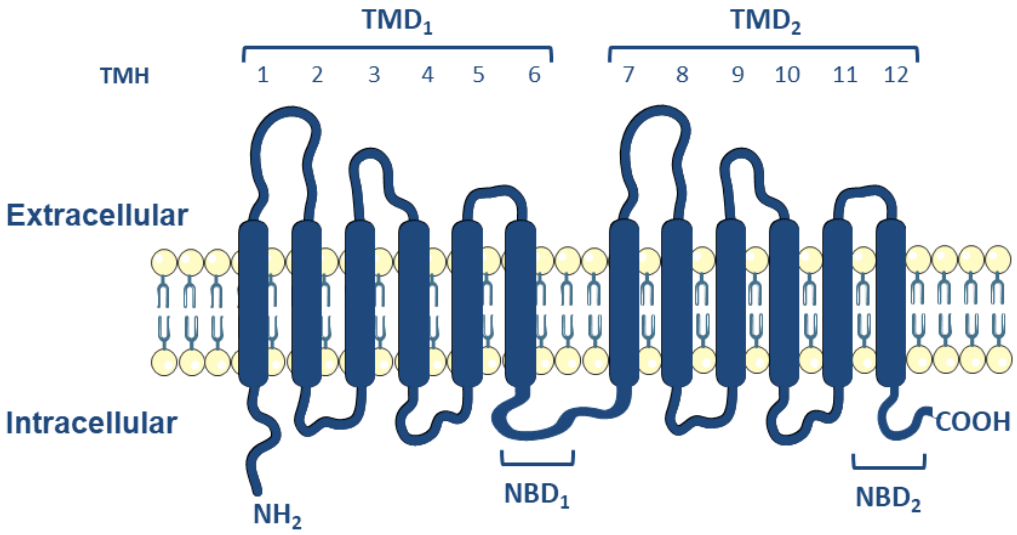

Figure 1. P glycoprotein (P-gp) topology. P-gp, a full-transporter, has twelve transmembrane α-helices (TMHs) distributed through two homologous transmembrane domains (TMD_1 and TMD_2), and two nucleotide binding domains (NBD_1 and NBD_2) located on the intracellular side. NBD - nucleotide binding domain; TMD - transmembrane domain; TMH – transmembrane α-helice. Adapted from (4, 5).

The TMDs form the ligand binding site, creating the translocation pathway through which the compounds cross the membrane and determining the substrate specificity (17). The role of the TMDs is to recognize and mediate the passage of structurally unrelated substrates across the cell membrane (27). For hydrophilic molecules, the TMDs shield the substrates from the lipids making up the bilayer, by providing a hydrophilic pathway across the cell membrane. In contrast, the TMDs are capable of recognizing and removing a large number of chemically unrelated hydrophobic drugs/toxins and lipids directly from the cell membrane for (27). The NBDs are located on the cytoplasmic side of the membrane and constitute the ATP binding sites, being the energy resulting from its hydrolysis responsible for the translocation of the substrates through the lipid bilayers against the concentration gradient (6). Each NBD consists of two core consensus motifs referred to as the Walker A and B motifs, that are at a distance of 100-200 amino acids from the NBD domain, and a C signature, unique of the superfamily of ABC transporters (22, 25). In the Walker A motif, there is a highly conserved lysine residue, which is involved in the binding of the phosphate group of ATP, and in the Walker B motif there is a highly conserved aspartate residue that appears to interact with the magnesium ion (Mg^{2+}), an ion that seems to be a mandatory cofactor in the hydrolysis of ATP (22, 28, 29). Mutations to either of these residues result in a non-functional P-gp (29). Furthermore, there is a third highly conserved amino acid sequence (LSGGQ) located between the Walker A and B motifs, assigned to as the ABC signature motif (or C motif or C signature) which, as previously mentioned, is unique to ABC proteins (4, 22). The Walker A and B motifs are generally found in a wide range of ATPases and are directly involved in the

binding and hydrolysis of nucleotides (25). The two homologous parts that constitute P-gp interact with each other. For this interaction to correctly occur there is a flexible binding region that joins the two halves allowing the communication between the two ATP binding sites, and this region is phosphorylated at various sites by protein kinase C (PKC) (25, 30).

1.2. Breast cancer resistance protein

BCRP is the second member belonging to the subfamily G of the ABC transporters, reason by which it is also called ABCG2 (31). Human BCRP is encoded by the *ABCG2* gene, which is located on chromosome 4q22 (32). BCRP is a 75 kDa membrane protein that was first identified in 1998 in a human breast cancer cell line, the MCF-7/AdrVp cells, which does not express other efflux transporters (33).

This protein is known to contribute to the resistance to multiple drugs, particularly those implicated in the cancer treatment (33, 34). The discovery and characterization of BCRP as an efflux transporter conferring MDR has unleashed a remarkable trajectory in understanding its role in physiology and cancer (6).

1.2.1. Structure of BCRP

BCRP is considered a half-transporter, presenting one NBD and one TMD consisting of six TMHs, and functioning as homodimer, oligomer and, possibly, as homotetramer (**Figure 2**) (6, 32, 34, 35).

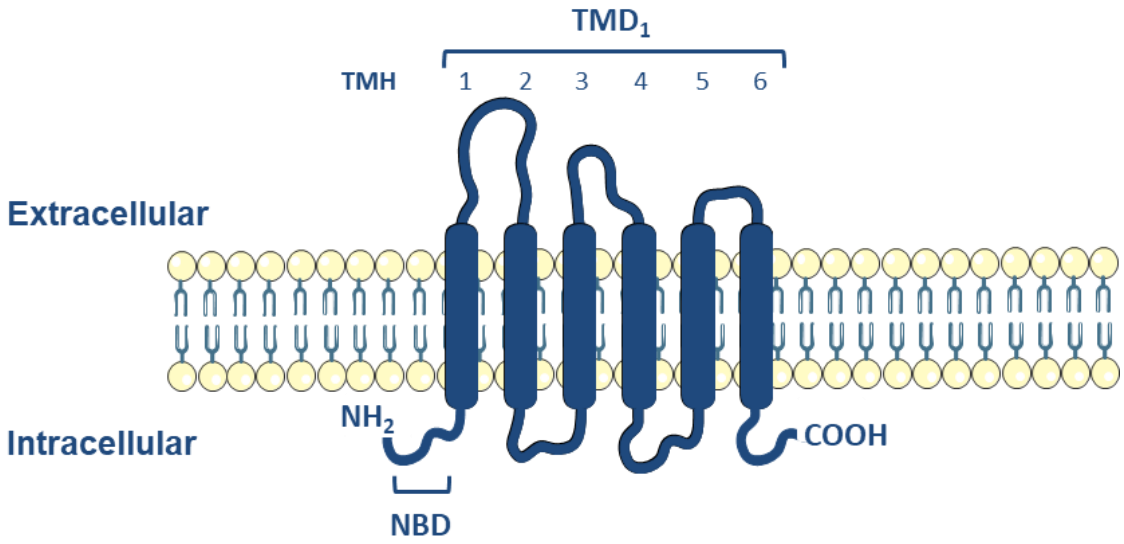

Figure 2. Breast cancer resistance protein (BCRP) topology. BCRP, a half-transporter, has one transmembrane domain (TMD_1) consisting of six transmembrane α-helices (TMH) and a nucleotide binding domain (NBD) containing the ATP binding site. Adapted from (5).

ABCG2 has been thought to function as a homodimer because its size is about half that of many other ABC transporters; its human homologous proteins ABCG5 and ABCG8 were reported to form heterodimers in the endoplasmic reticulum prior to being transported to the apical membranes (36); and its homologous proteins in *Drosophila* have been shown to work as heterodimers (35). However, some studies have suggested than human ABCG2 may also exist and function as an oligomer, such as a tetramer (35).

The human BCRP hydrophilic NBD is located between the amino acid residues 1 and 395, and is constituted by the Walker A and B domains and the conserved specific C domain, while the hydrophobic TMD is located between the amino acid residues 396 and 655 (6, 13, 31). The TMD and NBD communicate by a highly conserved domain located between TMH_2 and TMH_3. BCRP also contains an intracellular domain 1 (ICD1) that appears to be involved in the conformational changes suffered by the transporter (37).

1.3. Tissue distribution

ABC transporters are constitutively expressed in the most varied tissues of the human organism, but also in other mammals (17). In general, ABC transporters, namely P-gp, MRPs and BCRP, are expressed on the luminal (apical) and/or basolateral membrane of enterocytes (38-40), hepatocytes (40, 41), renal tubular epithelial cells (40, 42, 43) and relevant barrier tissues, notably the blood-brain barrier (BBB) (40, 44-46), the blood-cerebrospinal fluid barrier (BCSFB) (47), and in the blood-testis (48) and placental barriers (**Figure 3**) (40, 49, 50).

P-gp has an ubiquitous distribution in the body, with greater relevance in the barrier tissues (**Figure 3)**. P-gp has a polarized expression, *i.e.,* it is expressed in cells with a polarized surface constituted by both apical and basolateral membranes. The location of the protein allows a better understanding of the role of this carrier in the processes of distribution and elimination. Indeed, P-gp acts as a physiological barrier by conferring cellular protection through the efflux of endogenous and exogenous compounds, a phenomenon that explains its important role in altering the BA of xenobiotics recognized as its substrates, resulting in an extensive reduction of their action (26, 51, 52).

The polarized expression of BCRP in humans, similarly to P-gp, is very broad, covering several tissues and organs (**Figure 3**) (5, 6, 53).

The liver is an organ involved in the synthesis and excretion of bile acids, in the metabolism and transport of cholesterol, as well as in the efflux of endogenous and exogenous compounds (54). As the main organ of drug metabolism, the liver is involved in the first-pass effect and in the plasma clearance of compounds (55). Therefore, together with the kidneys, the liver is a detoxification organ (56). The hepatocytes present a

canalicular (apical) membrane and a basolateral membrane, which is in contact with the blood (57). Both membranes present a high expression of ABC transporters (**Figure 3 A**) (1, 5). P-gp and BCRP are located in the canalicular membrane and contribute to the biliary excretion of GSH, glucuronic acid and sulfate conjugates, monoanionic bile salts and bicarbonate (54, 58). It is also described the involvement of hepatic BCRP in biliary excretion of some therapeutically important drugs, such as methotrexate, the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA)-reductase inhibitors rosuvastatin and pitavastatin, and fluoroquinolones (59).

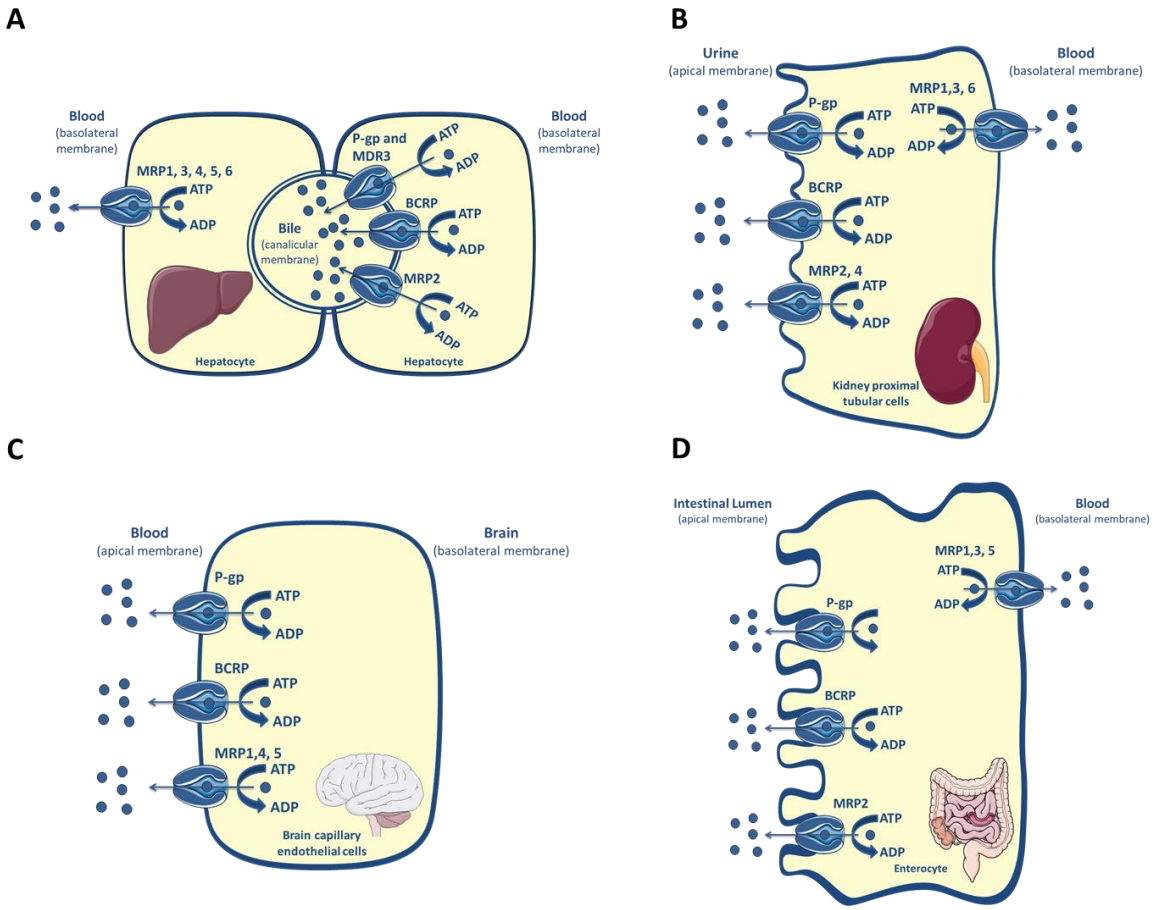

Figure 3. Main ABC transporters expressed in the hepatocytes (A), kidney proximal tubular cells (B), endothelial cells of the blood-brain barrier (C) and enterocytes (D), and their location. Adapted from (1, 5, 58, 60). ATP – Adenosine triphosphate; ADP – Adenosine diphosphate; P-gp – P-glycoprotein; BCRP – Breast cancer resistance protein; MDR3 – multidrug resistance protein 3; MRP – Multidrug resistance-associated protein.

The kidneys, like the liver, are organs involved in detoxification, being responsible for the maintenance of fluid and electrolyte homeostasis, but also maintaining the total body homeostasis by conserving essential nutrients and eliminating potentially toxic xenobiotics, xenobiotic metabolites, and metabolism products (42, 43). Therefore, the kidneys play a vital role in the body's defense against potentially toxic xenobiotics and

metabolic waste products through elimination pathways. Thus, secretory transporters in the proximal tubule are major determinants of the disposition of xenobiotics, including many prescription drugs (43). P-gp, as well as other efflux pumps, namely BCRP, are expressed on the apical membrane of renal tubular epithelial cells, where they mediate the efflux of the substrates found in the cytoplasm of renal tubular cells to the urine (**Figure 3 B**) (25, 61, 62).

The BBB regulates and protects the microenvironment of the central nervous system (CNS) as it is a physical and enzymatic barrier that separates the CNS from the systemic vascular environment, protecting the CNS from the exposure to circulating potentially harmful endogenous and exogenous substances (45, 63). BBB is composed by a monolayer of brain capillary endothelial cells (ECs) that form a polarized barrier, which regulates the diffusion of molecules across the BBB, and limits the entry of xenobiotics via paracellular pathways by intercellular tight junctions (46). P-gp and BCRP are expressed in the apical (luminal) membrane of the ECs that integrate the BBB, being blood-oriented (**Figure 3 C**). Therefore, these ABC transporters are responsible for the efflux of substrates from the ECs to the bloodstream against the concentration gradient, thus limiting the entry of some drugs into the CNS (**Figure 3 C**) (64).

The intestine is a very important organ since it regulates the absorption of orally administered drugs and it is involved in the first-pass removal (65). The absorption of compounds mainly occurs in the small intestine, especially in the polarized enterocytes of the duodenum and jejunum, due to the large surface area (38, 65). However, the role of the intestine in the absorption process is also explained by the presence of efflux and influx transporters, located at the apical and basolateral membranes of the intestinal cells (65, 66). P-gp and BCRP are highly expressed in the apical membrane of enterocytes, promoting the efflux of the compounds from the cells back into the intestinal lumen, thereby limiting the intestinal absorption of many clinically important drugs and dietary toxins (**Figure 3 D**) (67, 68). The expression of P-gp along the intestine is not uniform, since it presents high levels in the ileum and colon, in contrast to the jejunum and duodenum, where its expression is lower (67, 69). BCRP is also expressed at the apical membrane of the small and large intestine cells. However, unlike P-gp, the BCRP expression does not significantly differ along the small intestine (70) and, in the jejunum, the BCRP mRNA level is higher than other efflux transporters, namely P-gp (67, 71).

1.4. P-gp and BCRP substrates

1.4.1. P-gp substrates

P-gp has the ability to bind and transport a wide range of substrates, which can be chemically, structurally and pharmacologically very different from each other (14, 25, 26, 72). As such, the substrates that bind to P-gp greatly differ in size, structure and function, ranging from small molecules, such as organic cations, carbohydrates, amino acids and some antibiotics, to macromolecules, such as polysaccharides and proteins (25, 73). Most of the substrates transported by P-gp are slightly amphipathic, somewhat hydrophobic (26) and lipid-soluble molecules, with a molecular weight that varies between 300 and 1000 kDa (14), often containing aromatic rings and a positively charged nitrogen atom at physiological pH (26).

Due to the outstanding involvement of P-gp in pharmacokinetics and drug resistance, several studies have been conducted to elucidate the compounds structural characteristics important for the interaction between this efflux transporter and its substrates (25). Studies performed by Seelig *et al.* (1998) aimed to understand what defines a molecule as a substrate of this transport protein, and demonstrated that a compound can interact with P-gp if it contains: a) two electron donor groups with a spatial separation of 2,5 ± 3Å; or b) two electron donor groups with a spatial separation of 4.6 ± 0.6Å; or c) three electron donor groups with a spatial separation of the outer two groups of 4.6 ± 0.6Å (74). Another study has found that partitioning into the lipid membrane is the rate-limiting step for the interaction of a substrate with P-gp and that dissociation of the P-gp-substrate complex is determined by the number and strength of the hydrogen bonds formed between the substrate and this efflux transporter (75).

P-gp transports many xenobiotics, including drugs, belonging to numerous therapeutic classes, namely antiarrhythmics, anticancer drugs, antidepressants, cardiotonics, immunomodulators, among others described in **Table 3** (4, 5, 26). In addition to the exogenous substrates, P-gp has also the ability to transport endogenous substrates, including steroid hormones (4), phospholipids (76), simple glycosphingolipids (77), platelet-activating factors (PAF) (78), aldosterone (79), β-Estradiol 17-(β-D-glucuronide) (80), amyloid-β protein (81), peptides and various interleukins (**Table 3**) (82).

Table 3. P-gp substrates. Adapted from (4, 5, 14, 26, 83, 84).

P-gp substrates		
Endogenous substrates	**Exogenous substrates**	**Substrates used experimentally**
Amyloid-β (Aβ) peptide; **Bile acids;** **Bilirubin;** **Lipids;** **Steroids:** (aldosterone, corticosterone, cortisol).	**Antiarrhythmics** (amiodarone, propafenone, quinidine) **Antibacterial agents** (actinomycin D, amoxicillin, clarithromycin, doxycycline, erythromycin, gramicidin A, grepafloxacin, levofloxacin, minocycline, rifampin, sparfloxacin, tetracycline, valinomycin) **Antineoplastic drugs** • Anthracenes (bisantrene, mitoxantrone) • Anthracyclines (daunorubicin, doxorubicin) • Antimetabolites (methotrexate) • Cyclin-dependent kinase inhibitors (seliciclib) • Taxanes (paclitaxel, docetaxel) • Topoisomerase I inhibitors (irinotecan, topotecan) • Topoisomerase II inhibitors (etoposide, teniposide) • Tyrosine kinase inhibitors (gefitinib, imatinib mesylate, nilotinib, tandutinib) • Vinca alkaloids (catharanthine, vinblastine, vincristine) **Anticoagulant agents** (dabigatran) **Antidepressants** (amitriptyline, doxepin, nortriptyline, paroxetine, venlafaxine) **Antidiabetics** (berberine) **Antidiarrheals** (loperamide) **Antiemetics** (domperidone, ondansetron) **Antiepileptics and Anticonvulsants** (carbamazepine, felbamate, gabapentin, levetiracetam, lamotrigine, phenobarbital, phenytoin, topiramate) **Antifungal agents** (itraconazole, ketoconazole) **Antigout agents** (colchicine) **Antihelminthics** (abamectin, ivermectin) **Antihistamines** (fexofenadine, terfenadine) **Antihypertensives** (nicardipine, nifedipine, nimodipine, verapamil, azidopine, celiprolol, debrisoquine, prazosin, propranolol, reserpine, talinolol, losartan) **Antipsychotics** (chlorpromazine, trans-flupentixol, trifluoperazine) **Antiretrovirals** (amprenavir, atazanavir, darunavir, indinavir, lopinavir, maraviroc, nelfinavir, ritonavir, saquinavir) **Cardiotonics** (digitoxin, digoxin, quinidine) **Gastric secretion modifiers** (cimetidine, ranitidine) **Immunomodulators** (cyclosporin A, sirolimus, tacrolimus, valspodar) **Lipid modifying agents** (lovastatin, simvastatin) **Linear peptides**: ALLN, leupeptin, pepstatin A **Muscle relaxants** (vecuronium) **Narcotic analgesics** (meperidine, morphine, fentanyl, oxycodone, pentazocine) **Nature products** (curcuminoids, flavonoids, Rhei Rhizoma extract)	**Fluorophores:** Calcein-AM, CDCF, Hoechst 33342, Rhodamine 123, Rhodamine 6G; **Drugs:** Colchicine, Digoxin, Mercaptopurine, Methotrexate, Quinidine, Mitoxantrone, Cisplatina; **Pesticides:** Paraquat; **Others:** Amyloid-β (Aβ) protein, cAMP, cGMP, N-methyl-quinidine;

P-gp substrates		
Endogenous substrates	**Exogenous substrates**	**Substrates used experimentally**
	Other Nervous System Drugs (riluzole, disulfiram, methadone) **Pesticides** (cypermethrin, endosulfan, fenvalerate, methyl parathion, paraquat) **Steroid Hormones** (dexamethasone, hydrocortisone, methylprednisolone) **Vasodilators** (diltiazem)	

Aβ (amyloid β), ALLN (N-acetyl-L-leucyl-L-leucylnorleucinal)*, cAMP (cyclic adenosine monophosphate), CDCF (carboxydichlorofluorescein), cGMP (cyclic guanosine monophosphate), P-gp (P-glycoprotein).*

1.4.2. BCRP substrates

Similarly to P-gp, BCRP has also a wide variety of substrates, and it appears to transport both positively and negatively charged drugs (85, 86). However, unlike P-gp, there are not many studies elucidating the structural features that allow protein binding (32).

BCRP has some overlap of substrates with P-gp, in particular some anticancer agents such as mitoxantrone (MTX) (87), camptothecins (87, 88), methotrexate (89, 90) and tyrosine kinase inhibitors (91, 92) (**Table 4**). However, some chemotherapeutic agents that are P-gp substrates are not BCRP substrates, such as vinblastine, cisplatin and paclitaxel (32).

BCRP substrates also include other drugs, such as prazosin (87), rosuvastatin (93), sulfasalazine (94), cimetidine (95), indolocarbazole (96), pantoprazole (97), flavopiridol (98), nitrofurantoin (99), nelotinib (100), canertinib (CI1033) (101), zidovudine (AZT) and glibenclamide (**Table 4)** (5). In addition to the pharmacological substrates, BCRP has the ability to transport other compounds, particularly photosensitizers, which may suggest that BCRP is a possible cause of cell resistance to photodynamic therapy (31, 32).

Table 4. BCRP substrates. Adapted from (5, 14, 31, 32, 84, 102, 103)

BCRP substrates		
Endogenous substrates	**Exogenous substrates**	**Substrates used experimentally**
Dietary flavonoids; Estrone 3-sulfate; E217ßG; Porphyrins.	**Analgesics and antipyretics** (acetaminophen) **Antihypertensives** (prazosin, reserpine) **Antimicrobial agents** (ciprofloxacin, norfloxacin, ofloxacin) **Antineoplastic drugs** • Anthracenes (mitoxantrone) • Anthracyclines (epirubicin, daunorubicin, doxorubicin, idarubicinol) • Antimetabolites (methotrexate)	**Fluorophores:** BODIPY- prazosin, LysoTracker, Hoechst 33342, Rhodamine 123, Carboxy-dichlorofluorescein; **Drugs:** Mitoxantrone,

BCRP substrates		
Endogenous substrates	**Exogenous substrates**	**Substrates used experimentally**
	Antineoplastic drugs (cont.) • Flavonoid alkaloid CDK9 kinase inhibitors (alvocidib) • Topoisomerase I inhibitors (9-Amino Camptothecin, diflomotecan, irinotecan, J-107088, NB-506, SN-38, topotecan) • Topoisomerase II inhibitors (etoposide, teniposide) • Tyrosine kinases inhibitors (canertinib, erlotinib, gefitinib, imatinib, mesylate, lapatinib, tandutinib) **Antiretrovirals** (lamivudine, zidovudine) **Gastric secretion modifiers** (pantoprazole) **Immunomodulators** (cyclosporin A, sirolimus, tacrolimus) **Lipid modifying agents** (atorvastatin, cerivastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, simvastatin) **Metabolite conjugates** (2,4-dinitrophenyl-S-glutathione, acetaminophen sulfate, DHEA sulfate, *E217βG,* estrone-3-sulfate) **Natural products** (curcuminoids, flavonoids (genistein, quercetin), phytoestrogens) **Others** (K0143) **Other Nervous System Drugs** (riluzole) **Other Porphyrins** (protoporphyrin IX) **Porphyrins as photosensitizers for antineoplastic photodynamic therapy** (hematoporphyrin, pheophorbide a) **Toxins** (aflatoxin B1, fumitremorgin C, PhIP)	**Drugs (cont.)** Doxorubicin, Cisplatina;

BCRP (breast cancer resistance protein), E217βG (estradiol 17β-D-glucuronide), DHEA (dehydroepiandrosterone or prasterone), PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine), SN-38 (active metabolite of irotecane).

1.5. P-gp and BCRP ATP catalytic cycle and efflux mechanism

Most of the ABC transporters appear to move their substrates across the membrane by using the energy resulting from the hydrolysis of ATP. However, this mechanism is not fully understood (104).

The ABC transporters superfamily, as previously mentioned, has NBDs located in the cytoplasmic side of the cell membrane, which correspond to ATP binding and hydrolysis sites (105). The interaction of these domains appears to be essential for the transport of substrates, as found in studies that were based in the crystalline structure of the soluble Rad50 protein (catalytic domains of a deoxyribonucleic acid (DNA) repair enzyme), which dimerizes in the presence of ATP, since the two NBDs are in close contact to form a dimeric structure (105). Later, other studies based on the structures of bacterial ABC transporters emerged, such as *Vibrio cholera* MsbA (vcMsbA) (106) and BtuCD

(107). The crystalline structures of Rad50, vcMsbA and BtuCD indicated that the Walker A and B domains of one NBD cooperate with the signature C domain (LSGGQ) of the other NBD to form two nucleotide binding sites, yielding a “sandwich dimer", where two ATP molecules are bound at the dimer interface (14, 108). Thus, ATP binding and hydrolysis appeared to induce the formation and dissociation of a NBD dimer, respectively (109).

All ABC transporters exhibit basal ATPase activity, that is, constitutive activity that appears to be caused by decoupling of the transport and takes place in the absence of substrates (14). P-gp is the ABC transporter with the highest basal activity (3-5 μmol/min per mg of purified protein; affinity constant (Km) of 0.2-0.5 mM). It was suggested that this high level of basal ATPase activity in the absence of substrate (110) was due to the uncoupling of ATPase activity from drug transport (111). After substrate binding, ATPase activity is increased by 3-4 fold (112) and, in some cases, up to 20-fold (113). The basal ATPase activity of P-gp is modulated by substrates and shows a biphasic pattern, that is, some substrates stimulate its activity at low concentrations and inhibit it at high concentrations (114). BCRP seems to have a Km value similar to P-gp, however, the basal ATPase activity can only be stimulated up to twice by its substrates (31).

There are some difficulties in determining if all proteins belonging to the superfamily of ABC transporters exhibit the same efflux mechanism and ATP catalytic cycle. As it is known, there are differences in the molecular models of each specific protein, with variations in the number of TMDs, which may indicate that the different ABC transporters possess different mechanisms to transport their substrates (104).

P-gp presents, as previously mentioned, two TMDs and two NBDs, consisting of highly conserved domains called Walker A and B and C domain, which seem to play an essential role in the ATP hydrolysis reaction (104, 115). The catalytic cycle of P-gp is thought to occur as follows: the substrate binds to a site with high affinity in the TMDs at the level of the lipid bilayer’s internal leaflet, which induces an increase in the affinity of the opened NBD for ATP. The binding of ATP to the NBD causes a conformational change in the NBD leading to its dimerization (closed dimer). This modification causes a conformational change in P-gp, which results in a decrease in its binding affinity for the compound and in a change in the binding site, which is now oriented towards the outer leaflet. After ATP hydrolysis, but prior to the release of adenosine diphosphate (ADP) and inorganic phosphate (Pi), an additional conformational change occurs with dissolution of the closed NBD dimer. Immediately after the release of ADP + Pi, which causes P-gp to re-establish its original state (that is, it reorganizes and directs the ligand binding site to the internal leaflet of the lipid bilayer’s), the carrier becomes ready for the next catalytic cycle (4, 17, 104, 109, 116). It is suggested that, for P-gp, the conversion of the "inward" conformation (when binding the substrate) to the "outward" conformation (to release the transported

substrate) requires two ATP hydrolysis actions, which do not occur simultaneously (117). Studies indicate that the binding and hydrolysis of an ATP molecule results in a "power stroke" causing the protein to alter the state of binding to the substrate (from high to low affinity), which triggers its transport and release on the extracellular side (118). It is then necessary to hydrolyze a second ATP molecule to restore the high affinity state of the protein so that a new transport cycle can be initiated (108). Initially, it was common to defend the theory that ATP hydrolysis promoted conformational changes in the protein, making it functional for the transport of substrates (108). However, more recent studies indicated that it is the ATP binding, rather than its hydrolysis, that alters the protein and allows the transport of the substrates (109, 119, 120). However, the stoichiometry of the ATP hydrolysis relatively to the transport of its substrates is still a controversial subject, since it is not yet fully understood whether P-gp hydrolyzes one or two ATP molecules per each substrate that is transported (26). Furthermore, the human P-gp cryo–electron microscopy structure in an outward-facing conformation was recently reported, in which two ATP molecules are bound between the two NBDs. The substrate-binding site, located in the TMD, was reported to be open to the outside of the cell, and no substrate was bound, suggesting that ATP binding, rather than ATP hydrolysis, promotes the transition to the outward-facing conformation and the substrate release (121). It was also proposed that the subsequent ATP hydrolysis, which may occur at only one of the two catalytic sites stochastically, resets the transporter to the inward-facing conformation (121).

For BCRP, which is a half-transporter, it is assumed that the transport process also occurs by ATP binding and hydrolysis. Several studies using plasma membranes of insect cells with BCRP overexpression demonstrated the occurrence of ATP hydrolysis through BCRP photo-staining with 8-azido[α-^{32}P]ATP, in the presence of vanadate and Mg^{2+} or Cobalt ion (II) (Co^{2+}), under hydrolytic conditions (122, 123). There are still many doubts about how ATP binding and hydrolysis occurs, which favors the translocation of substrates mediated by BCRP. However, McDevitt *et al.* (2008) demonstrated that isolated ATP binding had the ability to convert the substrate binding site from high to low affinity (124). These results seem to meet the theory that supports ATP binding, and not its hydrolysis, as the driving force for the BCRP-mediated transport of the substrate (31).

1.6. Modulation of P-gp and BCRP expression and activity

1.6.1. General concepts

ABC transporters, as efflux pumps, are, from the toxicological point of view, useful tools to remove potentially harmful compounds from inside the cells. The fact that ABC transporters mediate the efflux of toxic substances from the cells should be emphasized as an important protection mechanism, since they decrease their intracellular concentration, thereby limiting their absorption and, consequently, reducing their toxicity by limiting the amount of xenobiotic that reaches the target tissue/organ (5). However, from the pharmacological point of view, these transporters will contribute to the decrease in drug BA, leading to the MDR phenomenon, which compromises the drug therapeutic efficacy (5, 17). In fact, the overexpression of ABC transporters is commonly associated with resistance to antineoplastic drugs, which limits the therapeutic efficacy of chemotherapy, since many chemotherapeutic agents are substrates of these transporters (125, 126). In this context, it is imperative to note that P-gp was the first efflux protein of the ABC superfamily to be associated with the MDR phenomenon in anticancer therapy (24).

MDR (one of the key problems in cancer therapy) is established as a phenomenon in which tumor cells, when exposed to a single drug, develop cross-resistance to other structurally and functionally unrelated cytotoxic agents (127). There are two categories of MDR, one of which may be termed as "intrinsic", when the tumor is refractory to chemotherapeutic agents since the beginning of the treatment, and the other is termed as "acquired" resistance, when the disease does not respond to the treatment after a relapse (128). MDR can be explained by several mechanisms, such as: the induction of efflux systems (e.g. P-gp) (117, 129); the expression and/or altered function of target proteins (e.g. tubulin and topoisomerase) (117); the induction of detoxification routes (130); an exaggerated DNA repair (127); and changes in the apoptotic signalling pathway (e.g., p53 mutation or Bcl-2 overexpression) (131).

Several clinical strategies may be undertaken in an attempt to overcome the problems associated with the MDR phenomenon (4). From these studies, the mechanism of inhibition of ABC efflux pumps was proposed, by using several compounds to block their activity (4). This finding emerged in 1981, few years after the isolation of the first cell line exhibiting the MDR phenomenon (Chinese hamster ovary cells), when verapamil (VER) was found to have a P-gp inhibitory effect, overcoming vincristine resistance in P388 leukaemia cells, thus potentiating the therapeutic use of such inhibitory compounds to increase drug BA and to achieve a treatment with an increased efficacy (132, 133). However, in some clinical trials, most of these chemosensitizers have shown to be unbeneficial due to the inherited toxicities or pharmacokinetic interactions, and only

showed limited or no benefits for cancer patients. Therefore, new inhibitors are being explored, which includes the development of new chemosensitizers and novel ways, such as ribonucleic acid (RNA) interference and epigenetic regulation (126).

In addition to ABC transporters inhibition, other mechanisms of modulation, namely induction and activation, also seem to present clinical relevance (4, 134). The induction and activation of the ABC transporters are not necessarily related, since an increased activity of the transporter (activation) may occur without a concomitant increase of its protein expression (induction), and increases in the P-gp protein expression (induction) may occur without concomitant increases in the activity of the protein (5, 134-136). In fact, an activator binds to the protein inducing a conformational alteration that stimulates the transport of a distinct substrate bound to a different binding site without interfering with the protein expression levels, and an inducer acts by promoting an upregulation in protein expression levels, from which an increased transport activity is expected, however, not necessarily observed (4, 5). Inducers and activators may be useful to decrease the amount of toxic compounds that reach the target tissues, and may be, in consequence, important tools in the treatment of intoxications by toxic substrates of these carriers (5, 137).

Therefore, in general, the compounds that interact with the ABC transporters may be classified as: **substrates**, compounds able of being transported by the efflux pumps; **inhibitors**, compounds that decrease the ABC transporters-mediated efflux of other compounds; **inducers**, compounds able to increase the expression of the transporter, from which a corresponding increase in its activity is expected; and **activators**, compounds that immediately increase the activity of the transporter without the need of increase its expression. However, a compound may present an overlap of the above-mentioned actions (4, 5, 137).

1.6.2. Inhibition

1.6.2.1. P-gp inhibition

Three different mechanisms are currently known for P-gp inhibition, namely blockade of the substrates binding site, in a competitive, non-competitive or allosteric mode (138); inhibition of ATP hydrolysis (139), and modification of the integrity of cell membrane lipids (140, 141).

Most drugs inhibit P-gp by blocking the substrates binding to the protein. However, the presence of multiple binding sites impairs the complete understanding of the transport mechanism and the elucidation of the mechanism(s) of P-gp inhibition (138). The transporter inhibition through the blockade of ATP hydrolysis proves to be more effective

than blocking the binding of substrates, since compounds acting through this mechanism are unlikely to undergo transport mediated by the protein. In addition, this mechanism has the advantage of not being necessary the administration of very high doses of compound to exert its P-gp inhibitory effect, namely in the intestinal lumen (4, 138). Other compounds, namely surfactants, act by altering the integrity of the cell membrane lipids, which modify the secondary and tertiary structure of the protein, leading to the modification of membrane fluidity, thus disturbing the hydrophobic environment (138).

Compounds that inhibit P-gp are categorized into four generations, according to their potency, selectivity and potential for triggering pharmacokinetic interactions (**Table 5**) (133).

The first-generation of P-gp inhibitors comprises pharmacologically active compounds that were already in clinical use or in clinical studies, and which later showed the ability to inhibit P-gp (133, 138). First-generation P-gp inhibitors include drugs such as antibiotics, anticancer, antifungal, antihistamines, anti-inflammatory, antimalarial, antiprotozoal, antiviral, anesthetics, cardiac/circulation drugs, CNS depressants, CNS stimulators, immunossupressants, steroid hormones and drugs for erectile dysfunction (**Table 5**) (133). Three of the first-generation P-gp inhibitors are VER (142), quinidine (143) and cyclosporine A (144). Although some of these compounds are P-gp substrates and, therefore, compete for protein binding, the low affinity between the substrate and the carrier limits their clinical use, since very high doses are necessary to achieve their inhibitory effect, which can cause toxicity (4, 133). In addition, many of these chemosensory inhibitors are substrates for other carriers and enzyme systems, resulting in unpredictable pharmacokinetic interactions in the presence of chemotherapeutic agents (133). In order to avoid this limitation, some studies have sought to improve the toxicity profile of these inhibitors, resulting in the second- and third-generations of P-gp inhibitors (133, 138).

The second-generation of P-gp inhibitors comprises compounds derived from other drugs with known clinical use, namely derived from the first-generation inhibitors, which underwent structural modifications in order to lose the pharmacological properties of the original molecule. These compounds have the ability to specifically inhibit P-gp, with less toxicity and greater potency (133, 145). For example, dexverapamil is the R-enantiomer of VER and its discovery was based on the toxicity profile and experimental potency of VER (146). The second-generation of P-gp inhibitors comprises derivatives of drugs with action in the cardiovascular system, immunossupressants, anticancer drugs, among others represented on **Table 5** (133).

Table 5. P-gp inhibitors. Adapted from (4, 5, 26, 133)

P-gp inhibitors		
First generation	**Therapeutic Classes and/or Clinical Use**	
	Analgesics	Meperidine, pentazocine
	Anesthetics	Chloroform, benzyl alcohol, diethyl ether, propofol
	Antibiotics	Cefoperazone, ceftriaxone, salinomycin, nigericin, erythromycin, azithromycin, brefeldin A, bafilomycin, clarithromycin, valinomycin
	Anticancer drugs	Tamoxifen, bicalutamide, mitotane, gefitinib, lapatinib, erlotinib, lonafarnib (SCH 66336), tipifarnib, vinblastine
	Antifungals	Itraconazole, ketoconazole, econazole, dihydroptychantol A, aureobasidin A
	Antihistamines	Benzquinamide, azelastine, tesmilifene, astemizole, terfenadine
	Anti-inflammatory drugs	Zomepirac, indomethacin, SC236, curcumin, ibuprofen, NS-398
	Antidepressants	Amoxapine, loxapine, sertraline, paroxetine, fluoxetine
	Antimalarial drugs	Quinine
	Antiprotozoal drugs	Hycanthone, monensin, metronidazole
	Antiviral drugs	Concanamycin A, ritonavir, nelfinavir, saquinavir
	Anxiolytics, sedative and hypnotics	Midazolam
	Cardiac/circulation drugs	Antiarrhythmics: amiodarone, propafenone, quinidine Calcium channel blockers: verapamil, emopamil, nifedipine, nicardipine, niguldipine, nitrendipine, nimodipine, felodipine, isradipine, lomerizine, tetrandrine, mibefradil, diltiazem, bepridil Antiplatelet drug: dipyridamole Antihypertensives: reserpine, prazosin, doxazosin, carvedilol
	Central nervous system stimulants	Caffeine, pentoxifylline, nicotine, cotinine
	Cholesterol-lowering drugs	Atorvastatin
	Dopaminomimetics drugs	Bromocriptine
	Drugs for treatment of drug addiction	Disulfiram, methadone
	Immunomodulators	Cyclosporin A, sirolimus, tacrolimus
	Neuroleptics and Anti-psychotics	Trans-Flupentixol, perphenazine, prochlorperazine, chlorpromazine, trifluoperazine, perospirone, haloperidol, Tetrabenazine
	Phosphodiesterase inhibitors	Vardenafil
	Steroid hormones	Progesterone, medroxiprogesterone, cortisol, methylprednisolone, medroxiprogesterone 17-acetate, mifepristone, tirilazad, U-74389 F, SB4723, SB4769

P-gp inhibitors	
Second generation	**Compounds (First generation origin)**
	BIBW22BS (Dipyridamole) **Biricodar (VX-710), timcodar (VX-853)** (Tacrolimus) **CGP 42700** (Staurosporine) **Cinchonine, hydro-cinchonine, quinine homodimer Q2** (Quinidine / Quinine) **Dexniguldipine, PAK-104P** (Niguldipine) **Dexverapamil, MM36, KR-30031, RO44-5912** (Verapamil) **Dofequidar (MS-209)** (Ciprofloxacin / levofloxacin) **S9788** (Almitrine) **SB-RA-31012** (tRA96023) (Paclitaxel) **Stipiamide homodimer** (Stipiamide) **Toremifene** (Tamoxifen) **Valspodar (PSC-833)** (Cyclosporine) **WK-X-34** (Tetrandrine)
Third generation	CBT-1, DP7, elacridar (GF120918), laniquidar (R101933), ontogen (OC144-093), PGP-4008, tariquidar (XR9576), zosuquidar (LY335979).
Fourth generation	**Dual ligands:** Dual inhibitors of P-gp and tumor cell growth [aminated thioxanthones such as 1-[2-(1H-benzimidazol-2yl)ethanamine]-4-propoxy-9H-thioxanthen-9-one] **Natural products** • **Alkaloids**: Ellipticine, pervilleine F • **Cannabinoids**: Cannabidiol • **Coumarins**: Cnidiadin, conferee, DCK, praeruptorin A, rivulobirin A • **Diterpenes**: Euphodendroidin D, jolkinol B, pepluanin A, portlanquinol • **Flavonoids**: Baicalein, heptamethoxyflavone, nobiletin, quercetin, sinensetin, tangeretin • **Ginsenosides**: 20S-ginsenoside • **Lignans**: Nirtetralin, schisandrin A, silibinin, • **Polyenes**: Pentadeca-(8,13)-dien-11-yn-2-one • **Sesquiterpenes**: Dihydro-β-agarofuran sesquiterpene • **Taccalonolides**: Taccalonolides A • **Triterpenes**: Oleanolic acid, sipholenol A, sipholenone E, uvaol, **Peptidomimetics:** Peptide 15, reversin 121, reversin 205, XR9051 **Surfactants and Lipids:** Cremophor EL, nonidet P40, pluronic P85, poly (ethylene glycol)-300 (PEG-300), triton X-100, tween-20.

P-gp (P-glycoprotein)

This second-generation of P-gp inhibitors has a better pharmacologic profile than the first-generation, although some of these compounds retain some characteristics that limit their use as transporter inhibitors, such as: a) these compounds significantly inhibit the metabolism and excretion of cytotoxic agents, thus leading to unacceptable toxicity, which requires chemotherapy dose reductions (133); b) many of the second-generation P-gp inhibitors (e.g. biricodar and valspodar) are substrates for cytochrome P450 (CYP 450**)**

and, therefore, the competition between chemotherapeutic agents and these P-gp inhibitors for CYP 450 activity has given rise to unpredictable pharmacokinetic interactions (133); c) several second-generation modulators may also inhibit other transporters, namely other ABC transporters (147), which may lead to a decreased capacity of normal cells to extrude toxic compounds or xenobiotics in the liver, kidneys or gastrointestinal tract (148, 149). Therefore, to overcome the limitations of the first- and second-generation inhibitors, several new third-generation inhibitors have been developed (133).

Third-generation P-gp inhibitors are compounds that specifically and potently inhibit P-gp, comprising this class the most selective and potent P-gp inhibitors known to date. These compounds were designed by using *quantitative structure-activity relationships* (QSAR) and combinatorial chemistry (145), which allowed the design of molecules with specific characteristics, such as lipophilicity, positive charge at neutral pH and with aromatic rings (150). In contrast to the second-generation P-gp inhibitors, the third-generation inhibitors do not interfere with CYP 450, namely with CYP 3A4, thus not affecting the pharmacokinetics of anticancer agents when given in combination, and thus not requiring a reduction in the dose of the chemotherapeutic drugs (4, 133). The third-generation inhibitors include zosuquidar (ZOS), elacridar, tariquidar, laniquidar, ontogen, DP7, PGP-4008 and CBT-1 (133). Third-generation inhibitors are summarized in **Table 5**.

A fourth generation of P-gp inhibitors was also created, which includes extracts of natural compounds and derivatives (151), surfactants and peptidomimetics, and other agents which, in addition to inhibit P-gp, also exhibit other beneficial biological activities (e.g. dual ligands) (**Table 5**) (133). Surfactants act by altering the integrity of membrane lipids leading to modifications in the secondary and tertiary structure of the protein (152). Other substances, such as rifampicin derivatives, as DiBenzRif, appear to limit P-gp activity by increasing the membrane fluidity at sub-toxic concentrations, thereby inhibiting the protein (153).

1.6.2.2. BCRP inhibition

BCRP is also involved in the MDR phenomenon and, thus, over the past years, an effort has been undertaken to develop compounds with an inhibitory effect of this transporter. BCRP inhibitors can be classified into four distinct classes: specific BCRP inhibitors; inhibitors of BCRP and other ABC transporters, namely P-gp and/or MRP1; natural products, such as flavonoids and their derivatives; and tyrosine kinase inhibitors (**Table 6**) (5, 154).

Specific BCRP inhibitors include fumitremorgin C (FTC) (155), novobiocin (156) and Ko143, which is a nontoxic synthetic analogue of FTC with high affinity for the protein and with the ability to inhibit it intensively (31, 155).

Among the broad-spectrum inhibitors category, *i.e.*, compounds that exhibit an inhibitory effect not only for BCRP but also for P-gp and/or MRP1, elacridar (GF120918) can be found, which is, as previously mentioned, a potent third-generation P-gp inhibitor (157, 158).

In what concerns flavonoids and their derivatives, those with BCRP inhibitory action include biochanin A and chrysin (159). The flavonoids are also known to inhibit P-gp and MRP1, but some show significantly higher affinity towards BCRP (31, 160, 161).

The last category includes tyrosine kinase inhibitors, such as gefitinib (162) and imatinib. These compounds can be classified as broad-spectrum inhibitors because they have demonstrated the ability to interact with P-gp, MRP1 and BCRP (163-165). In BCRP, tyrosine kinase inhibitors are able to inhibit the phosphatidylinositol 3 kinase–actin pathway, which could lead to relocation of BCRP to the intracellular compartment or to a decrease in BCRP expression (154).

Table 6. BCRP inhibitors. Adapted from (5, 63, 67, 154, 166-168)

BCRP inhibitors	
Class	**Examples**
Specific inhibitors	Eltrombopag, fumitremorgin C and analogues (Ko143, Ko132, Ko134, Cl1033),
Compounds that also inhibit P-gp and/or MRP1	Atazanavir, cyclosporine, elacridar (GF120918), epigallocatechin-3-gallate, ritonavir, tariquidar
Natural products	Flavonoids and derivatives, novobiocin, tryprostatin A
Tyrosine kinase inhibitors	Gefitinib and imatinib

BCRP (Breast cancer resistance protein)

1.6.3. Induction

Cells can adapt to the presence of xenobiotics through the positive regulation of ABC transporters expression, namely P-gp, promoting their long-term survival. In fact, the *MDR1* gene is activated and a stable MDR phenotype is induced, after exposure of the cells to a wide range of environmental factors (4, 25).

The genes coding to transporters are regulated at various levels, including membrane recovery and reinsertion, translation and transcription (25). Regulation of ABC transporters gene expression involves numerous nuclear receptors (169, 170). Nuclear

receptors are a family of transcription factors, which activity is modulated by binding to small signaling molecules, namely steroids, vitamin D3, thyroid hormone and retinoids (171, 172). The most relevant nuclear receptors for the expression of ABC transporters are *farnesoid X receptor* (FXR), *liver X receptor* (LXR), *pregnane X receptor* (PXR), *peroxisome proliferator-activated receptor α* and *peroxisome proliferator-activated receptor γ* (PPARα and PPARγ, respectively) (173).

Induction is a process that involves the positive regulation of protein expression levels from which an increased activity of the carriers is expected. However, it is possible to observe an increase in protein expression levels without a concomitant increase in its activity (5). Inducers of the ABC transporters appear to be a useful tool in reducing the access of potentially harmful compounds to specific target tissues, due to their ability to increase the expression of efflux transporters (5, 137).

1.6.3.1. P-gp induction

The P-gp induction has deserved special attention in studies related to cell protection, and there are several compounds that have shown to increase P-gp expression (**Table 7**). P-gp inducers present a wide structural diversity, which may indicate the existence of different signaling pathways able to regulate the transcription of the *MDR1* gene, as well as the strong ability of the gene to respond to a very broad range of stimuli, such as drugs, cytokines, free radicals, tumor suppressor genes and external stimuli, as environmental factors [gamma and ultraviolet (UV) radiation] (23, 25). Genetic and environmental factors have been shown to influence P-gp expression and also its activity. For example, the expression or function of P-gp is highly altered by some pathological conditions, such as diabetes, cancer and Alzheimer's disease (174-176).

Table 7. P-gp inducers. Adapted from (4, 25).

P-gp inducers	
Class	**Examples**
Antiarrhythmic drugs	Amiodarone, diltiazem, quinidine, verapamil
Antibacterial agents	Actinomycin D, doxycycline, erythromycin, rifampicin
Anticancer drugs	Alkylating agent (chlorambucil, cisplatin, cyclophosphamide, ifosfamide) Anthracenes (mitoxantrone, MX2) Anthracyclines (daunorubicin, doxorubicin, epirubicin, idarubicin) Anti-androgens (flutamide) Antimetabolites (5-fluorouracil, cytarabine, hydroxyurea, methotrexate) Others (m-amsacrine, daidzein, tamoxifen) Taxanes (paclitaxel, docetaxel) Topoisomerase I inhibitors (topotecan) Topoisomerase II inhibitors (etoposide)

P-gp inducers	
Class	**Examples**
Anticancer drugs (cont.)	Tyrosine kinase inhibitors (erlotinib, gefitinib, nilotinib, sorafenib, vandetanib) Vinca alkaloids (vinblastine, vincristine)
Antidepressants	Desvenlafaxine, nefazodone, trazodone, venlafaxine
Antiepileptics and anticonvulsants	Carbamazepine, pentylenetetrazole, phenobarbital, phenytoin
Antimalarial drugs	Artemisinin
Antiprotozoal agents	Emetine
Antipsychotics	Phenothiazine
Antifungals	Clotrimazole, trichostatin A
Anxiolytics, sedative and hypnotics	Midazolam
Antihelmintic	Avermectin, Ivermectin
Antihistamines	*R*-Cetirizine
Antihypertensive drugs	Ambrisentan, bosentan, celiprolol, diltiazem, nicardipine, nifedipine, propranolol, rescinnamine, reserpine
Nonsteroidal anti-inflammatory drugs (NSAIDs)	Diclofenac, fenbufen, indomethacin, meloxicam, nimesulide, sulindac
Antiviral drugs	Abacavir, amprenavir, atazanavir, daurunavir, efavirenz, indinavir, lopinavir, nelfinavir, nevirapine, rilpivirine, ritonavir, saquinavir
Cardiotonic drugs	Digoxin
Cholesterol-lowering drugs	Atorvastatin, berberine
Diuretics	Spironolactone
Dopaminomimetic drugs	Bromocriptine
Drugs used in erectile dysfunction	Sildenafil, Tadalafil
Drugs used for the treatment of gout	Colchicine, Probenecid
Endobiotics	1α,25-Dihydroxyvitamin D3 [1,25$(OH)_2D_3$]
Hormones	Insulin
Immunomodulators	Cyclosporine A, tacrolimus (FK-506), sirolimus
Central nervous system stimulants	Caffeine
Toxins	Aflatoxin B1
Natural products	Alkaloids (piperine) Catechin (epigallocatechin-3-gallate (EGCG)) Curcumins (*curcuma* extracts (extracts of *C. longa*, *C. zedoaria and C. aromatica*), curcumin, tetrahydrocurcumin) Diterpenes (ginkgolide-A, ginkgolide-B)

P-gp inducers	
Class	**Examples**
Natural products (cont.)	Flavonoids (chrysin, apigenin, catechin, eriodictyol, flavone, genistein, myricetin, naringenin, quercetin, tangeretin, taxifolin, isoxanthohumol) Phytochemicals (hyperforin, hypericin, *Hypericum perforatum extracts* (Saint John's-wort)) Polyphenols (norathyriol (polyphenol from *Mangifera indica*)) Quinones (rhinacanthin-C) Xanthonoids (Mangiferin (polyphenol from *Mangifera indica*)) Others (asiatic acid, capsaicin, parthenolide, oleocanthal, ouabain, retinoic acid, γ-tocotrienol, isosafrole).
Narcotic analgesics	Morphine, Oxycodone
Steroid hormones, and synthetic and semi-synthetic derivatives	Aldosterone, corticosterone, β-Estradiol, ethinylestradiol, 5β-Pregnane-3,20-dione Beclomethasone, betamethasone, budesonide, ciclesonide, dexamethasone, fluticasone, mifepristone (RU486), methylprednisolone, prednisolone
(Thio)xanthones	**Chiral aminated thioxanthones:** • (R)-1-((1-Hydroxypropan-2-yl)amino)-4-propoxy-9H-thioxanthen-9-one **[ATx 2 (−)]** **Dihydroxylated xanthones**: • 3,4-Dihydroxy-9H-xanthen-9-one **(X1)** • 1,2-Dihydroxy-9H-xanthen-9-one **(X2)** • 1,3-Dihydroxy-9H-xanthen-9-one **(X3)** • 2,3-dihydroxy-9H-xanthen-9-one **(X4)** • 3,6-dihydroxy-9H-xanthen-9-one **(X5)** **Thioxanthonic derivatives:** • 1-[(3-hydroxypropyl) amino]-4-propoxy-9H-thioxanthen-9-one **(TX1)** • 1-chloro-4-hydroxy-9H-thioxanthen -9-one **(TX2)** • 1-{[2-(1,3-benzodioxol-5yl) ethyl] amino-4-propoxy-9H-thioxanthen-9-one **(TX3)** • 1-[(2-methylpropyl) amino]-4-propoxy-9H-thioxanthen-9-one **(TX4)** • 1-(propan-2-ylamino) -4-propoxy-9H-thioxanthen-9-one **(TX5)**
Others	6,16α-dimethylpregnenolone, 2-acetylaminofluorene (2-AAF), N-acetoxy-2-acetylaminofluorene (AAAF), benzo(a)pyrene (BAP), benzo(e)pyrene (BEP), bilirubin, cadmium chloride, cembratriene, cholate, CITCO, , cyanidin, cycloheximide, depsipeptides (FR901228, FK228, NSC630176), dimethylformamide, dimethylsulfoxide, LY191401, *N*-hydroxy-2-(acetylamino)fluorene (*N*-OH-AAF, a metabolite of AAF derived via cytochrome P-450 dependent metabolism), phorbol 12-myristate 13-acetate (PMA), platelet-activating factor (PAF), pregnenolone-16α-carbonitrile (PCN), reduced rifampicin derivative (RedRif), sodium arsenite, sodium butyrate (NaB), SR12813 [[3,5-*bis*(1,1-dimethylethyl)-4-hydroxyphenyl]ethenylidene]*bis*-phosphonic acid tetraethyl ester, taurocholate, TCDD (2,3,7,8-tetraclorodibenzo-p-dioxina), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), triactyloleandomycin, trimethoxybenzoylyohimbine **Heat shock, UV-irradiation, X-irradiation**

P-gp (P-glycoprotein).

It is generally accepted that P-gp expression is increased as a consequence of an increase in the mRNA levels of the *MDR1* gene, which is observed in many cell lines and human metastatic sarcomas (23). This increase in the *MDR1* mRNA levels may be due to gene amplification and/or increased gene transcription (177, 178). These processes potentiate the decrease of the xenobiotic intracellular concentration, thus suggesting the induction of P-gp as a potential route for attenuation or prevention of the toxicity of its toxic substrates (23).

Studies have shown that the transcription of a gene is determined by the presence of response elements in the gene promoter, by their complexity and accessibility, and by the transcription factors that can interact with those response elements (179). The transcription process is, in fact, extremely complex and determined by the binding of transcription factors to distinct zones of the promoter. In this context, it is known that practically half of the genes transcribed by RNA polymerase II contain a TATA box and, in these cases, the first event that occurs is the recognition and binding of a transcription factor, TBP (*TATA binding protein*), to the TATA box (179). However, the promoter region of the human *MDR1* gene is atypical as it does not contain the TATA promoter sequence. Nevertheless, it has multiple response elements that support a complex regulation of P-gp expression (23). In the human *MDR1* gene, several studies have demonstrated the presence of several elements in its promoter, namely GC-box, Y-box, the p53 element, an inverted mediator-1 (invMED1) element, an activator protein 1 (AP-1), a heat shock element (HSE) and a steroid xenobiotic receptor (SXR) (4, 23, 180).

Although the mechanism underlying P-gp induction has not yet been fully elucidated, several transcription factors are now known to be involved in the regulation of P-gp expression, namely PXR, *constitutive androstane receptor* (CAR), *nuclear factor erythroid-derived 2-related factor* (Nrf2), *Y-box binding protein 1* (YB-1), *nuclear factor Y* (NF-Y), *nuclear factor kappa B* (NF-κB), LXR, FXR, PPARα and PPARγ (4, 5, 181, 182). These transcription factors regulate the transcription of the *MDR1* gene by binding to the above-mentioned promoter elements (4).

PXR is activated by a diverse number of P-gp inducers, including mifepristone, phenobarbital, and rifampicin in humans (183), and mediates the expression of human MDR1/P-gp (184). Geick *et al.* (2001) elucidated the molecular mechanism of MDR1 induction by rifampicin (bactericidal antibiotic used in the treatment of tuberculosis) (184). In this study, the human colon carcinoma cell line LS174T was used as an intestinal model to study induction because, in these cells, the endogenous *MDR1* gene is highly inducible by rifampin. Induction depends on the activation of the nuclear receptor PXR (NR1I2), which binds to a distinct DR4 motif in the *MDR1* regulatory region (184). In addition, rifampicin has been described to induce P-gp expression and activity in

lymphocytes, intestinal cells, hepatic cells and in renal cells, both *in vivo* and *in vitro* (185-188), via the PXR pathway (189). Some authors have recently reported that chronic treatment with rifampicin (repeated administration of 250 mg/kg for 9 days) induced P-gp overexpression, *in vivo*, in rat (maternal and fetal liver, heart and brain, as well as maternal jejunum, kidney and placentas, and fetal small intestine), via the PXR pathway (190). Since several of the ABC transporters, namely P-gp, are inducible by a PXR pathway and rifampicin is one of the ligands of PXR, the ability of induction of *Abcb1a* mRNA levels by rifampicin may be explained (190).

Arnold *et al.* (2004) have shown that the transcription factor CAR is expressed in the human small intestine and the expression of CAR is induced during the enterocytic differentiation of Caco-2 cells (191). Another research group has demonstrated that CAR is highly expressed in the differentiated epithelial cells of the villus tip, as shed enterocytes showed enhanced CAR mRNA expression when compared to small intestinal mucosa. In fact, the expression of CAR in enterocytes was nine-fold higher than in small intestinal mucosa, thus indicating that the enterocytes, especially terminally differentiated enterocytes, are the cells that predominantly express CAR in the intestine (192). The physiological relevance of CAR-dependent regulation of *MDR1* in the small intestine is suggested by the significant correlation between CAR and *MDR1* mRNA expression levels. In contrast, CAR and *MDR1* expression were not correlated in the liver. Accordingly, it appears that CAR may play a specific role in the regulation of intestinal *MDR1* expression. Therefore, CAR expression may be an important determinant of the interindividual variability of basal *MDR1* expression in the small intestine, since the intestine is the major site of drug interactions involving P-gp (192). This study clearly demonstrated that CAR binds to distinct nuclear receptor response elements and activates the transcription of the *MDR1* gene through DR4 motifs to which the receptor binds as a heterodimer with retinoid X receptor (RXR) or as a monomer, respectively (192).

1.6.3.2. BCRP induction

BCRP expression can be regulated at the transcriptional level (32). In humans, the promoter region of the *ABCG2* gene is designated E1A and E1B/C (32, 193). Bailey-Dell e*t al.* (2004) characterized E1B/C as the predominant promoter of BCRP (194). Like the human *MDR1* gene, this promoter region is atypical since it does not contain the TATA promoter sequence (32, 193, 194).

To date, several transcription factors and their respective regulatory elements in the BCRP promoter have been characterized, which include an estrogen response element (ERE), a progesterone response element (PRE), an hypoxia response element (HRE), an

antioxidant response element (ARE), an aryl hydrocarbon response element (AhRE), and the active nuclear factor *k*B subunit (NF*k*B) response element (32, 193). Thus, the *ABCG2* gene is upregulated under:

1) Hypoxic conditions, via the *hypoxia-inducible factor 1α* (HIF-1α) (by binding of HIF-1α to an HRE in the BCRP promoter) (195);
2) By estradiol, through estrogen receptor α (ERα) (by binding of ERα to ERE) (196);
3) By progesterone, via progesterone receptor B (PRB) (by binding of PRB to PRE) (197);
4) By aryl hydrocarbon receptor agonists, through the aryl hydrocarbon receptor (AhR) (198).

BCRP expression has also been shown to be induced via the PPARγ (199). Nrf2, an oxidative stress sensor, maintains the side population (SP) cell phenotype by increasing BCRP expression through its direct interaction with an ARE (200). In response to treatments with a histone deacetylase inhibitor (HDACI), namely romidepsin, AhR dissociates from its binding partner, the heat shock protein 90 (HSP90), binds to the AhRE element and stimulates BCRP transcription (198).

Several compounds are described in the literature as inducers of BCRP, namely efavirenz (67), oxotremorine, cetylpyridinium chloride, promazine, benzetimide, ifenprodil, etoposide, perphenazine, venlafaxine (201), phenobarbital (a CAR activator), rifampicin (a PXR activator), and oltipraz (a Nrf2 activator) (202), among others reported in **Table 8**.

Table 8. BCRP inducers. Adapted from (5, 67, 201-207)

BCRP inducers	
Class	**Examples**
Antibiotics	Rifampicin
Anticancer drugs	Etoposide, imatinib
Antidepressants	Venlafaxine
Antidiabetics drugs	Troglitazone
Antiepileptics and anticonvulsants	Phenobarbital
Anti-inflammatory drugs	Curcumin
Antiviral drugs	Efavirenz
Gastric anti-secretory drugs	Omeprazole

BCRP inducers	
Class	**Examples**
Neuroleptics and Anti-psychotics	Benzetimide, perphenazine, promazine,
Natural products	Flavonoids: Chrysin, flavone, naringin, quercetin,
Others	Benzopyrene conjugates, cetylpyridinium chloride dibenzoylmethane, ifenprodil, indole-3-carbinol, oltipraz, oxotremorine, resveratrol, TBHQ, 3-methylcholanthrene (3MC)

BCRP (Breast cancer resistance protein), TBHQ (tert-Butylhydroquinone)

1.6.4. Activation

A new class of compounds was recently identified and called P-gp activators, representing compounds with the ability to immediately increase the activity of the efflux pump through a conformational change of the carrier, without increasing its expression levels (4, 189, 208). This is, therefore, a faster process than the *de novo* synthesis of P-gp (induction), which may be a potential strategy in real-life intoxication scenarios, where a rapid approach is needed (136, 209, 210).

As noted above, the mechanism of activation of ABC transporters, namely P-gp, involves the ability of an activator compound to bind to the protein and induce a conformational change that stimulates the transport of a different substrate bound to a different binding site (5). In addition, the mechanism of P-gp activation seems to involve the NBDs, either due to an increase in the affinity for ATP, increasing its binding, or due to a higher transport speed, resulting in a higher ATP hydrolysis (189, 208).

It is important to note that the concept of "activator" is recent and was reported only for P-gp, although it is well-known that the binding of certain compound to a P-gp binding site stimulates the transport of substrates bound to another site of the protein (211-213). Indeed, Shapiro and Ling (1997) have demonstrated that P-gp exhibits at least two distinct binding sites that positively cooperate with each other. The researchers evaluated the transport of rhodamine 123 (RHO 123) and Hoechst 33342, two fluorescent P-gp substrates, using P-gp enriched plasma membranes vesicles isolated from Chinese hamster ovary CH'B30 cells. In addition to the fact that each substrate stimulated the transport of the other substrate, colchicine and quercetin were found to stimulate the transport of RHO 123 and inhibited the transport of Hoechst 33342, while the anthracyclines [daunorubicin and doxorubicin (DOX)] promoted the transport of Hoechst 33342 and inhibited the efflux of RHO 123. Vinblastine, actinomycin D and etoposide inhibited the transport of the two fluorescent compounds. It was concluded that RHO 123 and anthracyclines bind to the R binding site, while Hoechst 33342, Hoechst 33258,

quercetin and colchicine are H-site substrates. Vinblastine, actinomycin D and etoposide are substrates of both binding sites (211). These results were consistent with the functional model that P-gp exhibits at least two distinct substrates binding sites (H and R), which cooperate positively (211). Indeed, Shapiro *et al.* (1999) later demonstrated that P-gp has at least three positively cooperating drug binding sites, the H and R sites described above, and a third binding site for progesterone, which was reported as an allosteric binding site exhibiting a regulatory function (213). In this case, the activator binding to this third site may have a positive allosteric effect on the transport of substrates bound to the H and R sites (213).

Kondratov and co-authors (2001) tested the effect of a number of small molecules on the intracellular accumulation of DOX in mouse fibroblasts (214). The efflux of DOX occurred in cells expressing P-gp, in contrast to the protein-negative variants, in which no drug efflux was observed (214). The more potent compounds, QB102 and QB11, promoted the efflux of anthracyclines and RHO 123, but decreased the transport of Hoechst 33342 and paclitaxel (214). This study demonstrated that the effect of P-gp modulators seems to depend on the site of substrate binding (R or H) (214). Based on the structure of the QB102 and QB11 activators studied by Kondratov *et al.*, Sterz and co-authors (2009) synthetized and investigated the P-gp modulatory properties of 27 imidazobenzimidazoles and imidazobenzothiazoles, and their effect on the cellular accumulation of RHO 123 and daunorubicin. Most of the synthetized compounds exhibited the ability to promote both RHO 123 and daunorubicin efflux in a concentration-dependent manner. However, some of the tested compounds showed inhibitory effects, although these effects were shown to be weak (208). The novel compounds appear to be able of binding to the P-gp H-site, activating the efflux of specific substrates bound on the R site, whereas binding of substrates to the H-site was competitively inhibited (208).

A recent study aiming to evaluate the P-gp modulatory capacity of four enantiomeric pairs of newly synthetized chiral aminated thioxanthones (ATxs), in Caco-2 cells, and to elucidate the intervention of stereochemistry on P-gp activation and/or induction, showed the ability of ATxs 1-8 to immediately increase P-gp activity (significantly decreasing the intracellular accumulation of the fluorescent P-gp substrate, RHO123, when incubated simultaneously for 60 min), without interfering in its protein expression levels. Consequently, these compounds were considered P-gp activators (**Table 9**). ATx2(-) was the only derivative that, after 24 h of incubation, significantly increased P-gp expression, being able to differently modulate P-gp expression when compared to its ATx1(+) enantiomer. It was, thus, considered to be a P-gp activator and inducer, as described in **Table 7** and **Table 9** (215).

Other studies have demonstrated the P-activation ability of several compounds, namely thioxanthonic derivatives, dihydroxylated xanthones, and a reduced rifampicin derivative (RedRif) (**Table 9**) (134, 136, 189). These compounds, apart from exhibiting the ability to positively modulate P-gp expression behaving as P-gp inducers (**Table 7**), were also able to immediately increase the pump transport activity after a short contact with the cells, and therefore without interfering with its protein expression, and thus behaving as P-gp activators (**Table 9**) (134, 136, 189) .

P-gp activators that interfere with the intracellular accumulation of different substrates are important tools for studying the interaction between the multiple drug binding sites of the protein (4, 208).

To date, BCRP activation was not reported in the literature. However, the need for BCRP modulation studies as a potential therapeutic strategy is highlighted, given its polarized expression in barrier and excretory tissues and its efflux capacity of several substrates. The activators of ABC transporters are important tools in intoxications by toxic xenobiotics, as described in the following section.

Table 9. P-gp activators.

P-gp activators (by class of compounds)	
Chiral aminated thioxanthones (215)	(S)-1-((1-Hydroxypropan-2-yl) amino)-4-propoxy-9H-thioxanthen-9-one **[ATx1 (+)]**, (R)-1-((1-Hydroxypropan-2-yl) amino)-4-propoxy-9H-thioxanthen-9-one **[ATx2 (−)]**, (S)-1-((2-Hydroxypropyl) amino)-4-propoxy-9H-thioxanthen-9-one **[ATx3 (+)]**, (R)-1-((2-Hydroxypropyl) amino)-4-propoxy-9H-thioxanthen-9-one **[ATx4 (−)]**, (S)-1-((1-Hydroxy-4-methylpentan-2-yl) amino)-4-propoxy-9H-thioxanthen-9-one **[ATx5 (+)]**, (R)-1-((1-Hydroxy-4-methylpentan-2-yl) amino)-4-propoxy-9H-thioxanthen-9-one **[ATx6 (−)]**, (S)-1-((1-Hydroxy-3-methylbutan-2-yl) amino)-4-propoxy-9H-thioxanthen-9-one **[ATx7 (+)]**, (R)-1-((1-Hydroxy-3-methylbutan-2-yl) amino)-4-propoxy-9H-thioxanthen-9-one **[ATx8 (−)]**.
Dihydroxylated xanthones (136)	3,4-Dihydroxy-9H-xanthen-9-one **(X1)**, 1,2-Dihydroxy-9H-xanthen-9-one **(X2)**, 1,3-Dihydroxy-9H-xanthen-9-one **(X3)**, 2,3-dihydroxy-9H-xanthen-9-one **(X4)**, 3,6-dihydroxy-9H-xanthen-9-one **(X5)**.
Reduced rifampicin (189)	RedRif.

P-gp activators (by class of compounds)	
Thioxanthonic derivatives (134)	1-[(3-hydroxypropyl) amino]-4-propoxy-9H-thioxanthen-9-one **(TX1)**, 1-chloro-4-hydroxy-9H-thioxanthen -9-one **(TX2)**, 1-{[2-(1,3-benzodioxol-5yl) ethyl] amino-4-propoxy-9H-thioxanthen-9-one **(TX3)**, 1-[(2-methylpropyl) amino]-4-propoxy-9H-thioxanthen-9-one **(TX4)**, 1-(propan-2-ylamino) -4-propoxy-9H-thioxanthen-9-one **(TX5)**.

P-gp (P-glycoprotein)

1.7. Modulation of ABC transporters expression and activity as a potential antidotal pathway

As previously mentioned by significantly reducing the intestinal absorption of harmful xenobiotics and, consequently, avoiding their access to the target organs, the ABC transporters-mediated defense mechanism can be of particular relevance at the intestinal level. At the intestinal barrier, and as mentioned before, different transporters exist, namely those belonging to the ABC superfamily and also to the SLC superfamily, which are expressed at the apical and basolateral membranes of the enterocytes (**Figure 3 D**) (67). In the intestinal wall mucosa, specifically at the apical membrane of the enterocytes, there is a high expression of ABC transporters, which have the ability to bind and transport many endogenous and exogenous compounds, namely drugs, by moving them through a mechanism dependent on the ATP binding and hydrolysis (67). Due to their ability to pump their substrates against the concentration gradient and, in some cases (e.g. P-gp and BCRP), to their ability to transport them towards the lumen of the gastrointestinal tract, these proteins confer protection against toxic molecules, mediating their efflux and thereby avoiding their systemic absorption and, consequently, their access to the target organs (67, 216). Indeed, knowing that many intoxications by ABC transporters substrates may result from accidental or intentional ingestion, the ABC transporters-mediated defense mechanism is particularly relevant at the intestinal level.

However, in what concerns to drugs, their efflux into the lumen of the gastrointestinal tract prevents their passage into the bloodstream, thus reducing their intestinal absorption, thereby interfering with drug BA, metabolism and toxicity, and consequently, with their therapeutic efficacy (61, 62, 217, 218). P-gp and BCRP are the major barriers to intestinal absorption of drugs (5, 14, 38, 67, 219).

As a consequence, the inhibition of ABC transporters in the gut contributes to an increase in the oral BA of drugs and, consequently, to an increase in treatment efficacy (67). On the other hand, the induction/activation of the ABC transporters expressed at the intestinal barrier contributes to a decreased absorption of xenobiotics, by increasing their

efflux towards the intestinal lumen. From a toxicological point of view, this positive modulatory effect of increasing ABC transporters expression and/or activity is of particular interest since it can be exploited as a potential antidotal route in intoxication scenarios with several xenobiotics with appreciable human toxicity (4, 5, 209, 210). In the following sub-sections, several *in vivo* and *in vitro* studies reporting the therapeutic potential of P-gp and BCRP induction/activation as a promising antidotal pathway will be presented, as well as the potential of xanthonic derivatives for P-gp and BCRP modulation, since xanthones are a class known to interact with these transporters and are the main focus of the present dissertation.

1.7.1. *In vivo* and *in vitro* studies

Due to its polarized expression in tissues with excretory and barrier functions, its efflux capacity and its wide specificity for substrates, P-gp is, as previously mentioned, a potential mechanism of cellular defense against toxic compounds (4, 135). In this context, several studies have been carried out to evaluate the therapeutic applicability of using P-gp inducers and/or activators in the detoxification of harmful xenobiotics (4, 5), namely the herbicide paraquat (PQ) (220, 221).

PQ is an extremely toxic herbicide, used as a desiccant and defoliant in various crops, particularly in Asian countries, which is involved in thousands of human deaths due to accidental or voluntary exposure, which occur mostly orally (220, 221). A high oral dose of PQ in humans (> 30 mg/kg) causes rapid death due to multiorgan failure, with severe pulmonary lesions (220, 221). Lower PQ doses (16 mg/kg) may also lead to death, albeit more slowly, as a result of progressive pulmonary fibrosis that culminates in respiratory failure (220). Therefore, as the herbicide is a P-gp substrate, a therapeutic approach for PQ poisonings was proposed, which was based on the induction of this efflux pump (4, 220, 221).

Dinis-Oliveira and co-authors (2006) showed that dexamethasone (DEX), a synthetic glucocorticoid, intraperitoneally administered (100 mg/kg) to Wistar rats 2 h after exposure to PQ (25 mg/kg i.p.), apart from regulating neutrophil recruitment, collagenase activity and proliferation of type II pneumocytes, also demonstrated the ability to confer protection through the induction of *de novo* synthesis of P-gp at the pulmonary level, thus significantly reducing PQ concentration in the lung cells (to about 40% of the group only exposed to PQ), decreasing the intestinal absorption of PQ and, consequently, increasing its fecal excretion, thus reducing the toxic effects associated to the herbicide (220). This therapy also led to an improvement in tissue healing in just 24 h (220). Anti-inflammatory corticosteroid therapy, when administered early in lung injury,

has proven to reduce the morbidity and mortality associated with PQ. VER (10 mg/kg i.p.) was used as a P-gp competitive inhibitor and, when administered 1 h before DEX, prevented its protective effect leading to an increase in PQ concentration in the lung (up to about twice that of the group only exposed to PQ in just 24 h) and to an aggravation of PQ toxicity. This result confirmed the importance of P-gp in the efflux of PQ and in the PQ-mediated lung injury (220). The increased PQ fecal excretion can be explained by an increase in its biliary excretion, since there is a strong expression of P-gp in the canalicular membrane of hepatocytes (220). Furthermore, DEX reduced the lung infiltration by neutrophils observed in the interstitial space or within capillaries neighboring ECs, an effect that can be explained both by its anti-inflammatory properties and by its ability to induce P-gp. However, since in animals exposed to PQ + VER + DEX an increase in myeloperoxidase activity (MPO) was observed, when compared to the PQ + DEX group, it was suggested that the decrease in neutrophil infiltration was mainly due to PQ efflux from the lungs (220). Additionally, the results demonstrated that DEX promoted a significant improvement in the lung biochemical changes induced by PQ, reduced the lung lipid peroxidation (LPO) and reduced the protein carbonyl formation (221). It was also demonstrated that, contrary to the improvement in pulmonary and hepatic function after administration of DEX, the kidney and spleen presented increased LPO and carbonyl groups, as well as increased activity of MPO and aggravation of histological damage (221). Despite these discrepancies, treatment with DEX in PQ poisonings appeared to be a valuable therapeutic option, since the survival rate of the DEX-treated PQ-intoxicated animals was significantly higher than the observed for the PQ and PQ + VER + DEX groups of animals (only the PQ + DEX group had animals that survived beyond the 5th day, with 50% rats remaining alive by the 10th day) (221).

In agreement with the above-mentioned *in vivo* studies are the *in vitro* results reported following the exposure of Caco-2 cells (cellular model derived from human colorectal adenocarcinoma) to PQ and to the well-known P-gp inducer, DOX (210). Silva and co-authors (2011) evaluated the expression and activity of P-gp in the presence of DOX (0-100 μM) for up to 96 h, correlating the obtained results with PQ-mediated cytotoxicity. The observed results demonstrated a significant increase in P-gp expression and activity as soon as 6 h after exposure to DOX, being the effect dependent on the inducer concentration (135). The cytotoxicity of the herbicide (0-5000 μM) was further evaluated in cells previously exposed or not to DOX (5-100 μM) for 24 h, and a significant decrease in PQ cytotoxicity was observed in cells previously exposed to DOX, although the observed DOX-mediated protective effects against PQ cytotoxicity were not dependent on the inducer concentration. This can be justified by the fact that, when comparing the levels of expression and activity of the efflux pump, it was observed that the significant increase

in transporter expression did not correlate with the observed increases in its activity (135). Furthermore, the UIC2 antibody, a selective P-gp inhibitor, completely prevented DOX protective effects, thus confirming P-gp involvement in reducing PQ cytotoxicity (135). A similar study was also performed by the same authors but, in this case, the inducer was added only 6 h after exposure to the herbicide, mimicking a real-life intoxication scenario where the potential antidote is administered after PQ contact with the target tissues. In this study, DOX also protected against PQ cytotoxicity, by preventing the intracellular accumulation of the herbicide. However, the protective effects exerted by DOX were not completely prevented by the presence of the specific inhibitor, the UIC2 antibody, suggesting that mechanisms other than P-gp induction could be involved in the observed DOX-mediated protection (210). Indeed, DOX reduced the intracellular PQ accumulation by two distinct mechanisms: increasing PQ efflux by increasing the levels of P-gp expression/activity, and decreasing the herbicide influx by inhibiting the choline transport uptake system. The authors suggest that compounds capable of having this double behavior, limiting the intestinal absorption and promoting the xenobiotics efflux, can be used as antidotes in intoxication scenarios (210).

Hypericin (HYP), a natural substance present in *Hypericum perforatum,* also increased P-gp expression and activity in the same cellular model, according to the time and concentration tested (209). The results showed a HYP-mediated protective effect against PQ cytotoxicity in three distinct protocols of HYP incubation (pre-exposure, simultaneous exposure to both HYP and PQ and HYP exposure 6 h after PQ). Noteworthy, the observed protective effects were completely prevented in the presence of the P-gp specific inhibitor, the UIC2 antibody (209), which indicates that the induction of P-gp is the main mechanism involved in the HYP-mediated protection (**Table 5**) (209).

The same investigators sought to evaluate the effect of newly synthetized thioxanthonic derivatives as potential modulators of P-gp expression and/or activity, and to analyse the potential protection against PQ-induced toxicity (134, 136). Indeed, the five tested thioxanthones (20 μM) significantly increased P-gp expression and activity 24 h after exposure, being the PQ-mediated toxicity significantly reduced in the presence of four of the tested thioxanthones (TXs 2-5) (134). The results were similar when five dihydroxylated xanthones were tested, which significantly increased both P-gp expression and activity in Caco-2 cells, thus conferring protection against PQ cytotoxicity (136), being these the first reports on the ability of such compounds to act as P-gp inducers (134, 136). Furthermore, all the tested xanthonic and thioxanthonic compounds demonstrated the ability to rapidly and significantly increase P-gp activity, after a short (45 min) contact with the cells, thus not reflecting a possible contribution of an increased P-gp expression, being this effect explained by the previously described P-gp activation phenomenon.

Therefore, given the obtained results demonstrating, *in vitro*, the therapeutic potential of both xanthones and thioxanthones, new experiments should be performed to assess their toxicity, toxicokinetics, toxicodynamics and potential use as antidotes, both *ex vivo* and *in vivo* (5).

Noteworthy, the P-gp-mediated detoxification mechanism against PQ-induced toxicity was also corroborated in other *in vitro* studies (222, 223), even when using a different cellular model and a different P-gp inducer (223). Indeed, methylprednisolone (200 μg/mL), a synthetic corticosteroid, dramatically decreased PQ cytotoxicity in the alveolar A549 cell line, an effect attributed to P-gp induction. The corticosteroid significantly increased both *MDR1* mRNA and P-gp protein levels as soon as 3 h after incubation, and also increased P-gp activity. However, although a remarkable increase in P-gp expression was observed at 3, 6 and 12 h, it did not follow a time dependency (223). Vilas Boas *et al*. (2013) synthetized a group of three rifampicin derivatives and evaluated their effect in P-gp activity and expression in RBE4 cells (mouse brain immortalized ECs) and assessed if the potential increases in the protein expression/activity could afford protection against a toxic P-gp substrate, PQ. As a result, one of the tested derivatives significantly increased P-gp expression and activity, resulting in a significant decrease in PQ-induced cytotoxicity, an effect reverted by elacridar, a P-gp inhibitor, corroborating P-gp involvement in the observed protection (189).

The use of inducers and/or activators of ABC transporters, namely P-gp, may be a promising and effective antidotal pathway against the toxicity of multiple xenobiotics, as has been demonstrated in the above-mentioned *in vitro* and *in vivo* studies (5). However, the concept of using ABC transporters modulation as a therapeutic strategy has been mainly focused on P-gp. Concerning other ABC transporters, and as mentioned above, a lack of studies demonstrating their induction/activation as potential antidotal pathways against the toxicity of their substrates is evident, highlighting the need of new studies focusing e.g. BCRP.

1.7.2. Xanthones as potential modulators of P-gp and BCRP

Over time, several studies were conducted with the purpose of discovering new chemical entities with P-gp induction and/or activation capacity. In fact, several xanthonic, thioxanthonic and rifampicin derivatives were synthetized and tested *in vitro* as potential antidotes in detoxification pathways (134, 136, 189).

Xanthones are oxygenated heterocyclic compounds belonging to the dibenzo-γ-pyrone class, which can be found as secondary metabolites in plants, fungi, lichens and bacteria (**Figure 4**) (224). These compounds may occur naturally, being subdivided,

depending on the nature of the substituents on the dibenzo-γ-pyrone structure, into: oxygenated simple xanthones, glycosylated xanthones, xanthones and their prenylated derivatives, xanthone dimers and xanthonolignoids (225, 226). On the other hand, it is possible to create an enormous number of derivatives from a xanthonic scaffold by adding different side chains to the backbone, and thus the xanthones of synthetic origin can have more complex substituents such as epoxide, azole, methylidenebutyrolactone, aminoalcohol, sulfamoyl, methylthiocarboxylic acid, and dihydropyridine moieties, as well as simple groups such as hydroxyl, methoxyl, methyl and carboxyl groups (225).

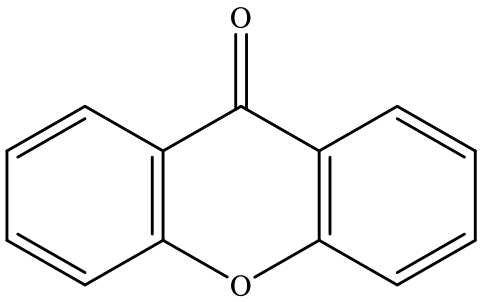

Figure 4. Chemical structure of a xanthonic scaffold (drawn with chemdraw professional 15.0)

Given their pronounced pharmacological and biological activity, within a notably broad spectrum of disease states, as a result of their interaction with a correspondingly diverse range of target biomolecules, this class of compounds (natural and synthetic derivatives) is described as a class of "privileged structures" (224, 225, 227). The study of the properties of xanthones demonstrated their hepatoprotective, anti-inflammatory and chemopreventive actions since, as phenolic compounds, xanthones have been described for their antioxidant properties (225, 228) and can act as metal chelators, free radical scavengers, as well as inhibitors of LPO (225). Thioxanthones, sulfur analogs of xanthones, also represent an interesting class of molecules, which are traditionally synthetized via benzophenone, diarylthioether or diarylthioester intermediates with proved biological properties. Miracil D, an antischistosomal agent, was the first TX introduced in therapy, in 1945, but later other biological properties of the thioxanthones were discovered, namely antitumor activity and modulation of P-gp (229).

Two potential applications already described in the literature for xanthones and aminated thioxanthones are their ability to induce apoptosis in human leukemia cells (230) and their ability to act as dual inhibitors of cell growth and P-gp, thus conferring a new opportunity for MDR reversal (231). However, in those studies, some of the tested thioxanthones demonstrated an opposite effect in P-gp activity, causing a significant

decrease in the RHO 123 accumulation ratio, an effect compatible with an increased P-gp function (231).

As mentioned in the previous section, the ability of five newly synthetized thioxanthonic derivatives and five newly synthetized simple oxygenated xanthones to increase P-gp expression and/or activity in Caco-2 cells has been recently evaluated (134, 136). According to the reported results, all the tested xanthones (136) and thioxanthones (134) significantly increased the expression and activity of the efflux pump, 24 h after exposure. Although it is known that an increase in protein expression does not always translates into a proportional increase in its activity (4, 135, 232), the detected increases in the cell surface P-gp expression mediated by these compounds were accompanied by similar increases in P-gp transport activity (134, 136). In addition, all xanthonic and thioxanthonic compounds were able to rapidly and significantly increase P-gp activity (45 min), without increasing its expression, which may indicate that the mechanism involved in this process is the activation of P-gp (134, 136).

The results described suggested that the xanthonic and thioxanthonic derivatives tested are, thus, P-gp inducers and activators, so it is possible to face them as potential antidotes in poisoning scenarios. Indeed, in those studies, and as previously mentioned, the applicability of P-gp induction/activation as a potential antidotal pathway was demonstrated, *in vitro*, with all the five tested xanthones and four out of the five tested thioxanthones significantly reduced the cytotoxicity of PQ, an herbicide used as a model of a toxic P-gp substrate. Furthermore, it was demonstrated that the observed protective effect was mediated by P-gp, as it was prevented in the presence of a P-gp inhibitor. Therefore, these derivatives are a potential source of new compounds with the ability for P-gp modulation that worth to be further explored.

II. AIMS OF THE WORK

2. AIMS OF THE WORK

The ABC superfamily of transporters represents one of the largest families of carrier proteins that mediate the efflux of substrates across cell membranes and their organelles, against the concentration gradient, by using the energy resulting from ATP hydrolysis. ABC transporters are constitutively expressed in the most varied tissues of the human organism. Specifically, in the intestinal wall mucosa, at the apical membrane of the enterocytes, there is a high expression of ABC transporters, namely P-gp and BCRP, which have the ability to bind and transport many endogenous and exogenous compounds, moving them through a mechanism dependent on the ATP binding and hydrolysis. Due to their ability to pump their substrates against their concentration gradient, transporting them towards the lumen of the gastrointestinal tract, and to their broad substrate specificity, P-gp and BCRP confer protection against toxic molecules by avoiding their systemic absorption. In fact, P-gp and BCRP promote the extrusion of toxic substances from cells, reducing their intracellular concentration, limiting the amount of xenobiotics that reach the target organ/tissue and, consequently, are able to reduce their toxicity.

Therefore, considering the important physiological role of these transporters in the elimination of harmful compounds from de body, the main aim of the present work is to discover new chemical entities with the ability to potently increase P-gp and BCRP expression and activity, to be further used as potential antidotes in intoxications caused by toxic P-gp and/or BCRP substrates. For that purpose, the objectives of this work were:

- **To evaluate the effect of six newly synthetized xanthones** (X1, X2, X5, X6, X12 and X16) **on P-gp and BCRP expression and activity**, in **SW480 and Caco-2 cells**.

- **To evaluate the potential of the new xanthonic derivatives** (X1, X2, X5, X6, X12 and X16) **to afford protection against** the **cytotoxicity of P-gp and BCRP substrates**. For that purpose, **Mitoxantrone** (MTX), a potent and extremely toxic anthracenedione antineoplastic agent, was used as a model of a toxic P-gp and BCRP substrate, and the potential protective effects against MTX cytotoxicity were correlated with the corresponding ability of the tested compounds for P-gp and BCRP induction/activation.

- **To evaluate, ex vivo, the effect of the most promising compound on P-gp activity, using everted intestinal sacs of Wistar-Han rats.**

III. EXPERIMENTAL PROCEDURES

3. EXPERIMENTAL SECTION

In this chapter, all the materials and laboratory procedures performed to develop the proposed laboratory work are presented.

3.1. Materials

Reagents used in cell culture including Dulbecco's modified Eagle's medium (DMEM) without glucose, Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, nonessential amino acids (NEAA), L-glutamine, rhodamine 123 (RHO 123), zosuquidar (ZOS), Ko143, mitoxantrone (MTX), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), neutral red (NR) solution and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO, USA). Sodium bicarbonate and glucose were purchased from Merck (Darmstadt, Germany). Triton™ X100 detergent solution was acquired from Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS), antibiotic (10,000 U/mL penicillin, 10,000 μg/mL streptomycin), hank's balanced salt solution without calcium and magnesium (HBSS (-/-)), hank's balanced salt solution with calcium and magnesium (HBSS (+/+)), phosphate-buffered saline solution (PBS) and 0.25% trypsin/1 mM ethylenediamine tetraacetic acid (EDTA) were purchased from Gibco Laboratories (Lenexa, KS, USA). P-gp monoclonal antibody (clone UIC2) conjugated with phycoerythrin (PE), was purchased from Abcam (Cambridge, United Kingdom). Reagents used in flow cytometry, namely, the cleaning solution, decontamination solution and flow cell extended clean solution were purchased from BD Biosciences (San Jose, CA, USA). All the reagents used were of analytical grade or of the highest grade available.

3.2. Synthesis of Xanthones

In the scope of the present work, six xanthonic derivatives (**X1**, **X2**, **X5**, **X6**, **X12** and **X16**) were synthetized and made available from a library of compounds from the Organic and Pharmaceutical Chemistry Laboratory, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto. **Figure 5** illustrates the chemical structure and the IUPAC nomenclature of the tested xanthonic derivatives used in the present study.

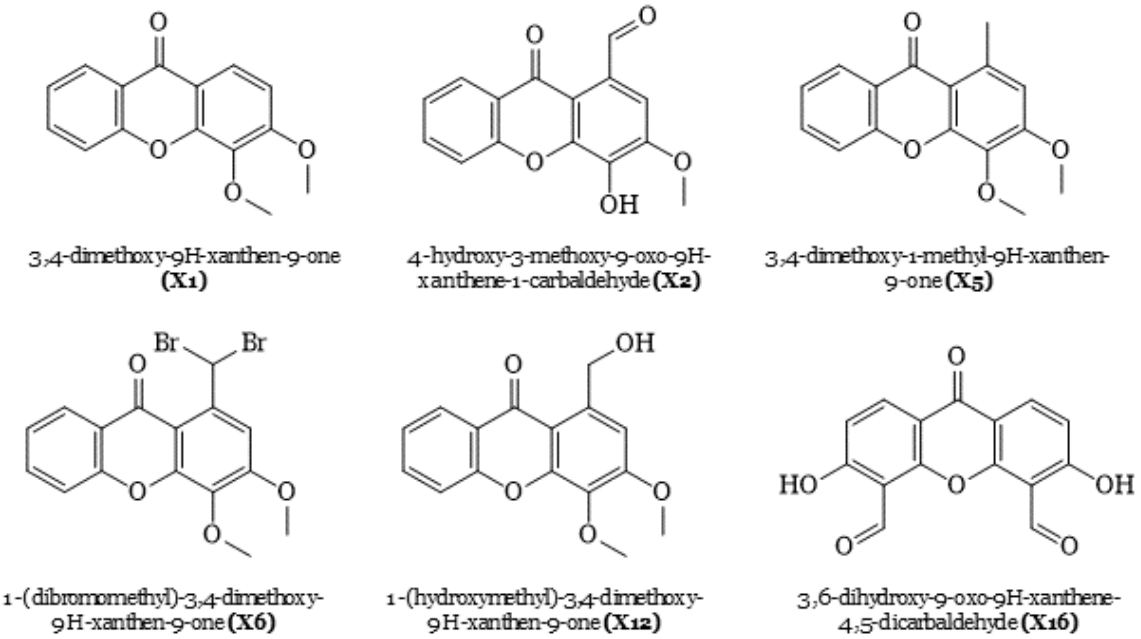

Figure 5. Chemical structure and IUPAC nomenclature of the xanthonic derivatives under investigation in the present study.

3.3. Cellular models: SW480 and Caco-2 cells

The role of the gut in the absorption of orally administered xenobiotics (accidentally or intentionally) is influenced by the presence of efflux transporters located at the apical and basolateral membranes of intestinal cells, namely P-gp and BCRP (**Figure 3 D**) (65, 66). Thus, the protective role of these efflux pumps is particularly important at the intestinal level, with a significant reduction in the intestinal absorption of the toxic compound, decreasing its concentration in the target organs and, consequently, resulting in the reduction of its toxicity (5). On the other hand, pharmacologically, and specifically in cancer therapy, the action of these transporter proteins promotes the decrease in drug BA, contributing to MDR (5, 17). In fact, and as previously mentioned, the MDR phenomenon is, at least in part, due to the efflux of the antineoplastic agent mediated by ABC transporters that are overexpressed in cancer cells, which contributes to the decrease in drug BA and, consequently, compromises the therapeutic efficacy (125, 126).

The study of intestinal transporters has been difficult due to the fragility and limited viability of intestinal enterocytes (upon isolation, the enterocytes lose their polarity) (233). However, the cell lines established from human colorectal carcinomas are useful tools for predicting drug intestinal absorption and excretion in humans. Among several established colon cancer cell lines, the SW480 line is one of the most well characterized (234). This cell line was isolated, by Leibovitz *et al.* in 1976, from a Duke's B colon adenocarcinoma

resected from a 50-year-old male patient (235). Several studies have already demonstrated the presence of ABC transporters, particularly P-gp and BCRP, in the SW480 cell line (236-241), which justifies its selection for the present study.

Caco-2 cells, an *in vitro* model of human colorectal adenocarcinoma cells, form monolayers of differentiated epithelial cells linked by intercellular junctions, which prevent the paracellular diffusion of solutes (233). Thus, this *in vitro* model represent the model of choice used in *in vitro* studies that allows to predict the permeability and intestinal absorption of drugs in humans, being used to clarify of transepithelial transport of compounds, to elucidate the involved transport routes, to determine the optimal physicochemical characteristics for passive diffusion of drugs, to assess potential toxic effects of drug candidates or formulation components in the intestine. In addition, it may allow the study of the pre-systemic metabolism of drugs since differentiated Caco-2 cells express various CYP 450 isoforms and phase II enzymes, such as UDP-glucuronosyltransferases, sulfotransferases and glutathione-S-transferases (242-245). The biochemical, morphological and functional properties of these cells resemble the enterocytes of the small intestine epithelium, expressing several transporters involved in drug absorption and excretion, including P-gp and BCRP, that may affect the stability and transport of the compounds through the cell membranes, thus mimicking the absorption process at the small intestine (210, 246-250). The levels of P-gp expression in Caco-2 cells are in good agreement with those of the normal human jejunum (71). However, the same does not occurs with BCRP. In fact, several studies have reported that BCRP expression levels are higher in normal human jejunum (71), duodenum, ileum and colon than in Caco-2 cells (251). Despite these discrepancies, P-gp and BCRP were already characterized as having an apical membrane localization in this intestinal cell line (247, 249). In addition, several studies have validated Caco-2 cells as a suitable model for the screening of new P-gp inducers/activators and inhibitors (134, 135, 210). Thus, the Caco-2 cell line was used in this work given its capacity to mimic the gastrointestinal barrier, constituting an useful *in vitro* model for the studies predicted in the scope of this laboratory work.

3.3.1. SW480 cell culture

SW480 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), and were routinely cultured in 75 cm^2 flasks (T75) using DMEM supplemented with 0.9 g/L glucose, 3,7 g/L $NaHCO_3$, 10% FBS (provides growth factors), 1% L-glutamine and 1% antibiotic (10,000 U/mL penicillin, 10,000 µg/mL streptomycin, to prevent bacterial proliferation). Cells were maintained in a 5% CO_2 - 95% O_2 air atmosphere at 37◦C and, to maintain an adequate cell growth, the medium was changed

every 2-3 days. Cultures were passed weekly by trypsinization (0.25% trypsin/1 mM EDTA). In all experiments, the cells were seeded at a density of 50,000 cells/cm² (cells were counted using a Neubauer chamber) and used 3 days after seeding, when confluence was reached. The cells used in all experiments were taken between the 12th and 22nd passages. A protocol for SW480 cells trypsinization and seeding is shown in **Figure 6.**

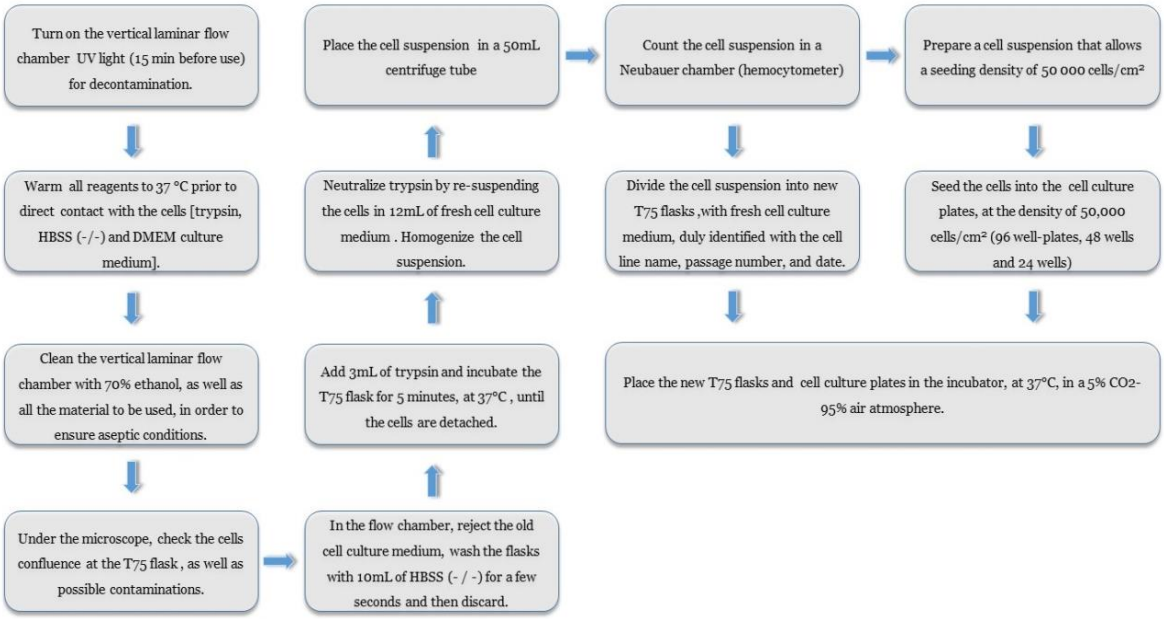

Figure 6. Experimental protocol for cell *sub culturing* and cell seeding.

3.3.2. Caco-2 cell culture

Caco-2 cells were obtained from the European Collection of Cell Culture (ECACC, UK) and were routinely cultured in 75 cm² flasks using DMEM with 4.5 g/L glucose supplemented with 10% heat-inactivated FBS, 100 μM NEAA and 1% antibiotic (10,000 U/mL penicillin, 10,000 μg/mL streptomycin). Cells were maintained in a 5% CO_2 - 95% O_2 air atmosphere at 37◦C and, to maintain a good cell growth, the medium was changed every 2-3 days. Cultures were passaged weekly by trypsinization (0.25% trypsin/1 mM EDTA). In all experiments, the cells were seeded at a density of 60,000 cells/cm² (cells were counted using a Neubauer chamber) and used 3 days after seeding, when confluence was reached. The cells used in all experiments were taken between the 54th and 64th passages. The protocol for trypsinization and seeding of Caco-2 cells is similar to the protocol performed for trypsinization and seeding of SW480 cells, shown in **Figure 6** of the section 3.3.1 - SW480 cell culture.

3.4. Xanthones cytotoxicity

Xanthones (0 – 50 μM) cytotoxicity was evaluated, in SW480 cells, by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction and neutral red (NR) uptake assays, 24 h after exposure. The cytotoxicity of the tested compounds towards Caco-2 cells was previously evaluated, by the NR uptake assay, in a previous work performed in the Toxicology Laboratory (unpublished data). In the present cytotoxicity assays, the SW480 cells were seeded at a density of 50,000 cells/cm^2 into 96-well plates and exposed, after reaching confluence, to X1, X2, X5, X6, X12 and X16 (0 – 50 μM), in fresh cell culture medium.

3.4.1. MTT reduction assay

General principle:

The MTT reduction assay is a versatile, rapid and quantitative assay that can be used to determine cytotoxicity, cell proliferation or cellular activation since it detects only living cells and the signal generated is dependent on the degree of activation of the cells (252). This test was developed at the DNAX Research Institute of Molecular and Cellular Biology as a possibility to use a colour reaction as a measure of the number of viable cells (252).

The MTT reduction assay is a colorimetric assay based on the reduction of the water-soluble yellow tetrazolium dye, MTT, to a water-insoluble blue formazan product, by mitochondrial succinic dehydrogenases. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, reducing the tetrazolium dye into purple and water-insoluble formazan crystals, which can be solubilized in an organic solvent and measured at 550 nm. Thus, it is predictable that formazan formation is directly proportional to the number of metabolically viable cells (253).

Experimental protocol:

After the exposure period (24 h), the cell culture medium was removed, followed by the addition of fresh cell culture medium containing 0.3 mg/mL MTT, and incubation at 37°C, in a humidified 5% CO_2 – 95% O_2 air atmosphere, for 45 minutes. After this incubation period, the cell culture medium was removed, and the formed formazan crystals dissolved in 100% DMSO. The absorbance was measured at 550 nm in a multiwell plate reader (PowerWaveX BioTek Instruments, Vermont, US). The percentage of MTT reduction relative to that of the control cells (0 μM) was used as the cytotoxicity measure.

3.4.2. Neutral Red uptake assay

General principle:

The NR uptake assay is a rapid, simple, sensitive, inexpensive, and environment-friendly assay for the determination of the number of viable cells in culture (254). This test is one of the most commonly used cytotoxicity tests with many biomedical and environmental applications (255) and was developed at the Rockefeller University as a cell viability chemosensitive assay (256).

The NR uptake assay has been used to measure the relative cytotoxicity of a large spectrum of agents, including surfactants, pharmaceutical, shampoos, preservatives, antibacterial agents, industrial chemicals, inorganic metals, organotins and other neurotoxins and aquatic pollutants (253).

It is based on the ability of viable cells to incorporate and bind the supravital dye NR into the lysosomes and, thus, the amount of NR dye incorporated into the cells represents their lysosomal functionality, providing a quantitative estimation of the number of viable cells in a culture (134). This weakly cationic dye penetrates the cell membranes by non-ionic passive diffusion and concentrates in the lysosomes, where it binds, by electrostatic hydrophobic bonds, to anionic and/or phosphate groups of the lysosomal matrix. The uptake of NR depends on the cell's capacity to maintain pH gradients, through the production of ATP, and, at physiological pH, the dye presents a net charge close to zero, enabling it to penetrate the membranes of the cell. Inside the lysosomes, there is a proton gradient to maintain a pH lower than that of the cytoplasm, so the dye becomes charged and is retained inside the lysosomes. The dye is extracted from the viable cells using an acidified ethanol solution, and the absorbance of the solubilized dye is quantified using a spectrophotometer (255).

Experimental protocol:

After exposure to the tested xanthones for 24 h, the cell culture medium was removed, and the cells incubated with NR (50 μg/mL in cell culture medium) at 37°C, in a humidified 5% CO_2 – 95% O_2 air atmosphere, for 1 h. After this incubation period, the cell culture medium was removed, the dye absorbed only by viable cells extracted with ethyl alcohol absolute/distilled water (1:1) with 5% acetic acid, and the absorbance measured at 540 nm in a multiwell plate reader (PowerWaveX BioTek Instruments, Vermont, US). The percentage of NR uptake relative to that of the control cells (0 μM) was used as the cytotoxicity measure.

3.5. P-gp modulation studies

The studies aiming the evaluation of the potential P-gp modulatory effect of the tested compounds were performed both *in vitro* and *ex vivo*. However, the *in vitro* experimental studies on the efflux transporter, P-gp, were only performed on the SW480 cell line, since the P-gp studies in Caco-2 cells had been previously performed in another research project (unpublished results).

3.5.1. Evaluation of P-glycoprotein expression

For the *in vitro* evaluation of P-gp expression, the SW480 cells were seeded onto 48-well plates, at a density of 50,000 cells/cm^2 to obtain confluent monolayers at the day of the experiment, and exposed, 3 days after seeding, to a noncytotoxic (20 μM) concentration of the tested X1, X2, X5, X6, X12 and X16, in fresh cell culture medium, for 24 h. Twenty-four hours after exposure, the cells were washed with HBSS (-/-) and harvested by trypsinization (0.25% trypsin/1 mM EDTA) to obtain a cell suspension. The cell suspension was transferred to eppendorf tubes, centrifuged (130g for 7 minutes, at 4°C) and re-suspended in HBSS (+/+) buffer containing the P-gp antibody (UIC2 clone conjugated with PE). The antibody dilution used in this experiment was applied according to the manufacturer's instructions for flow cytometry. The cells were then incubated for 1 h, at 37°C, in the dark and under gentle shaking. After this incubation period, the cells were washed twice with HBSS (+/+), with intercalated centrifugations (130g, for 7 min, at 4°C) and kept on ice until analysis. Cells were then re-suspended on ice-cold PBS (-/-) buffer immediately before the cytometer analysis. Fluorescence measurements of isolated cells were taken with a BD Accuri™ C6 flow cytometer (BD Biosciences, CA, USA), with the FCS Express™ analysis software. The fluorescence of the PE-UIC2 antibody was measured by a 585 ± 40 nm band-pass filter (FL2). Logarithmic fluorescence was recorded and displayed as a single parameter histogram and based on the acquisition of data for at least 20,000 cells. The parameter used for comparison was the mean of fluorescence intensity (MFI), calculated as percentage of control cells (0 μM). The cells autofluorescence (unlabelled cells with or without the tested xanthones) was also evaluated in every experiment in order to eliminate its potential contribution to the analysed fluorescence signals. Six independent experiments were performed in duplicate. Mouse IgG2a-PE was used as an isotype-matched negative control to estimate non-specific binding of the PE-labelled anti-P-gp antibody [UIC2-PE].

3.5.2. Evaluation of P-glycoprotein transport activity

The xanthones effect on P-gp transport activity was evaluated in a multiwell plate reader using RHO 123 (5 μM) as a P-gp fluorescent substrate. In fact, RHO 123 accumulation/efflux assays are common experiments used to directly evaluate P-gp activity by measuring RHO 123 intracellular fluorescence (134-136, 189, 257). For that purpose, two different protocols were used: A) RHO 123 accumulation in the presence of the tested xanthones; B) RHO 123 accumulation in cells pre-exposed to the xanthones for 24 h.

In protocol A, the test xanthones were added (except in the controls) 30 minutes prior to addition of the fluorescent substrate, RHO 123, and remained in contact with the cells during all the substrate accumulation period. This protocol aims to detect immediate effects of the tested compounds on P-gp activity as a result of a direct activation (or inhibition) of the pump. Therefore, in this case, the measured RHO 123 intracellular fluorescence reflects a direct P-gp activation (or inhibition), without the possible contribution of an increased (or decreased) expression of the pump, given the short period contact between the tested compounds and the cells (2 h), which occurs only during the RHO 123 accumulation phase (134, 136).

In protocol B, RHO 123 accumulation was evaluated in cells pre-exposed to the tested xanthones for 24 h. In this protocol, the cell culture medium containing the xanthones was removed (24 h after exposure), the cells washed with HBSS (+/+) and, subsequently, submitted to a RHO 123 accumulation protocol in the absence of the test compounds. Therefore, the measured RHO 123 intracellular fluorescence allows to assess potential changes in P-gp activity that may be due to changes in P-gp expression induced by the tested xanthones, given the long period of pre-incubation with the tested compounds (24 h) (136). For both protocols, P-gp activity was assessed in the presence and absence of ZOS (5 μM), a third-generation P-gp inhibitor.

3.5.2.1. Zosuquidar and RHO 123 cytotoxicity

Prior to the evaluation of the effects of the xanthones under study on P-gp activity, the cytotoxicity of both the P-gp fluorescent substrate (RHO 123) and P-gp inhibitor (ZOS) was assessed. For that purpose, the SW480 cells were seeded at a density of 50,000 cells/cm^2 into 96-well plates and exposed, after reaching confluence, to ZOS (0 – 10 μM) and RHO 123 (0 – 5 μM) in fresh cell culture medium. Subsequently, the plates exposed to ZOS were incubated at 37°C in a humidified 5% CO_2 - 95% O_2 air atmosphere for 24 h, while for the plates exposed to RHO 123, the cytotoxicity was evaluated at two time-points, 2 and 24 h after exposure. Following the respective incubation periods, the cytotoxicity of

both ZOS and RHO 123 was evaluated by using the MTT reduction and NR uptake assays, as described in the sections - 3.4.1 MTT reduction assay and 3.4.2 - Neutral Red uptake assay, respectively.

3.5.2.2. RHO 123 accumulation assay in the presence of xanthones (Protocol A)

For the *in vitro* evaluation of P-gp activity through protocol A, SW480 cells were seeded in 24-well plates at a density of 50,000 cells/cm² to obtain confluent monolayers on the day of the experiment. After reaching confluence (3 days after seeding), the old cell culture medium was removed, and cells were subjected to the following procedures:

- **RHO 123 accumulation under normal conditions** (**NA**): the cells were exposed to a noncytotoxic concentration of xanthones (20 μM), prepared in HBSS (+/+), for 30 minutes, and then further incubated with RHO 123 (5 μM) for 90 minutes, at 37ºC, in a humidified atmosphere with 5% CO_2 - 95% O_2 air. Control cells were only exposed to the P-gp fluorescent substrate.

- **RHO 123 accumulation in the presence of the P-gp inhibitor** (**IA**): the cells were simultaneously exposed to a noncytotoxic 20 μM concentration of the tested xanthones and to the specific P-gp inhibitor, ZOS (10 μM), both prepared in HBSS (+/+), for 30 minutes, and then further incubated with RHO 123 (5 μM) for 90 minutes, at 37ºC, in a humidified atmosphere with 5% CO_2 - 95% O_2 air. Control cells were only exposed to the P-gp inhibitor and to the P-gp fluorescent. It should be noted that each of the previously mentioned exposure conditions (**NA** and **IA,** in the presence or absence of the tested compounds) were performed in triplicate (3 wells for each condition).

After the incubation period, the cells were washed twice with HBSS (-/-) and lysed with 0.1% Triton™ X100 for 30 min, in the dark, and at room temperature. After the cells lysis, the RHO 123 intracellular fluorescence was measured at excitation/emission wavelengths of 485/528 nm, respectively, in a multi-well plate reader (PowerWave-X, BioTek Instruments, Vermont, USA) and expressed as MFI. The ratio between the MFI after inhibited RHO 123 accumulation (IA) and the MFI of non-inhibited RHO 123 accumulation (NA) was the parameter used for comparison, and the results were expressed as percentage of control cells **(Equation 1)**. As the P-gp activity increases, the amount of RHO 123 that is effluxed from the cells is higher and, consequently,

accompanied by a decrease in the intracellular fluorescence intensity due to the corresponding decrease in the intracellular content of RHO 123. Therefore, a higher RHO 123 accumulation ratio (**Equation 1**) is a consequence of a smaller MFI under NA conditions, which results from a higher P-gp activity since the dye is being effluxed out of the cells during the accumulation phase.

$$\boldsymbol{RHO123\ Accumulation} = \frac{MFI\ of\ RHO123\ accumulation\ under\ P-gp\ inhibition\ (IA)}{MFI\ of\ RHO123\ accumulation\ under\ normal\ conditions\ (NA)}$$

Equation 1: P-gp activity assessed by the ratio between the amount of RHO 123 accumulated in the presence of the inhibitor (10 μM ZOS) and the amount of RHO 123 accumulated in the absence of P-gp inhibition. MFI (mean fluorescence intensity).

3.5.2.3. RHO 123 accumulation assay in cells pre-exposed to xanthones for 24 hours (Protocol B)

For the evaluation of the effects of the tested xanthones on P-gp activity 24 h after exposure, SW480 cells were seeded onto 24-well plates, at a density of 50,000 cells/cm² to obtain confluent monolayers at the day of the experiment. Three days after seeding, and after reaching confluence, the old cell culture medium was removed, and the cells exposed to X1, X2, X5, X6, X12 and X16 (20 μM) in fresh cell culture medium for 24 h. After this 24 h incubation period, the cell culture medium containing the compounds was removed, the cells washed with HBSS (+/+), and then submitted to the previously mentioned **NA** and **IA** RHO 123 accumulation procedures, but in the absence of xanthones. Briefly, the cells were first incubated with (**IA**) or without (**NA**) ZOS (10 μM), a specific P-gp inhibitor, for 30 minutes at 37 °C, in a humidified 5% CO_2 - 95% O_2 air atmosphere, and then RHO 123 (5 μM, prepared in HBSS (+/+)) was added, followed by an additional 90 minutes incubation, at 37 °C, in a humidified 5% CO_2 - 95% O_2 air atmosphere. After the 90 min RHO 123 accumulation period, the cells were washed twice with HBSS (-/-) and lysed with 0.1% Triton-X100, for 30 min, in the dark, at room temperature. After the lysis period, the RHO 123 intracellular fluorescence was measured at excitation/emission wavelengths of 485/528nm, respectively, in a multi-well plate reader (PowerWave-X, BioTek Instruments, Vermont, USA) and expressed as MFI. The analysis of the results was performed as described in the previous section, also using **Equation 1** [3.5.2.2 - RHO 123 accumulation assay in the presence of xanthones (Protocol A)].

3.5.3. *Ex vivo* studies

General principle:

The *ex vivo* assays described below aimed to evaluate the modulatory effect of one of the tested compounds, **X12** (for which the effects observed *in vitro* were more pronounced), in the activity of P-gp expressed at the apical membrane of rat ileum enterocytes. For that purpose, everted intestinal sacs were prepared and placed in a Krebs-Henseleit (KH)-containing chamber. Each sac was filled with the fluorescent substrate of the efflux transporter under study that is expressed at the apical membrane of the enterocytes. The serosal (basolateral) to mucosal (apical) transport of the substrate was evaluated, in the presence and in the absence of a specific inhibitor of the protein and the test compound, by sampling aliquots of the buffer every 5 min for a 45-min period. The concentration of the fluorescent substrate was determined, by spectrofluorometry, in samples of mucosal medium. This approach allows the evaluation of a putative role of the test compound over the protein activity (inhibition or activation), since the measured effect results from an acute and direct contact between the test compound and the protein and, thus, does not result from a change in the expression pattern of the protein, for which a long-term contact is needed.

Animals:

Eight to ten-week-old adult male Wistar-Han rats (238g – 316g) were used. Animals were obtained from Rodent Animal House Facility, from the Abel Salazar Biomedical Sciences Institute, University of Porto (Portugal). Animals were kept under standard laboratory conditions (12/12h light/dark cycles, 22±2ºC room temperature, 50–60% humidity) and had free access to water and pellet food. Before the experiments, animals were fasted for 12 h and water containing 1% sugar was provided *ad libitum*. Animal experiments were approved by the *Organismo Responsável pelo Bem-Estar Animal* (ORBEA; protocol number 250/2018) from the Abel Salazar Biomedical Sciences Institute and submitted to evaluation and licensing to the Portuguese General Directorate of Veterinary Medicine. Housing and experimental treatment of the animals were in accordance with the guidelines defined by the European Council Directive (2010/63/EU). Two to four animals were used per experiment. The abdominal cavity was opened under deep anesthesia and the distal portion of the ileum was dissected out and gently cleaned in saline solution. Euthanasia was performed by sternotomy of the animals deeply anesthetized followed by excision of the heart.

Reagent preparation:

- **X12**, prepared in DMSO (50 mM stock solution).

- **ZOS,** prepared in DMSO (5 mM stock solution).
- **RHO 123** prepared in distilled water (1500 μM stock solution) and diluted in KH buffer on the day of the experiment.
- **KH buffer** (pH 7.4) prepared in distilled water (120 mM NaCl; 9 mM KCl; 20 mM $NaHCO_3$; 1 mM KH_2PO_4; 1 mM $MgSO_4.7H_2O$; 2 mM $CaCl_2$; 40 mM glucose). Vigorous oxygenation was promoted before use.
- **Saline solution** prepared in distilled water (NaCl 0.9%).

System preparation (**Figure 7**):

- Fill the bath with distilled water and heat to 37ºC (confirm with thermometer placed and kept in the bath throughout the experimental period);
- Place the chambers in the bath at T = 37ºC, controlled by thermometer;
- Fill each chamber with 5 mL KH buffer (or volume adjusted as a function of the volume of the drugs to be placed in each chamber);
- Oxygenate the KH buffer placed in each chamber individually and maintain the oxygenation throughout the experimental period.

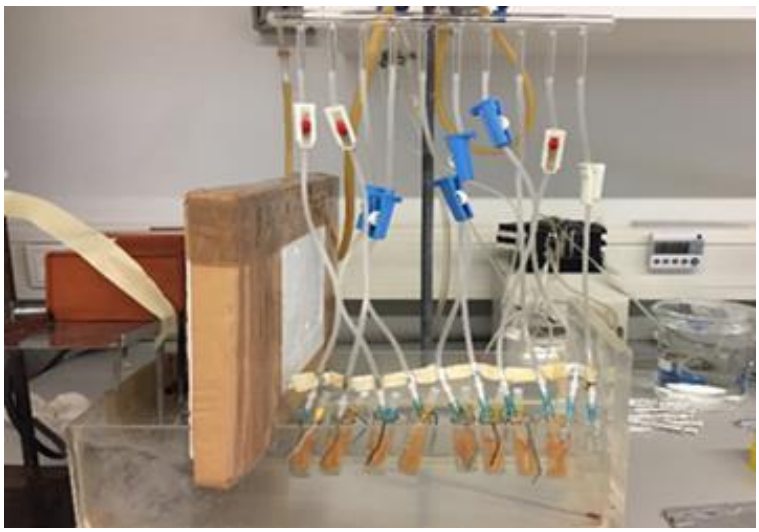

Figure 7. System used in the *ex vivo* experimental studies to evaluate the effect of X12 on P-gp activity.

Experimental protocol:

To evaluate*, ex vivo,* the effect of X12 on P-gp activity, the rats were deeply anesthetized with ketamine/xilazine (270/30 mg/kg, respectively; i.p.) and the distal portion of the ileum (20 cm) was removed, gently rinsed with ice-cold saline solution and immediately used to prepare everted intestinal sacs. For that, the intestine portions were carefully everted and each sac (approximately 10 cm length) was placed in a 5 mL KH-containing chamber (40 mM glucose, pH 7.4), with or without the addition of X12 (20

μM), and in the presence or absence of ZOS (10 μM), a known third-generation P-gp inhibitor. The mucosal medium was continuously gassed with 95% O_2 – 5% CO_2 and maintained at 37 ºC. After a 10 minutes stabilization period, the serosal compartment was filled with 1 mL RHO 123 (300 μM) diluted in KH buffer, which was used as a model of a P-gp fluorescent substrate. After the addition of the P-gp substrate to each sac, aliquots of 100 μL of mucosal medium were sampled every 5 min for a 45-min period. Then, the sacs were emptied and gently dried and weighed (**Figure 8**). RHO 123 concentration was determined by spectrofluorometry (excitation/emission wavelengths of 485/528 nm, respectively) in a multi-well plate reader (PowerWave-X, BioTek Instruments, Vermont, USA), in all samples taken from mucosal medium, using a RHO 123 calibration curve prepared in KH buffer. The amount of RHO 123 was normalized per milligram of tissue.

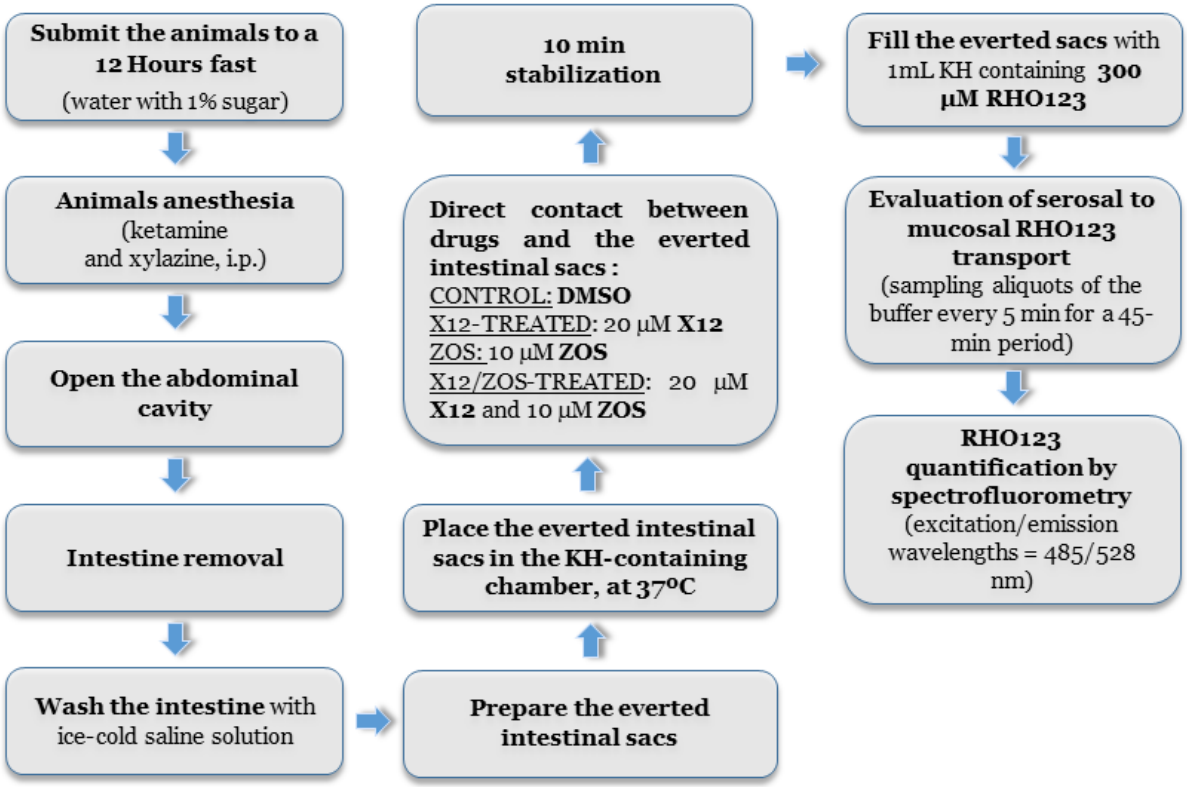

Figure 8. Experimental protocol to evaluate, *ex vivo*, the effect of X12 on P-gp activity.

3.6. BCRP studies

In vitro experimental studies concerning the efflux transporter, BCRP, were performed on two different cell lines: the SW480 cell line and the Caco-2 cell line. In both cases, only the immediate effects of the tested compounds on the pump activity were evaluated, therefore aiming to assess their potential as BCRP activators. Consequently, the experimental procedure used to evaluate the effect of the tested compounds on BCRP

activity was similar to the performed for evaluation of their effect on P-gp activity, with the compounds being present only during the accumulation phase of the fluorescent substrate [section 3.5.2.2 - RHO 123 accumulation assay in the presence of xanthones (Protocol A)].

3.6.1. Evaluation of BCRP transport activity

As mentioned above, the assays performed to study BCRP activity were based on the experimental procedure previously described for P-gp, in the section 3.5.2.2 - RHO 123 accumulation assay in the presence of xanthones (Protocol A), but using MTX and Ko143 as BCRP fluorescent substrate and inhibitor, respectively. However, some adjustments in the protocol were made, namely the concentration of both substrate and inhibitor, and the time of incubation with the BCRP substrate. The changes made during the preliminary tests aimed to develop a final procedure to evaluate BCRP activity in a simple, efficient and reproducible way, to further evaluate the effects of the xanthonic derivatives on the activity of the transporter. However, to ensure a sensitive determination of BCRP activity using MTX as the fluorescent substrate, the intracellular amount of the substrate was evaluated by flow cytometry. For that purpose, the xanthones were added (except at the controls) 30 minutes prior to the addition of the fluorescent substrate, MTX, and remained in contact with the cells during all the incubation period with the substrate. As in Protocol A described in the RHO 123 efflux assay in the presence of xanthones (section 3.5.2.2), the present assay aimed to detect the immediate effects of the tested compounds on BCRP activity as a result of direct activation or inhibition of the pump, since the intracellular accumulation of MTX is evaluated in the presence or absence of compounds only during the accumulation of the fluorescent substrate. Therefore, in this case, the measured intracellular MTX fluorescence reflects a direct activation or inhibition of BCRP, without the possible contribution of an increase or decrease of its expression, given the short period of contact between the tested compounds and the cells (2 h). Also, BCRP activity evaluation using this experimental approach was assessed in the presence and absence of Ko143, a specific BCRP inhibitor (31, 154). This assay was performed on both cell lines, SW480 and Caco-2 cells, and the cytotoxicity of both MTX and the BCRP specific inhibitor, Ko143, was initially evaluated.

3.6.1.1. SW480 cells studies

3.6.1.1.1. Ko143 and Mitoxantrone cytotoxicity

In the present cytotoxicity assays, the cells were seeded at a density of 50,000 cells/cm^2 into 96-well plates and exposed, after reaching confluence, to Ko143 (0 – 10 μM)

or MTX (0 – 10 μM) in fresh cell culture medium. Subsequently, the plates exposed to Ko143 were incubated, at 37°C in a humidified 5% CO_2 - 95% O_2 air atmosphere, for 24 h, while MTX cytotoxicity was evaluated after two different incubation times: 2 h and 24 h. In both cases, the cytotoxicity of Ko143 and MTX was evaluated by MTT reduction and NR uptake assays, as described in the sections 3.4.1 - MTT reduction assay and 3.4.2 - Neutral Red uptake assay, respectively.

3.6.1.1.2. Mitoxantrone accumulation assay in the presence of xanthones

For the *in vitro* evaluation of BCRP activity in the presence of the tested xanthones, SW480 cells were seeded onto 75 cm^2 flasks and, after reaching confluence, the old culture medium was removed, the cells washed twice with HBSS (-/-) and harvested by trypsinization (0.25% trypsin/1 mM EDTA) to obtain a cell suspension. This cell suspension was then divided into aliquots of 500,000 cells/mL. The cells were centrifuged (130g, for 7 min, at 4°C) and subjected to the following procedures:

- **Accumulation of MTX under normal conditions** (**NA**): cells were exposed to a noncytotoxic concentration of the tested xanthones (20 μM), prepared in HBSS (+/+), for 30 minutes, and then incubated with MTX (10 μM), for 90 minutes, at 37ºC, in a humidified atmosphere with 5% CO_2 - 95% O_2 air. Control cells were exposed only to the BCRP substrate.
- **Accumulation of MTX in the presence of the BCRP inhibitor** (**IA**): cells were simultaneously exposed to a noncytotoxic concentration of the tested xanthones (20 μM) and to the specific BCRP inhibitor, Ko143 (5 μM), both prepared in HBSS (+/+), for 30 minutes, and then incubated with MTX (10 μM), for 90 minutes, at 37ºC, in a humidified atmosphere with 5% CO_2 - 95% O_2 air. Control cells were only exposed to the BCRP inhibitor and to the BCRP substrate.It should be noted that each of the exposure conditions tested (**NA** and **IA,** in the presence or absence of the tested compounds) were performed in triplicate.

After this incubation period with the BCRP substrate, the cells were washed twice with in HBSS (+/+), with intercalated centrifugations (130g, for 7 min, at 4ºC) and kept on ice until analysis. Cells were then re-suspended on HBSS (+/+) immediately before the analysis. Fluorescence measurements of isolated cells were taken with a BD Accuri™ C6 flow cytometer (BD Biosciences, CA, USA), with the FCS Express™ analysis software. The MTX intracellular fluorescence was measured by a 675 ± 25 nm band-pass filter (FL4) and

the logarithmic fluorescence was recorded and displayed as a single parameter histogram. The MFI was obtained based on the acquisition of data for at least 20,000 cells. The ratio between the MFI after inhibited MTX accumulation (IA) and the MFI of non-inhibited MTX accumulation (NA) was the parameter used for comparison, and the results were expressed as percentage of control cells **(Equation 2)**. As the BCRP activity increases, the amount of MTX that is effluxed from the cells is higher accompanied by a decrease in fluorescence intensity due to the corresponding decrease in the intracellular MTX content. Therefore, a higher MTX accumulation ratio (**Equation 2**) is a consequence of a smaller MFI under NA conditions, which results from a higher BCRP activity since the dye is being effluxed out of the cells during the accumulation phase.

$$\textbf{MTX Accumulation} = \frac{MFI\ of\ MTX\ accumulation\ under\ BCRP\ inhibition\ (IA)}{MFI\ of\ MTX\ accumulation\ under\ normal\ conditions\ (NA)}$$

Equation 2: BCRP activity assessed by the ratio between the amount of MTX accumulated in the presence of the inhibitor (5 μM Ko143) and the amount of MTX accumulated in the absence of BCRP inhibition. MFI (mean fluorescence intensity).

3.6.1.2. Caco-2 cells studies

3.6.1.2.1. Ko143 and Mitoxantrone cytotoxicity

In the present cytotoxicity assays, the cells were seeded at a density of 60,000 cells/cm² into 96-well plates and exposed, after reaching confluence, to Ko143 (0 – 10 μM) or MTX (0 – 10 μM) in fresh cell culture medium. Subsequently, the plates exposed to Ko143 were incubated, at 37°C in a humidified 5% CO_2 - 95% O_2 air atmosphere, for 24 h, while MTX cytotoxicity was evaluated 2 h after incubation. In both cases, the *in vitro* evaluation of Ko143 and MTX cytotoxicity was assessed by the MTT reduction and NR uptake assays, as described in the sections 3.4.1 - MTT reduction assay and 3.4.2 - Neutral Red uptake assay, respectively.

3.6.1.2.2. Mitoxantrone accumulation assay in the presence of xanthones

For the *in vitro* evaluation of BCRP activity, Caco-2 cells were seeded onto 75 cm² flasks and, after reaching confluence, the old culture medium was removed, the cells washed twice with HBSS (-/-) and harvested by trypsinization (0.25% trypsin/1 mM EDTA) to obtain a cellular suspension. This cell suspension was then divided into aliquots of 500,000 cells/mL. The cells were centrifuged (2000 rpm, for 5 min, at 4 °C) and subjected to the following procedures:

- **Accumulation of MTX under normal conditions** (**NA**): cells were exposed to a noncytotoxic 20 μM concentration of the tested xanthones prepared in HBSS (+/+), for 30 minutes, and then incubated with MTX (5 μM) for 60 minutes at 37ºC in a humidified atmosphere with 5% CO_2 - 95% O_2 air. Control cells were exposed only to the BCRP substrate.

- **Accumulation of MTX in the presence of the BCRP inhibitor** (**IA**): cells were simultaneously exposed to a noncytotoxic concentration of the xanthones (20 μM) and to specific BCRP inhibitor, Ko143 (5 μM), both prepared in HBSS (+/+), for 30 minutes, and then incubated with MTX (5 μM) for 60 minutes at 37ºC in a humidified atmosphere with 5% CO_2 - 95% O_2 air. Control cells were only exposed to the BCRP inhibitor and to the BCRP substrate. It should be noted that each of the exposure conditions tested (**NA** and **IA,** in the presence or absence of the tested compounds) were performed in triplicate.

After this incubation period, the cells were washed twice with in HBSS (+/+), with intercalated centrifugations (130g, for 7 min, at 4ºC) and kept on ice until analysis. Cells were then re-suspended on HBSS (+/+) immediately before the analysis performed in a BD Accuri™ C6 flow cytometer (BD Biosciences, CA, USA), with the FCS Express™ analysis software. The fluorescence measurements of isolated cells were performed as described in section 3.6.1.1.2 - Mitoxantrone accumulation assay in the presence of xanthones.

3.7. Xanthones protective effects against MTX-induced cytotoxicity

Given the observed effects of the tested compounds on both P-gp and BCRP expression and/or activity, their potential protective effects against the cytotoxicity of substrates of these efflux pumps were further evaluated. For that purpose, MTX was used a model of a substrate of both P-gp and BCRP (258, 259). The cytotoxicity of the selected substrate was evaluated in both SW480 and Caco-2 cells, in the presence or absence of the tested xanthonic derivatives. For that purpose, SW480 cells were seeded in 96-well plates at a density of 50,000 cells/cm² and exposed, after reaching confluence (three days after seeding), to MTX (0; 0,1; 0,5; 1; 2,5; 5,0; 10; 25; 50; 100 and 150 μM) in the presence or absence of the studied xanthones (20 μM). Concerning the Caco-2 cells, the cells were seeded at a density of 60,000 cells/cm² into 96-well plates to obtain confluent monolayers at the day of the experiment, and exposed, 3 days after seeding, to MTX at the

concentrations of 500, 1000 and 1500 μM, in the presence or absence of the tested xanthones (20 μM). Twenty-four hours after exposure, the MTX cytotoxicity was evaluated by the NR uptake assay. Briefly, the cell culture medium was removed, and the cells incubated with NR (50 μg/mL in cell culture medium) at 37°C, in a humidified 5% CO_2 - 95% O_2 air atmosphere, for 1 h. After this incubation period, the cell culture medium was removed, the dye absorbed only by viable cells extracted with ethyl alcohol absolute/distilled water (1:1) with 5% acetic acid, and the absorbance measured at 540 nm in a multiwell plate reader (PowerWaveX BioTek Instruments, Vermont, US). The percentage of NR uptake relative to that of the control cells (0 μM) was used as the cytotoxicity measure.

3.8. Statistical analysis

GraphPad Prism 7 for Windows (GraphPad Software, San Diego, CA, USA) was used to perform all statistical calculations. Three tests were performed to evaluate the normality of the data distribution: KS normality test, D'Agostino & Pearson omnibus normality test and Shapiro-Wilk normality test. For data with parametric distribution One-way ANOVA was used to perform the statistical comparisons, followed by the Dunnett's multiple comparisons test. For data with only two groups (e.g. RHO 123 cytotoxicity), the statistical comparisons were made using the unpaired *t* test. Statistical comparisons between groups in experiments with two variables (e.g. *ex vivo* assays) were made using Two-way ANOVA, followed by the Sidak's multiple comparison post hoc test or by the Tukey's multiple comparisons test. In all cases, *p* values lower than 0,05 were considered significant.

IV. RESULTS and DISCUSSION

4. RESULTS AND DISCUSSION

4.1. Xanthones cytotoxicity assays

Xanthones (0 – 50 μM) cytotoxicity towards SW480 cells was evaluated by the MTT reduction and NR uptake assays, 24 h after exposure, to select a non-cytotoxic working concentration to be used in the subsequent studies. In the MTT reduction assay, and as observed in **Figure 9**, no significant cytotoxicity was detected for any of the tested concentrations (0 – 50 μM) and up to 24 h of exposure to **X2**, **X5**, **X6** and **X16**. For **X1** and **X12**, no significant effects were observed for concentrations up to 20 μM, but a significant decrease in the MTT reduction was observed for the 50 μM concentration (the MTT reduction decreased to 87 and 85% for **X1** and **X12**, respectively, when compared to control cells).

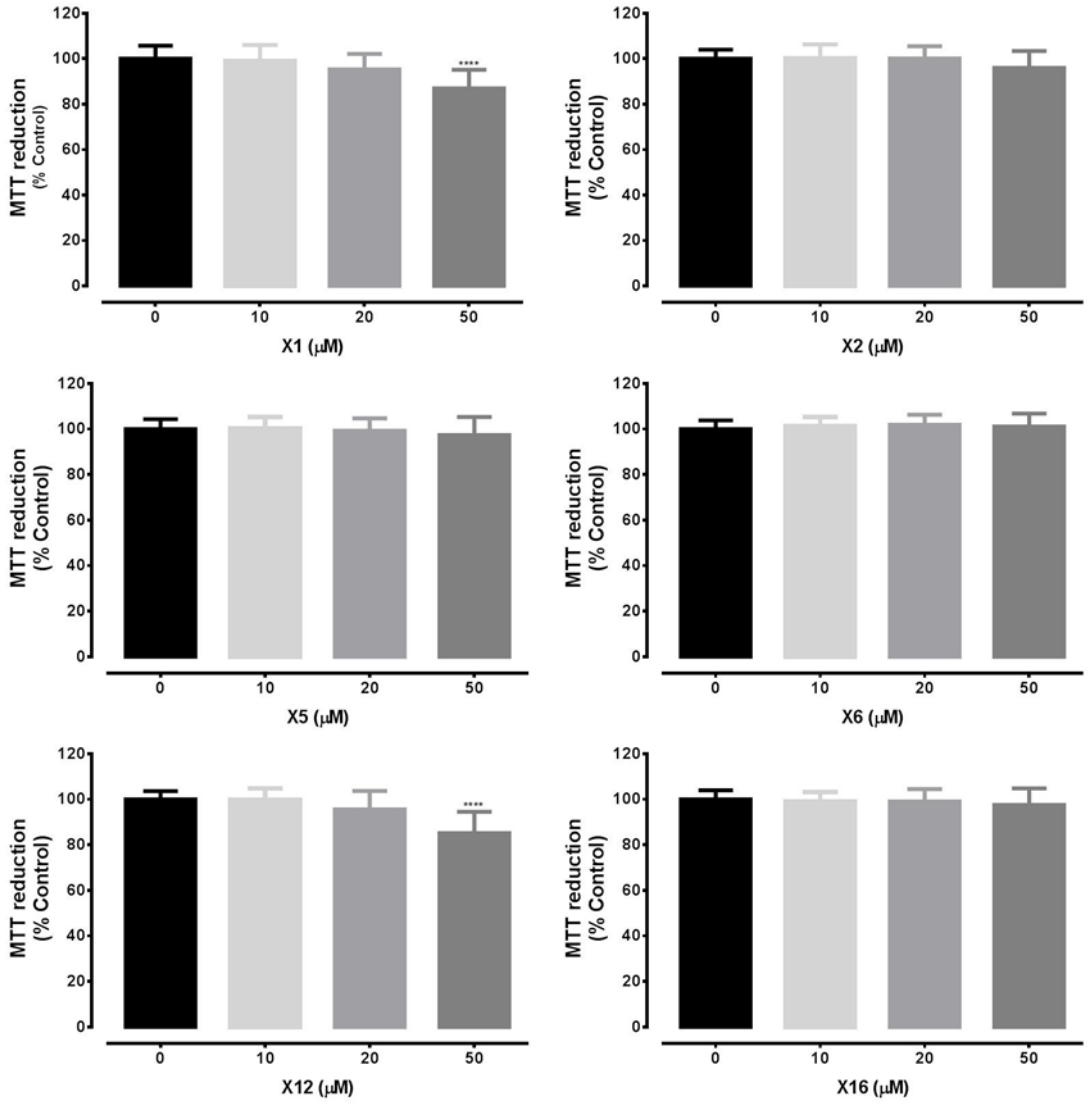

Figure 9. Xanthones (0-50 μM) cytotoxicity in SW480 cells evaluated by the MTT reduction assay, 24 h after exposure. Results are presented as mean ± SD from 6 independent experiments, performed in triplicate. Statistical comparisons were made using the parametric method of One-way ANOVA, followed by the Dunnett's multiple comparisons test [****$p < 0.0001$ vs. control (0 μM)].

Concerning the NR uptake assay (**Figure 10**), no significant effects on the NR uptake were observed after 24 h of exposure to **X2**, **X6** and **X12**, for all the tested concentrations (0 – 50 μM). However, for **X1**, **X5** and **X16**, a small but significant decrease in the NR uptake was observed only for the highest tested concentration (50 μM). Indeed, after 24 h of exposure to 50 μM **X1**, **X5** and **X16**, the NR uptake significantly reduced to 96, 94 and 93%, respectively, when compared to control cells.

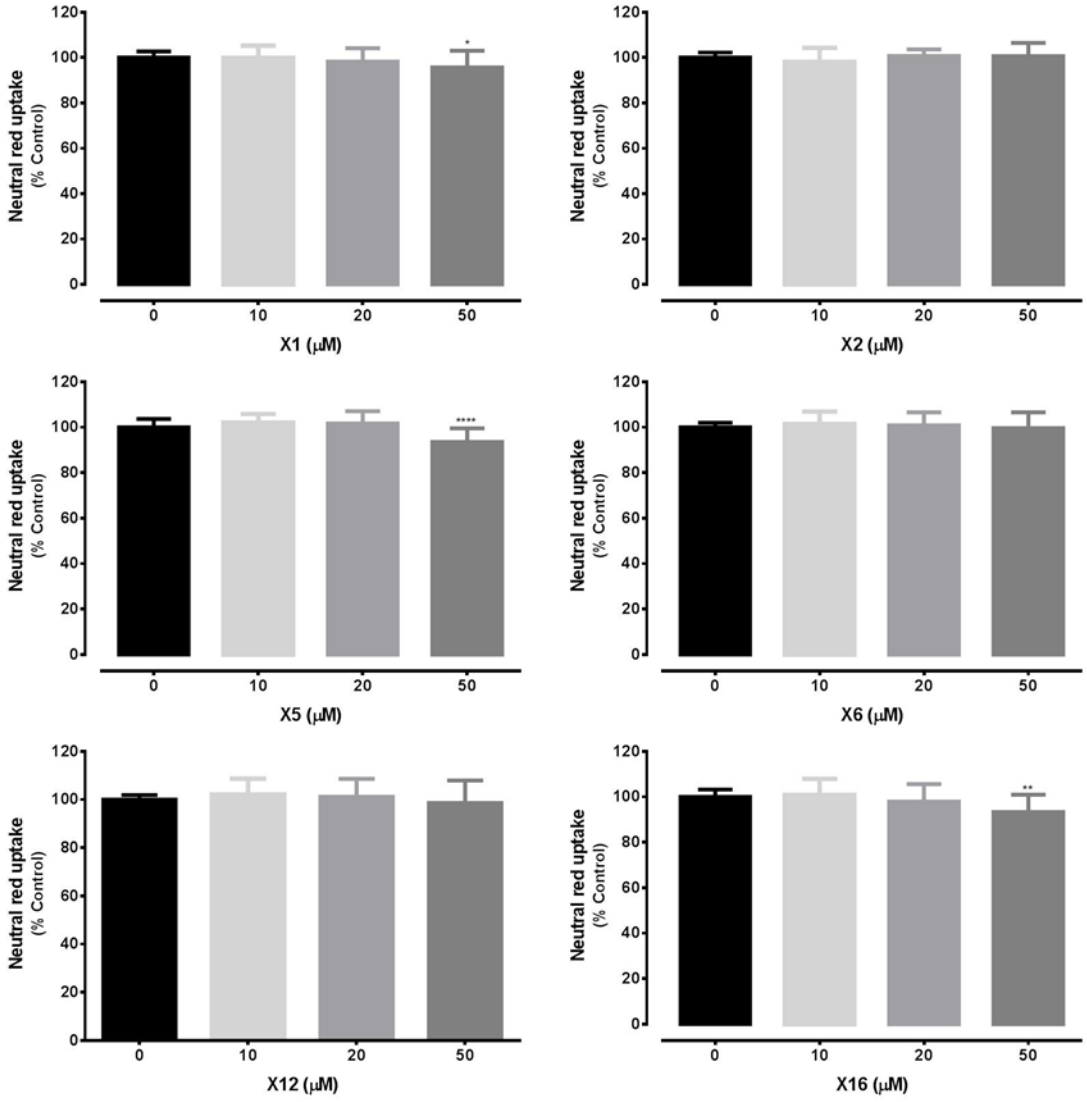

Figure 10. Xanthones (0-50 μM) cytotoxicity in SW480 cells evaluated by the neutral red uptake assay, 24 h after exposure. Results are presented as mean ± SD from 6 independent experiments, performed in triplicate. Statistical comparisons were made using the parametric method of One-way ANOVA, followed by the Dunnett's multiple comparisons test [*$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$ vs. control (0 μM)].

According to the obtained results, the concentration of 20 μM was selected for the subsequent experiments given the lack of significant cytotoxicity, and given that this non-cytotoxic concentration was already reported for similar xanthonic and thioxanthonic

derivatives as a concentration able to cause a significant increase in P-gp expression and activity (134, 136, 215). Furthermore, and concerning the cytotoxicity of the tested xanthones towards Caco-2 cells, no significant cytotoxicity was reported for any of the tested concentrations (0 - 50 μM) and up to 24 h of exposure, as evaluated by the NR uptake assay (unpublished data), further supporting the selection of the 20 μM concentration as a non-cytotoxic concentration to be used in the following experiments.

4.2. P-gp studies

4.2.1. Flow cytometry analysis of P-gp expression

The ability of the newly synthetized compounds to induce P-gp expression in SW480 cells was evaluated by flow cytometry, using a P-gp monoclonal antibody [UIC2] conjugated with PE, 24 h after exposure to the synthetic xanthones (20 μM), as previously described (134, 136, 215).

As shown in **Figure 11**, the xanthonic derivatives **X6** and **X12** significantly increased P-gp expression by 139 and 122%, respectively, when compared to control cells (0 μM). Compounds **X1** and **X2**, although to a lower extent, also increased P-gp expression to 113 and 119%, respectively. For the remaining xanthonic derivatives, namely **X5** and **X16**, no significant effect on P-gp expression was observed after 24 h of exposure. Thus, from the obtained results, **X6** was the most potent P-gp inducer among the tested compounds (**Figure 11**). Furthermore, it is important to note that the monoclonal antibody recognizes an external P-gp epitope, thus allowing to assess the cell surface P-gp expression (257, 260).

Concerning the Caco-2 cells model, previous studies performed in the Laboratory of Toxicology reported that some of the tested compounds were also able to increase P-gp expression 24 h after incubation (unpublished data). Furthermore, this *in vitro* model was previously validated as a suitable model for the screening of several P-gp inducers, including xanthonic derivatives (134-136, 209, 215, 260). Indeed, at the same 20 μM concentration, five dihydroxylated xanthones previously demonstrated, in Caco-2 cells, to significantly increase cell surface P-gp expression, 24 h after exposure (136). Noteworthy, to the best of our knowledge, this is the first report on the ability of xanthonic derivatives to significantly increase P-gp expression on SW480 cells, thus behaving as inducers of this efflux pump, and validating SW480 cells as a suitable *in vitro* model for the screening and selection of P-gp inducers.

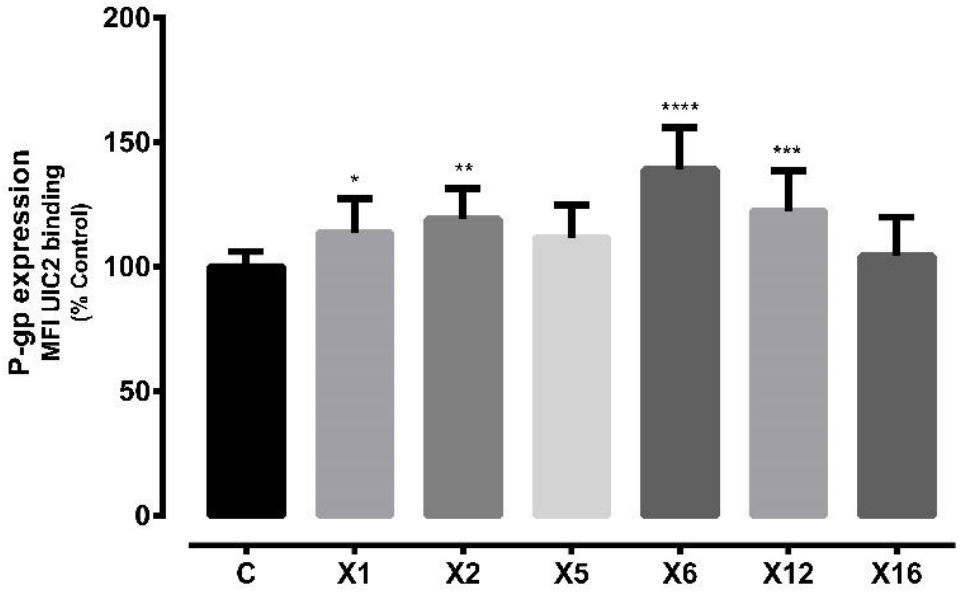

Figure 11. P-glycoprotein expression evaluated by flow cytometry in SW480 cells, 24 h after exposure to the tested xanthones (20 μM). Results are presented as mean ± SD from 6 independent experiments, performed in duplicate. Statistical comparisons were made using the parametric method of One-way ANOVA, followed by the Dunnett's multiple comparisons test [*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ vs. control (0 μM)].

4.2.2. Evaluation of P-gp transport activity

Based on previous studies, the effect of the newly synthetized compounds on P-gp transport activity was evaluated by two different experimental approaches, both using the well-known P-gp fluorescent substrate, RHO 123 (134, 136, 189, 215, 257) and a specific third-generation P-gp inhibitor, ZOS.

In the first approach, RHO 123 accumulation was evaluated in the presence of the tested compounds (20 μM) during the accumulation phase of the fluorescent substrate that lasted 90 minutes, aiming to evaluate potential immediate effects of the xanthonic derivatives on P-gp activity as a result of a direct activation or inhibition of the pump. The second approach, by evaluating the effects of the tested compounds on P-gp activity after a 24 h pre-exposure period, aimed to evaluate whether potential increases in the P-gp protein expression can be translated into the corresponding increases in the pump activity. In both protocols, P-gp activity was assessed in the presence and absence of ZOS (5 μM). Correspondingly, and in both cases, P-gp activity was evaluated through the ratio between the MFI after inhibited RHO 123 accumulation (IA – in the presence of ZOS,) and the MFI of non-inhibited RHO 123 accumulation (NA, in the absence of the P-gp inhibitor), and the results were expressed as percentage of control cells (0 μM). When P-gp activity increases, the amount of RHO 123 that is effluxed from the cells is higher and accompanied by a decrease in the fluorescence intensity due to the corresponding

decrease in the intracellular RHO 123 content (134). Thus, the ratio MFI_{IA}/MFI_{NA} will be higher as a result of a MFI_{NA} decrease, as the dye is being pumped out of the cells during the accumulation phase. In contrast, when P-gp activity decreases, the ratio MFI_{IA}/MFI_{NA} will be lower, since a higher fluorescence intensity under normal conditions (MFI_{AN}) will be detected, because of the decreased RHO 123 efflux.

Prior to the evaluation of the effects of the tested compounds on P-gp activity, the cytotoxicity of both the P-gp substrate (RHO 123: 0 - 5 μM) and inhibitor (ZOS: 0 - 10 μM) was evaluated, by the MTT reduction and NR uptake assays. The ZOS and RHO123 concentrations were selected based on previous studies performed in the Laboratory of Toxicology (unpublished data) aiming the evaluation of P-gp activity.

In what concerns to ZOS (0 - 10 μM), and as observed in **Figure 12 (A**), no significant cytotoxicity relative to control cells (0 μM) was observed for any of the tested concentrations (0 - 10 μM), 24 h after exposure to the P-gp inhibitor. Similar results were observed in the NR uptake assay, where no significant cytotoxicity was detected at any of the tested ZOS concentrations (**Figure 12 (B)**).

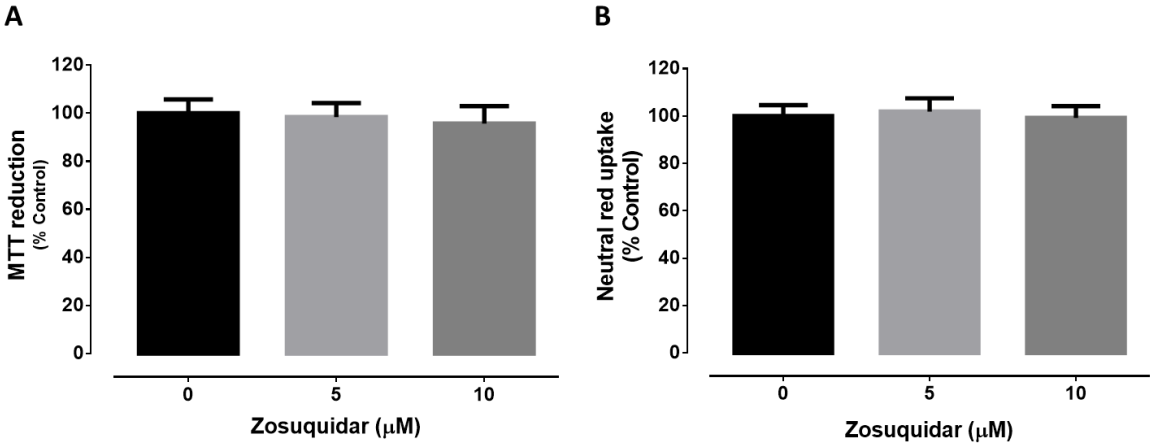

Figure 12. Zosuquidar (0 – 10 μM) cytotoxicity in SW480 cells, evaluated by the MTT reduction (A) and NR uptake (B) assays, 24 h after exposure. Results are presented as mean + SD from 4 independent experiments, performed in triplicate. Statistical comparisons were made using the parametric method of One-way ANOVA, followed by the Dunnett's multiple comparisons test [$^{****}p < 0.0001$ vs. control (0 μM)].

In what concerns to RHO 123, the cytotoxicity of the P-gp fluorescent substrate was initially evaluated 24 h after exposure, also by the MTT reduction and NR uptake assays. However, and as observed in **Figure 13**, after 24 h of incubation with 5 μM RHO 123, a significant decrease in both the MTT reduction and NR uptake was observed, when compared to control cells (21% and 15%, respectively).

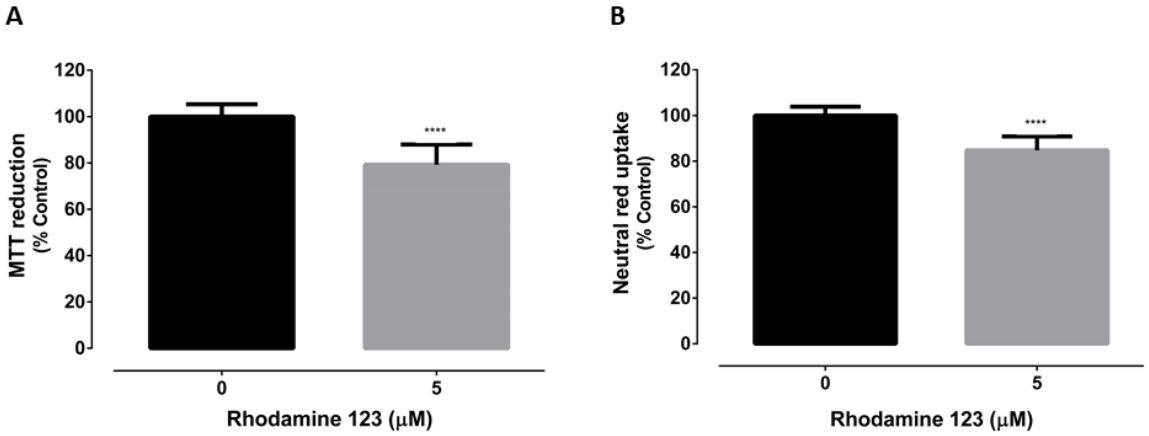

Figure 13. Rhodamine 123 (0 – 5 μM) cytotoxicity in SW480 cells, evaluated by the MTT reduction (A) and NR uptake (B) assays, 24 h after exposure. Results are presented as mean ± SD from 5 independent experiments, performed in triplicate. Statistical comparisons were made using the Unpaired *t*-test [****$p < 0.0001$ vs. control (0 μM)].

Knowing that, in most studies reported in the literature aiming to evaluate P-gp activity, the accumulation with the P-gp substrate lasts less than 2 h (134-136, 189, 260), the cytotoxicity of the fluorescent substrate was then evaluated at this time-point. Accordingly, as observed in **Figure 14**, no significant effect on both MTT reduction and NR uptake was observed 2 h after exposure of SW480 cells to 5 μM RHO, when compared to control cells (0 μM).

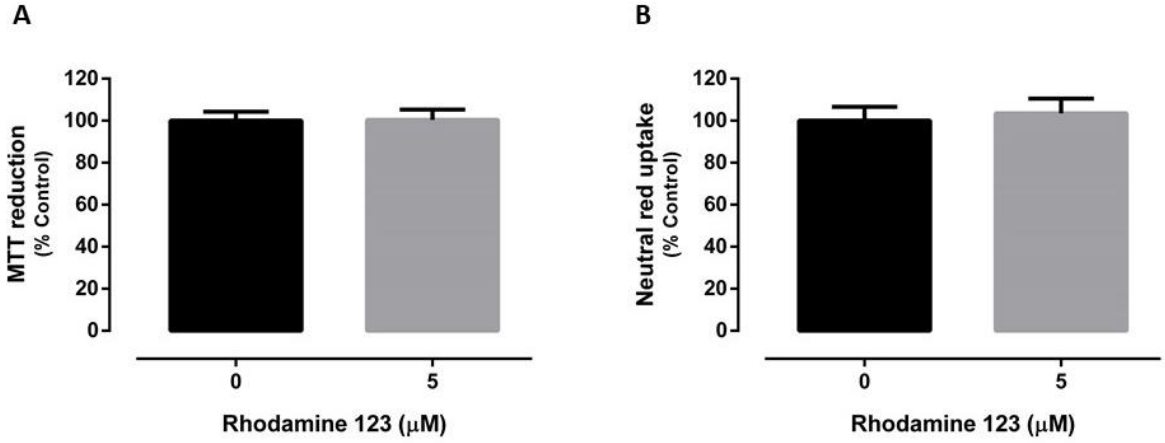

Figure 14. Rhodamine 123 (0 – 5 μM) cytotoxicity in SW480 cells, evaluated by the MTT reduction (A) and NR uptake (B) assays, 2 h after exposure. Results are presented as mean ± SD from 6 independent experiments, performed in triplicate. Statistical comparisons were made using the Unpaired *t*-test.

Based on these results (**Figure 12** and **Figure 14**), P-gp transport activity can be safely assessed using ZOS and RHO 123 at the concentrations of 10 μM and 5 μM, respectively, since no significant cytotoxicity was observed towards SW480 cells. It is also important to mention that, in the present studies performed to evaluate P-gp activity,

apart from the ZOS and RHO 123 concentrations used, the 90 min incubation with the P-gp fluorescent substrate was also selected based on previous studies performed in the Laboratory of Toxicology (unpublished data).

4.2.2.1. P-gp transport activity in the presence of the tested xanthones

In the protocol A, where RHO 123 accumulation was assessed in the presence of the tested compounds (20 μM) during the 90 minutes accumulation with the fluorescence substrate, the results demonstrated that the simultaneous incubation of SW480 cells with the xanthonic derivatives **X1**, **X2**, **X5**, **X6** and **X12** resulted in a significant increase in P-gp activity (**Figure 15**). From the tested xanthones, **X5**, **X6** and **X12** were the most efficient P-gp activators, as revealed by the significantly increased P-gp activity (128%, 126% and 135%, respectively), when compared to control cells (0 μM). The derivatives **X1** and **X2**, although to a lower extent, also significantly increased P-gp activity to 121% and 120%, respectively, thus also indicating a potential for P-gp activation, even in a modest way. On the other hand, the derivative **X16** caused a small but significant decrease in P-gp activity. Indeed, the derivative **X16** demonstrated the ability to reduce RHO 123 efflux, increasing the intracellular accumulation of the fluorescent substrate, thus slightly reducing P-gp activity to 93%, when compared to control cells.

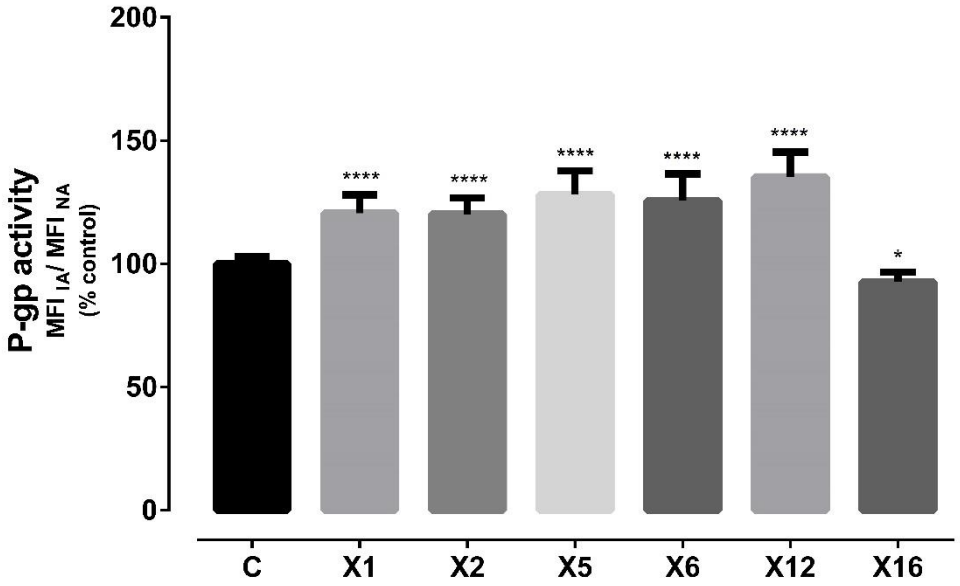

Figure 15. P-glycoprotein activity evaluated by fluorescence spectroscopy in SW480 cells exposed to the tested xanthones (20 μM) only during the 90 minutes incubation period with the fluorescent substrate (5 μM RHO 123). Results are presented as mean ± SD from 5 independent experiments, performed in triplicate. Statistical comparisons were made using the parametric method of One-way ANOVA, followed by the Dunnett's multiple comparisons test [*p < 0.05; ****p < 0.0001 vs. control (0 μM)].

In accordance with the obtained data, similar results were observed in Caco-2 cells, with all the tested xanthones, except **X16**, significantly and immediately (given the short incubation period) increasing P-gp activity, when compared to control cells (P-gp activity significantly increased to 130, 121, 130, 130 and 132%, when RHO 123 accumulation was performed in the presence of X1, X2, X5, X6 and X12, respectively). For X16, no significant effect on P-gp activity was observed, when compared to control cells (unpublished data).

Therefore, the obtained results clearly demonstrated the ability of the tested compounds, except **X16,** to immediately increase P-gp activity, thus demonstrating to be activators of this efflux pump, and a promising source of new activators. Furthermore, although previous studies already reported the potential of xanthonic derivatives, namely dihydroxylated xanthones, for P-gp activation in Caco-2 cells (136), to the best of our knowledge this is the first report on the ability of xanthones to immediately increase P-gp activity in SW480 cells, further validating the use of this *in vitro* model in the screening of P-gp activators.

4.2.2.2. P-gp transport activity in cells pre-exposed to the tested xanthones

In the second experimental approach (Protocol B), RHO 123 accumulation was evaluated in SW480 cells pre-exposed to the tested compounds (20 μM) for 24 h. Therefore, and before the RHO 123 accumulation phase, the cell culture medium containing the tested xanthones was removed and the cells washed with HBSS. Through this second protocol, it is possible to evaluate whether the potentially increased P-gp expression caused by the xanthonic derivatives could be translated into a significantly increased P-gp activity. This fact it is of utmost importance since an increased P-gp protein expression does not necessarily translates into an increased transport activity (134, 232, 260).

As observed in **Figure 16**, among the tested compounds, **X1** and **X5** significantly increased P-gp activity relative to control cells (0 μM). In fact, P-gp activity significantly increased to 130 and 133% in SW480 cells pre-exposed to **X1** and **X5** for 24 h, respectively. The xanthonic derivatives **X6** and **X12**, although to a lower extent, also significantly increased P-gp activity to 113 and 119%, respectively. Contrarily, the **X2** and **X16** compounds showed no ability to increase the activity of the efflux pump, since no significant effects on P-gp activity were observed after 24 h of pre-exposure.

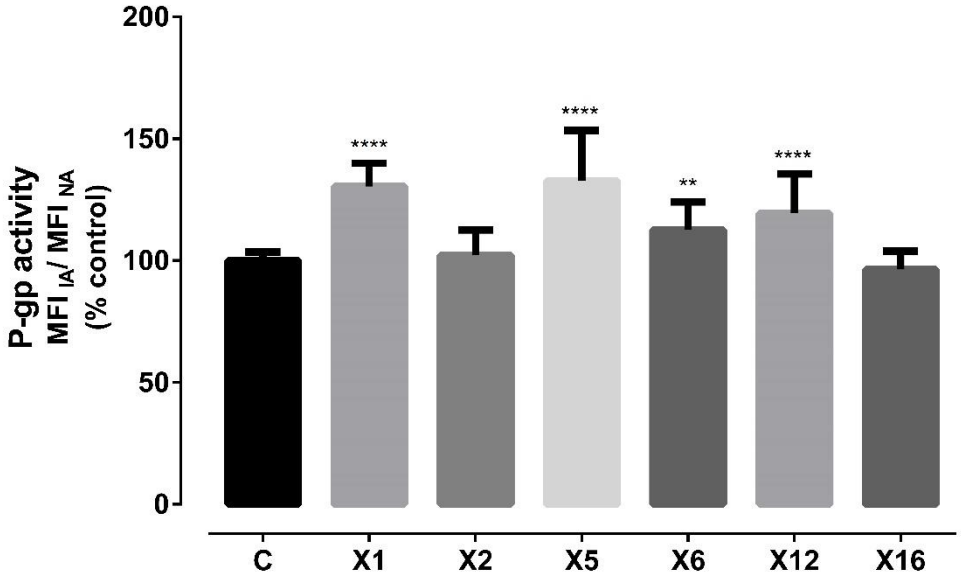

Figure 16. P-glycoprotein activity evaluated by fluorescence spectroscopy in SW480 cells pre-exposed to the tested xanthones (20 μM) for 24 h. Results are presented as mean ± SD from 6 independent experiments, performed in triplicate. Statistical comparisons were made using the parametric method of One-way ANOVA, followed by the Dunnett's multiple comparisons test [$^{**}p < 0.01$; $^{****}p < 0.0001$ vs. control (0 μM)].

For the compounds **X1**, **X6** and **X12** the increase in P-gp activity observed after the 24 h pre-exposure period (**Figure 16**) may result from an increase in the expression of this efflux pump, since a significant increase in cell-surface P-gp expression was also observed 24 h after exposure to these xanthonic derivatives (**Figure 11**). On the other hand, and according to the **Figure 16**, the increase in P-gp activity observed after 24 h of pre-exposure to the xanthonic derivative **X5** does not result from an increase in the pump expression, since no significant increase in cell-surface P-gp expression was observed 24 h after pre-exposure to this compound (**Figure 11**). However, **X5** demonstrated the ability to increase P-gp activity as a consequence of direct pump activation (**Figure 15**). Thus, the increased activity observed in SW480 cells pre-exposed to this compound for 24 h may be the result of a direct activation of the pump caused by the compound that may have remained intracellularly after the exposure period and the subsequent washing of the cells.

In addition, no significant increase in P-gp activity was observed in SW480 cells pre-exposed to compound **X2** (**Figure 16**), although this xanthonic derivative was able to significantly increase the cell-surface expression of the protein (**Figure 11**). Thus, the results demonstrated that, although the *de novo* synthetized P-gp is already incorporated into the plasma membrane (since the UIC2 antibody recognizes an external epitope of the protein), it may not yet be fully functional. Taken together, these results reinforce the importance of the simultaneous determination of both P-gp expression and activity since, and as previously mentioned, an increased protein expression does not necessarily

translates into an increased transport activity, and increases in activity can be observed independently of the level of expression, like has been already demonstrated in several studies (135, 257, 260).

Silva and co-authors (2011) evaluated the expression and activity of P-gp in the presence of DOX (0-100 μM) for up to 96 h, correlating the obtained results with PQ-mediated cytotoxicity. The observed results demonstrated a significant increase in P-gp expression and activity as soon as 6 h after exposure to DOX, being the effect dependent on the inducer concentration and on the time of exposure. However, when comparing the levels of expression and activity of the efflux pump, it was observed that the significant increases in the transporter expression did not correlate with the observed increases in its activity. For example, after 6 h of exposure to 50 μM DOX, P-gp expression levels significantly increased to 300%, although protein activity increased only to 120% (135). Therefore, these results suggest that although P-gp is expressed at higher levels and incorporated into the cell membrane, it may not yet be fully functional. Another study reported similar results in Caco-2 cells exposed to colchicine. In this study, colchicine was shown to significantly increase the expression of P-gp in a concentration-dependent manner, behaving as an inducer. In fact, 24 h after exposure to 0.5, 1, 5, 10, 50 and 100 μM colchicine, P-gp expression significantly increased to 129%, 135%, 145%, 150%, 154% and 183%, respectively. However, this increase in P-gp expression was not associated with a concomitant increase in the protein activity at any of the tested colchicine concentrations. In this study, the lack of effect of colchicine on P-gp activity was explained by its action as a P-gp competitive inhibitor, as supported by the performed *in silico* studies (260). In addition, Vilas-Boas *et al.* (2011), when evaluating the activity and expression of P-gp in isolated whole blood lymphocytes from 65 Caucasian donors, divided according to their age, found that the activity of P-gp in human lymphocytes did not follow the significant increase in its expression observed during aging (257).

4.2.3. *Ex vivo* study - Evaluation of P-gp transport activity in rat everted intestinal sacs

Ex vivo methodologies are an experimental approach where an organ or tissue is removed from the animal and placed in chambers where physiological conditions found in the living body are mimicked, namely the access to nutrients and oxygen, allowing the viability of the organ or tissue during the experimental period.

Many studies use *ex vivo* approaches to accurately evaluate the function of ABC transporters (261-263). Indeed, the modulatory effect of different compounds over ABC carriers expressed in intestinal epithelia can be studied by using *ex vivo* methodologies in

which everted intestinal sacs are prepared, filled with a specific ABC protein fluorescent substrate and placed in aerated buffer-containing chambers, at 37ºC. Serosal to mucosal transport of the fluorescent substrate, in the presence or absence of the putative ABC carrier modulator, is evaluated in each intestinal sac by determining the substrate concentration, by spectrofluorometry, in samples of the mucosal medium, over time. RHO 123 is a dye usually used as P-gp substrate (261-263).

P-gp is highly expressed in the apical membrane of enterocytes, promoting the efflux of the compounds from the cells back to the intestinal lumen (67). The expression of P-gp along the intestine is not uniform, as it has high levels in the ileum and colon, in contrast to the jejunum and duodenum, where its expression is lower (67). The *ex vivo* assays aimed to evaluate the modulatory effect of one of the test compounds over P-gp activity expressed on the apical membrane of enterocytes from the distal portion of Wistar-Han rat intestine. To achieve this purpose, the distal portion of the ileum (portion where P-gp expression is the highest) was removed and everted to prepare intestinal sacs. After inversion, the P-gp expressed on the apical membrane of the enterocytes is turned towards the sac exterior, in close contact with the KH medium, promoting the transport of the fluorescent substrate, placed inside the sac (serosal to mucosal transport). Thus, the transport of the fluorescent substrate, RHO 123, was evaluated by its quantification in all medium samples, by spectrofluorometry. For the accomplishment of this experimental approach, it was initially necessary to select the most promising xanthone. The compound was selected based on the results obtained from the *in vitro* evaluation of the effect of the tested xanthones on P-gp transport activity (**Figure 15**). In fact, as previously described, simultaneous incubation of SW480 cells with the **X12** xanthonic derivative during the accumulation of the fluorescent substrate resulted in a significant increase in P-gp activity (135%, when compared to control cells), being X12 the most efficient P-gp activator, which justified its selection for the *ex vivo* assays.

As observed in **Figure 17**, **X12** was able to significantly increase the cumulative efflux of RHO 123, when compared to controls. This tendency to increase serosal to mucosal transport of the fluorescent substrate was present since minute 5, however, the significant differences occurred from minute 25 on, when controls and X12-treated sacs are compared. This increase was blocked by ZOS, a specific P-gp inhibitor, which proves the involvement of the protein in the efflux of RHO 123 (**Figure 17**). The RHO 123 concentration at the mucosal side of the intestinal membrane significantly increased from 12.6±3.4, 16.8±4.9, 21.8±6.1, 30.4±5.9 and 32.2±7.6 in control everted intestinal sacs to 24.1±4.4, 31.6±4.7, 38.8±5.4, 46.7±6.9 and 54.1±6.8 pmol/mg tissue in the presence of X12, at sampling times of 25 to 45 min, respectively).

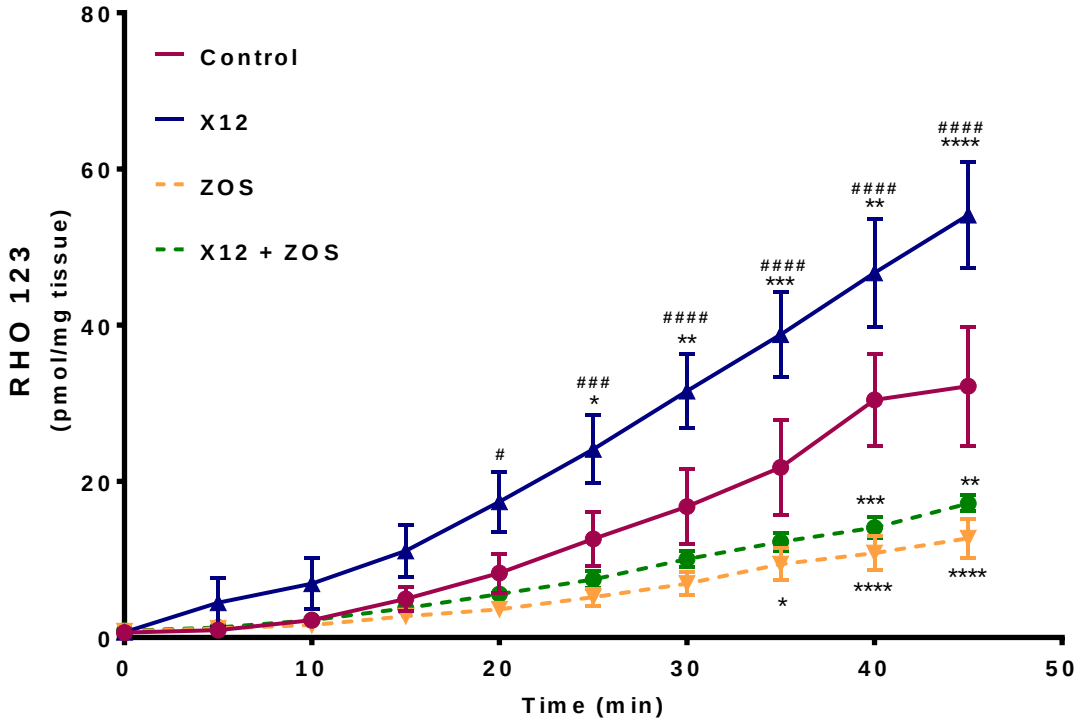

Figure 17. Short-term and direct effect of X12 on P-gp activity, evaluated *ex vivo*. P-gp-mediated RHO 123 efflux was measured using 10 cm everted sacs from distal ileum. The sacs were filled with 300 µM RHO 123 (serosal side). The dye secreted into the outside compartment (mucosal side) was assessed every 5 min for 45 min, in the presence or absence of 20 µM X12 and/or 10 µM zosuquidar (ZOS). Data are expressed as means ± SEM of five to eight rats per group. The excreted amounts of RHO 123 into the mucosal side were evaluated by spectrofluorometry, using a RHO 123 calibration curve, and expressed as pmol of RHO 123 transported *per* mg of tissue. Statistical comparisons were made using Two-way ANOVA followed by the Tukey's multiple comparisons post hoc test (*p <0.05; **p <0.01; ***p < 0.001; ****p<0.0001 vs. Control; #p<0.05; ###p<0.001; ####p<0.0001 X12 vs. X12 + ZOS).

The observed significantly higher serosal to mucosal transport of RHO 123 in X12-treated everted sacs, effect inhibited by ZOS, reveals that P-gp expressed in the enterocytes of the rat ileum distal portion is activated by X12. In fact, the increase in the transport of RHO 123 results from an immediate increase in its activity since a direct and a short-term contact between P-gp and the test compound, X12, occurred. These results are in agreement with an activation affect, rather than with an increase in P-gp expression levels, since the increase in the activity of the transport protein associated to an increase in its expression levels requires a long-term contact between the test compound and the protein. In fact, changes in protein expression levels, namely an upregulation, require the *de novo* synthesis of the protein, which necessarily implies a longer time of contact between protein and compound. For this reason, our results show that the increase in P-gp activity, revealed by a higher cumulative efflux of RHO 123 quantified in mucosal samples, is related to its direct activation by the test compound. Furthermore, this effect was selectively blocked by ZOS, a third-generation inhibitor of the efflux pump. In accordance to the results obtained *in vitro*, it is possible to again conclude that X12

behaves as a P-gp activator *ex vivo*, in the conditions tested, being able to immediately activate P-gp at the intestinal barrier, when a short contact between the drug and the everted intestinal sac occurs.

4.3. BCRP studies

The *in vitro* experimental studies of the efflux transporter, BCRP, were performed on both SW480 and Caco-2 cells. In both cases, only the potential of the tested compounds for BCRP activation was evaluated. Therefore, BCRP activity was assessed with the xanthones present during the accumulation phase of the BCRP substrate. For that purpose, MTX and Ko143 were used as substrate and inhibitor of the efflux pump, respectively, and their cytotoxicity towards both SW480 and Caco-2 cells assessed prior the evaluation of BCRP activity.

4.3.1. BCRP transport activity in the presence of the tested xanthones - SW480 cells

As previously mentioned, the experimental procedure adopted for the evaluation of the effect of the tested compounds on BCRP activity was based on the experimental procedure adopted for the evaluation of their effect on P-gp activity. For that purpose, MTX was used as a fluorescent BCRP substrate and Ko143 as a specific BCRP inhibitor (31, 154, 264), as mentioned above.

To select a non-cytotoxic concentration of MTX and Ko143 to be used in the evaluation of BCRP activity, SW480 cells were exposed to MTX and Ko143 for 24 h and the compounds cytotoxicity was then evaluated by the NR uptake and the MTT reduction assays. As stated in **Figure 18**, no significant effects in both MTT reduction and NR uptake were observed 24 h after exposure of SW480 cells to any of the tested Ko143 concentrations (0 – 10 μM), and when compared to control cells (0 μM).

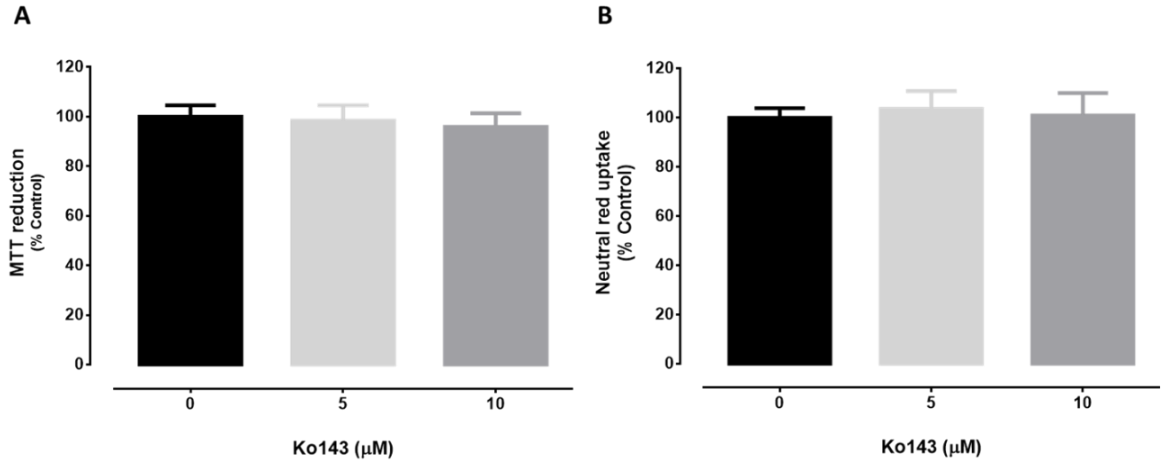

Figure 18. Ko143 (0 – 10 μM) cytotoxicity in SW480 cells, evaluated by the MTT reduction (A) and NR uptake (B) assays, 24 h after exposure. Results are presented as mean ± SD from at least 4 independent experiments, performed in triplicate. Statistical comparisons were made using the parametric method of One-way ANOVA, followed by the Dunnett's multiple comparisons test.

In what concerns to MTX, a significant cytotoxic effect was observed 24 h after exposure to both tested MTX concentrations, and as measured either by the NR uptake or by the MTT reduction assays (**Figure 19)**. Indeed, the exposure of SW480 cells to 10 μM MTX significantly reduced the MTT reduction by 50% and NR uptake by 49%, when compared to control cells (**Figure 19)**. For the 5 μM concentration, a pronounced cytotoxicity to SW480 cells was also observed after 24 h of incubation, an effect observed in both cytotoxicity assays (40% reduction in the MTT reduction assay and 30% reduction in the NR uptake assay).

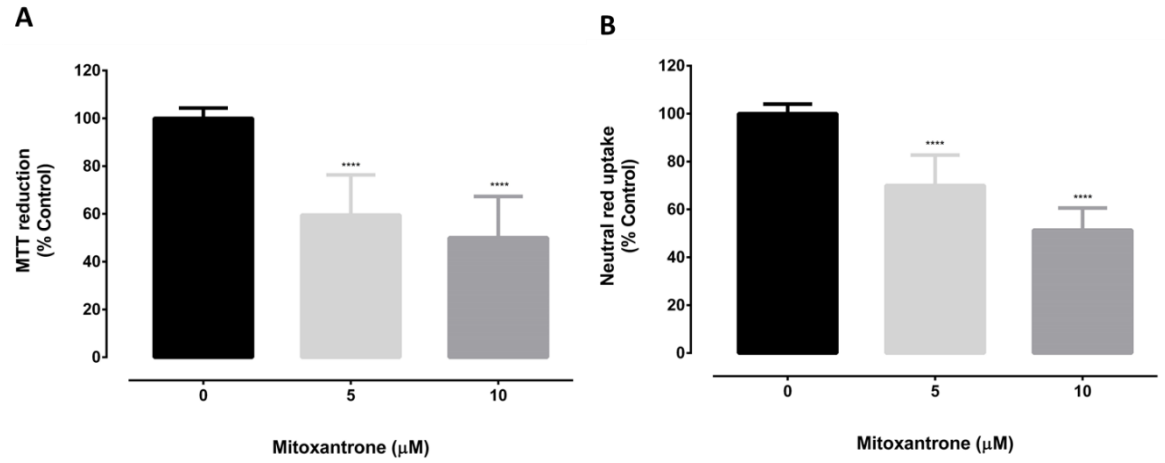

Figure 19. Mitoxantrone (0 – 10 μM) cytotoxicity in SW480 cells, evaluated by the MTT reduction (A) and NR uptake (B) assays, 24 h after exposure. Results are presented as mean ± SD from 4 independent experiments, performed in triplicate. Statistical comparisons were made using the parametric method of One-way ANOVA, followed by the Dunnett's multiple comparisons test [****$p < 0.0001$ vs. control (0 μM)].

Given the obtained results, the exposure to MTX was reduced to 2 h since, in the subsequent experiments performed to evaluate BCRP activity, the time of contact of the substrate with the cells was always smaller than 2 h. As observed in **Figure 20**, none of the tested MTX concentrations (0 – 10 μM) caused a significant toxicity towards SW480 cells, as compared to control cells (0 μM), an effect observed in both performed cytotoxicity assays.

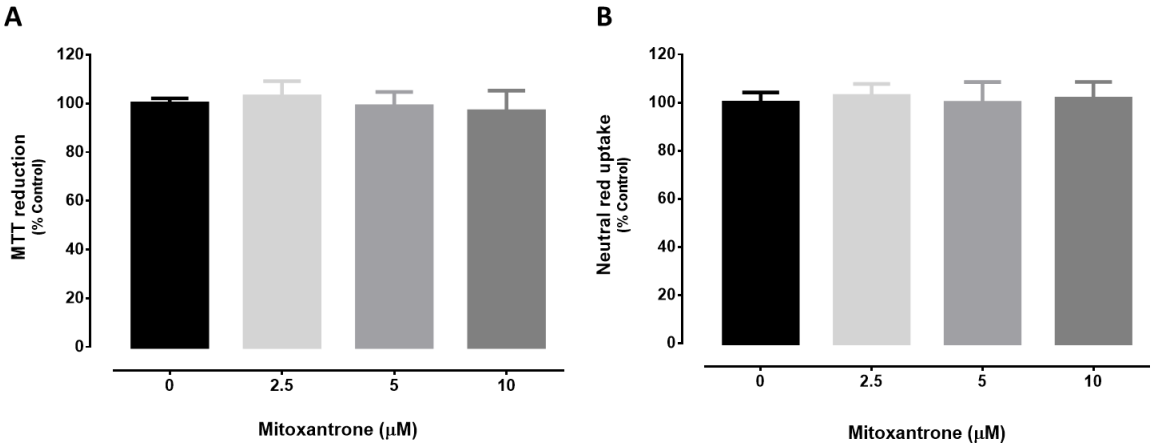

Figure 20. Mitoxantrone (0 – 10 μM) cytotoxicity in SW480 cells, evaluated by the MTT reduction (A) and NR uptake (B) assays, 2 h after exposure. Results are presented as mean ± SD from 4 independent experiments, performed in triplicate. Statistical comparisons were made using the parametric method of One-way ANOVA, followed by the Dunnett's multiple comparisons test.

Based on these results (**Figure 18** and **Figure 20**), BCRP transport activity assays in SW480 cells can be safely performed using Ko143 and MTX at concentrations up to 10 μM.

For the evaluation of BCRP transport activity, MTX intracellular content was measured by flow cytometry, in the presence or absence of the tested xanthonic derivatives (20 μM) during the accumulation phase of the fluorescence substrate, and in the presence or absence of the BCRP inhibitor. However, preliminary assays were needed to select the most suitable MTX and Ko143 concentrations, as well as to define the duration of the substrate accumulation phase, aiming to set a final procedure that allows a simple, efficient and reproducible determination of BCRP activity, and which enables to detect the potential effects of the xanthonic derivatives tested on the activity of this efflux pump. Accordingly, MTX was tested at the final concentration of 2.5, 5 and 10 μM, Ko143 was tested at the 5 and 10 μM concentrations, and the incubation with the fluorescent substrate was performed during 60, 90 and 120 minutes. According to the results obtained in those preliminary studies, and in this cell model, BCRP activity was set to be evaluated incubating the cells with 10 μM MTX, in the presence or absence of 5 μM Ko143, for 90 minutes, being the xanthonic derivatives present during all the incubation period with the BCRP substrate. This protocol allows to detect the xanthones immediate effects on BCRP

transport activity, either a direct activation or inhibition. BCRP activity was evaluated through the ratio between the MFI after inhibited MTX accumulation (IA – in the presence of Ko143) and the MFI of non-inhibited MTX accumulation (NA, in the absence of the BCRP inhibitor) and the results were expressed as percentage of control cells (0 μM), as described in section 3.6.1.2.2 - Mitoxantrone accumulation assay in the presence of xanthones. As shown in **Figure 21**, none of the tested xanthones was able to significantly increase BCRP activity when compared to control cells (0 μM). This indicates that these xanthones do not have the ability to directly activate BCRP, in this *in vitro* model and in the present experimental conditions, thus not behaving as activators of this efflux pump. In what concerns to **X16**, a slight but significant decrease in BCRP activity was observed (BCRP activity decreased by 13% in the presence of X16, when compared to control cells). Thereby, this xanthonic derivative seems to behave, although in modestly magnitudes, as a BCRP inhibitor, opening new perspectives to overcome MDR in cancer cells, namely representing a potentially new source of derivatives with BCRP inhibition ability.

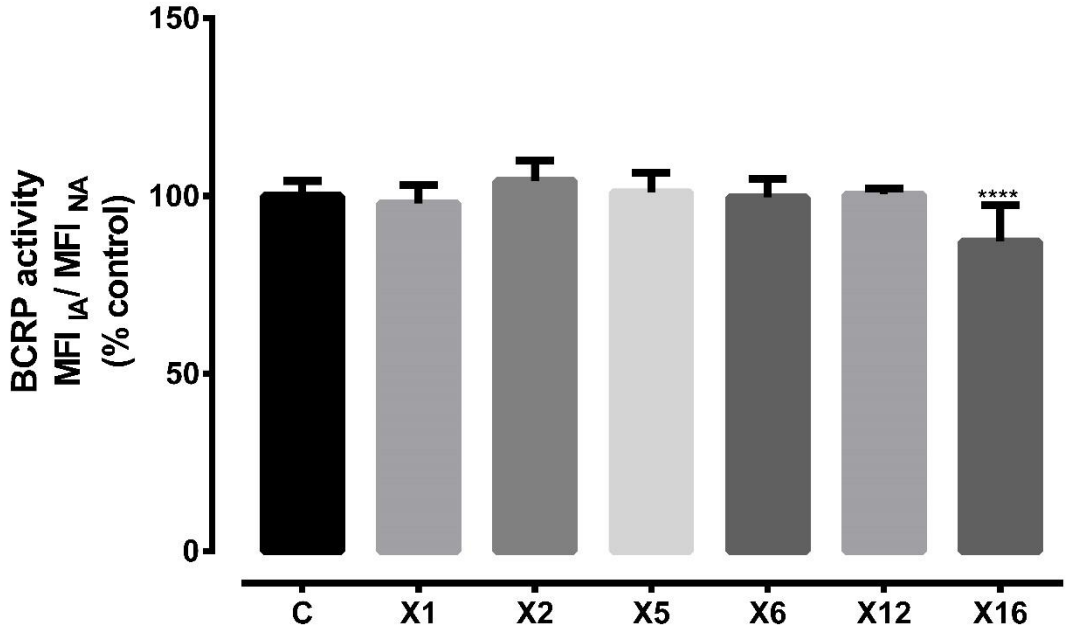

Figure 21. BCRP activity evaluated by flow cytometry in SW480 cells exposed to the tested xanthones (20 μM) only during the 90 minutes incubation period with the fluorescent substrate (10 μM MTX). Results are presented as mean ± SD from 4 independent experiments, performed in triplicate. Statistical comparisons were made using the parametric method of One-way ANOVA, followed by the Dunnett's multiple comparisons test [****$p < 0.0001$ vs. control (0 μM)].

The fact that none of the tested xanthones was able to increase MTX efflux, and, therefore, did not demonstrate a direct BCRP activation capacity, may be justified by the low expression of the efflux pump in the SW480 cell line. In fact, it is reported in the

literature that SW480 cells express low levels of ABCG2 (264). Thus, since the BCRP is expressed at low levels in the SW480 cell line, the BCRP-mediated MTX efflux will be low and, consequently, small differences in MTX intracellular content will be obtained between control cells and cells under BCRP inhibition (inhibiting the carrier with Ko143). It should also be noted that the fluorescent substrate used, MTX, is not specific for BCRP and can also be carried by P-gp (4, 14). However, as the inhibitor used, Ko143, is described as specific for BCRP (91, 123), differences in the MTX intracellular fluorescence in the absence and presence of this inhibitor will reflect the activity of this efflux pump. Taking into account the observed results, and the possible justification for them, the effect of the tested xanthones on BCRP transport activity was then evaluated in another cell line, specifically in Caco-2 cells, which are used in several studies to investigate the BCRP transport activity (265-267).

4.3.2. BCRP transport activity in the presence of the tested xanthones - Caco-2 cells

The assays performed to study the activity of BCRP in Caco-2 cells were based on the experimental procedure previously described for SW480 cells.

To select a non-cytotoxic concentration of both MTX and Ko143 to be used in the BCRP activity assays, Caco-2 cells were exposed to MTX and Ko143 for 24 h and the compounds cytotoxicity was then evaluated by the NR uptake and MTT reduction assays. As shown in **Figure 22**, no significant cytotoxicity towards Caco-2 cells was observed 24 h after exposure to any of the tested Ko143 concentrations (0 – 10 μM), relative to control cells (0 μM), as assessed by both the NR uptake and MTT reduction assays.

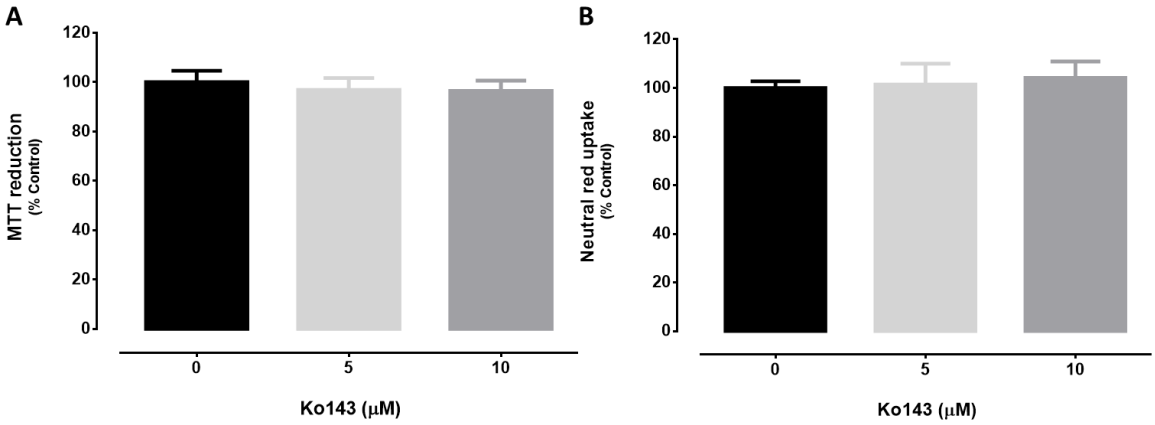

Figure 22. Ko143 (0 – 10 μM) cytotoxicity in Caco-2 cells, evaluated by the MTT reduction (A) and NR uptake (B) assays, 24 h after exposure. Results are presented as mean ± SD from at least 4 independent experiments, performed in triplicate. Statistical comparisons were made using the parametric method of One-way ANOVA, followed by the Dunnett's multiple comparisons test.

Regarding MTX, the cytotoxicity of this substrate was assessed, in Caco-2 cells, by NR uptake and MTT reduction assays, 24 h after exposure only to the 5 μM MTX concentration. Contrarily to the study performed in SW480 cells, the 10 μM MTX concentration was not tested in this cell line given the significant toxicity observed 24 h after exposure of SW480 cells to this MTX concentration. As shown in **Figure 23**, the exposure of Caco-2 cells to 5 μM MTX resulted in a significant decrease in both MTT reduction and NR uptake (24 % decrease in MTT reduction and 31% decrease in NR uptake), when compared to control cells (0 μM).

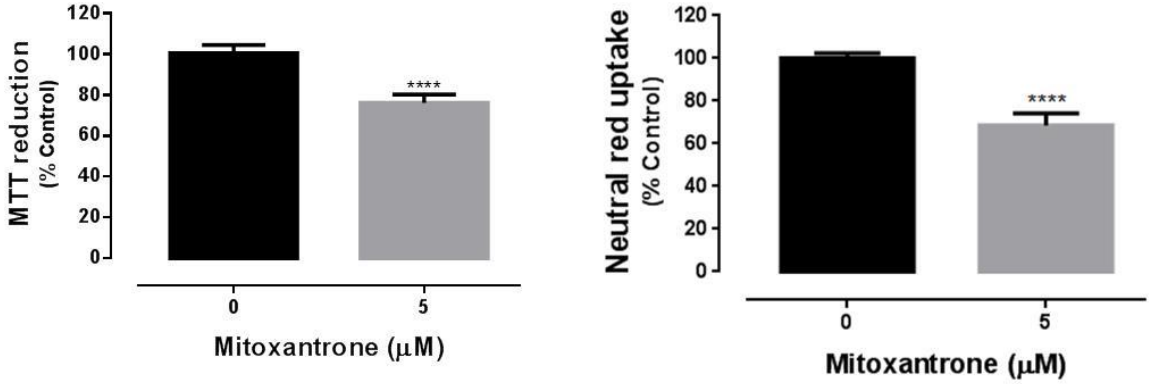

Figure 23. Mitoxantrone (0 – 10 μM) cytotoxicity in Caco-2 cells, evaluated by the MTT reduction (A) and NR uptake (B) assays, 24 h after exposure. Results are presented as mean ± SD from 2 independent experiments, performed in triplicate. Statistical comparisons were made using the Unpaired *t* test [****$p < 0.0001$ vs. control (0 μM)].

Based on these results, and as performed for the SW480 cell line, the exposure to MTX was reduced to 2 h since, as previously mentioned, in the experiments performed to evaluate BCRP activity, the time of contact of the substrate with the cells was never higher than 2 h. As shown in **Figure 24**, none of the tested MTX concentrations (0-10 μM) significantly decreased either MTT reduction or NR uptake, when compared to control cells (0 μM), thus demonstrating the lack of cytotoxicity towards Caco-2 cells in these experimental conditions.

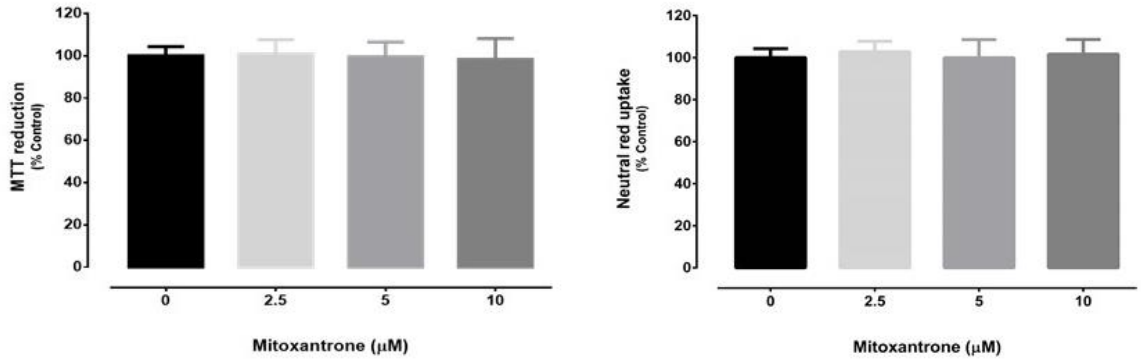

Figure 24. Mitoxantrone (0 – 10 μM) cytotoxicity in Caco-2 cells, evaluated by the MTT reduction (A) and NR uptake (B) assays, 2 h after exposure. Results are presented as mean ± SD from at least 5 independent experiments, performed in triplicate. Statistical comparisons were made using the parametric method of One-way ANOVA, followed by the Dunnett's multiple comparisons test.

Considering the results obtained in the previous cytotoxicity assays (**Figure 22** and **Figure 24**), BCRP transport activity can also be safely assessed in Caco-2 cells using Ko143 and MTX at concentrations up to 10 μM.

Like for the SW480 cell line, the evaluation of BCRP transport activity in Caco-2 cells was also performed using MTX, and the incubation with this fluorescent substrate performed in the presence or absence of the tested xanthonic derivatives (20 μM), and with or without simultaneous incubation with the BCRP inhibitor, Ko143. MTX intracellular content was, then, measured by flow cytometry. However, and as performed for SW480 cells, preliminary assays were needed to select the most suitable MTX and Ko143 concentrations, as well as to define the duration of the substrate accumulation phase. MTX was tested at the final concentration of 2.5, 5 and 10 μM, Ko143 was tested at the 5 and 10 μM concentrations, and the incubation with the fluorescent substrate was performed during 30, 60 and 90 minutes. In the Caco-2 cell line, the results obtained in those preliminary studies demonstrated that, to ensure an efficient and reproducible determination of BCRP activity, the cells needed to be incubated with 5 μM MTX, in the presence or absence of 5 μM Ko143, for 60 min, being the xanthonic derivatives present during all the incubation period with the BCRP substrate.

As demonstrated in **Figure 25**, xanthones **X1**, **X5** and **X6** significantly increased BCRP activity to 112, 119 and 112% of control cells (100%), respectively. This indicates that these xanthones, even though in a modest way, have the capacity to directly activate BCRP in this cell line, thereby behaving as BCRP activators. With respect to **X16**, this xanthonic derivative significantly reduced the efflux of MTX, thus reducing BCRP activity in about 17%, when compared to the control cells. This result, which is in agreement with the results obtained for this compound in the SW480 cell line (**Figure 21**), demonstrated the ability of X16 to inhibit BCRP.

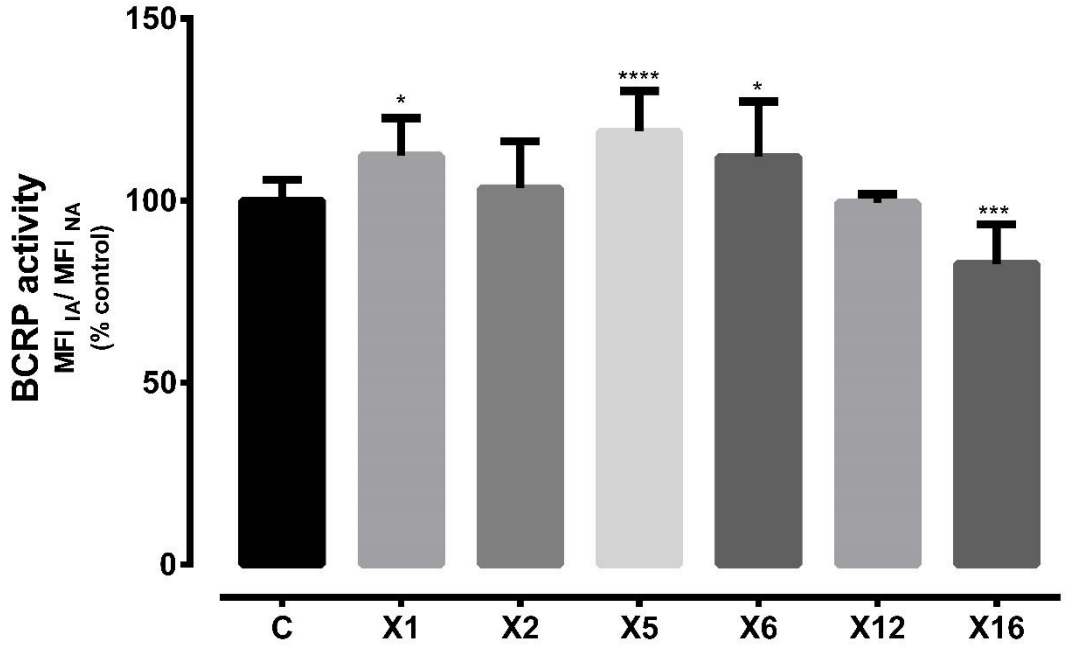

Figure 25. BCRP activity evaluated by flow cytometry in Caco-2 cells exposed to the tested xanthones (20 μM) only during the 60 minutes incubation period with the fluorescent substrate (5 μM MTX). Results are presented as mean ± SD from 4 independent experiments, performed in triplicate. Statistical comparisons were made using the parametric method of One-way ANOVA, followed by the Dunnett's multiple comparisons test [*$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$ vs. control (0 μM)].

The results obtained concerning the BCRP activation potential of the tested compounds highlighted differences between both cell lines, as the significant and immediate increases in the efflux pump activity were only observed using Caco-2 cells. However, concerning P-gp, no differences were observed between both cell lines, as similar results were obtained in what concerns to the P-gp activation effect caused by the tested compounds. Therefore, although Caco-2 cells express BCRP at low levels when compared with those found on the normal human jejunum (71), an increased responsiveness of these cells to potential BCRP modulators was observed, when compared to the SW480 cell line. Consequently, the obtained results demonstrated that Caco-2 cells are more suitable for the *in vitro* evaluation of the effects of potential BCRP modulators on the activity of this efflux pump, when compared to SW480 cells, and represent an useful model of jejunal drug efflux. However, the low expression of BCRP in this *in vitro* model needs to be always taken into account, especially when screening potential BCRP inducers.

4.3.3. Potential protective effects of the xanthonic derivatives against Mitoxantrone-induced cytotoxicity

As previously mentioned, by significantly reducing the intestinal absorption of harmful xenobiotics and hence preventing their access to target organs, the defense mechanism mediated by P-gp and BCRP may be of particular relevance at the intestinal level. Noteworthy, this relevance is further reinforced by the knowledge that many intoxications by toxic P-gp and/or BCRP substrates may result from accidental or intentional ingestion. Indeed, due to their ability to pump their substrates against the concentration gradient and to their ability to transport them towards the lumen of the gastrointestinal tract, these proteins confer protection against toxic molecules, mediating their efflux and, thereby, avoiding their systemic absorption and, consequently, their access to the target organs. Therefore, and given the observed P-gp and BCRP modulatory effects of the tested compounds, the present study aimed to evaluate their potential protective effect against the cytotoxicity caused by MTX, a model of a substrate of both P-gp and BCRP.

For this purpose, and concerning the SW480 cells, to verify whether the previously observed increases in P-gp expression and activity caused by the xanthonic derivatives under study could translate into a cellular protection against MTX-induced toxicity, SW480 cells were incubated with the toxic substrate, MTX (0; 0,1; 0,5; 1; 2,5; 5; 10; 25; 50; 100 and 150 μM), in the presence or absence of tested xanthones (20 μM), for 24 h. Given the complexity of the study, the xanthonic derivatives were divided into two groups of experiments: group 1, comprising xanthones X5, X6 and X12, and group 2, comprising xanthones X1, X2 and X16. Indeed, xanthones X5, X6 and X12 were initially selected for these experiments as they proved to be the most promising P-gp activators among the tested compounds, resulting in a significant increase in the protein transport activity to 128%, 126% and 135%, respectively, when compared to control cells (**Figure 15**). However, considering that X16 showed a significant capacity to slightly decrease P-gp activity (**Figure 15**), it became interesting to verify if this xanthonic derivative could worsen the cytotoxicity induced by MTX, behaving as a potential inhibitor of the efflux pump. Therefore, the group 2 of experiments was later performed, comprising also X1 and X2. Despite this, it was possible to verify a high similarity between the MTX cytotoxicity curves obtained in both groups of experiments (that is, the MTX concentration-response curves obtained in the absence of xanthonic derivatives, in group 1 and group 2 of experiments) (**Figure 26**). Thus, given the similarity between the obtained MTX concentration-response curves, it is possible to compare the results obtained for the MTX cytotoxicity in the presence of xanthonic derivatives tested in different groups of experiments.

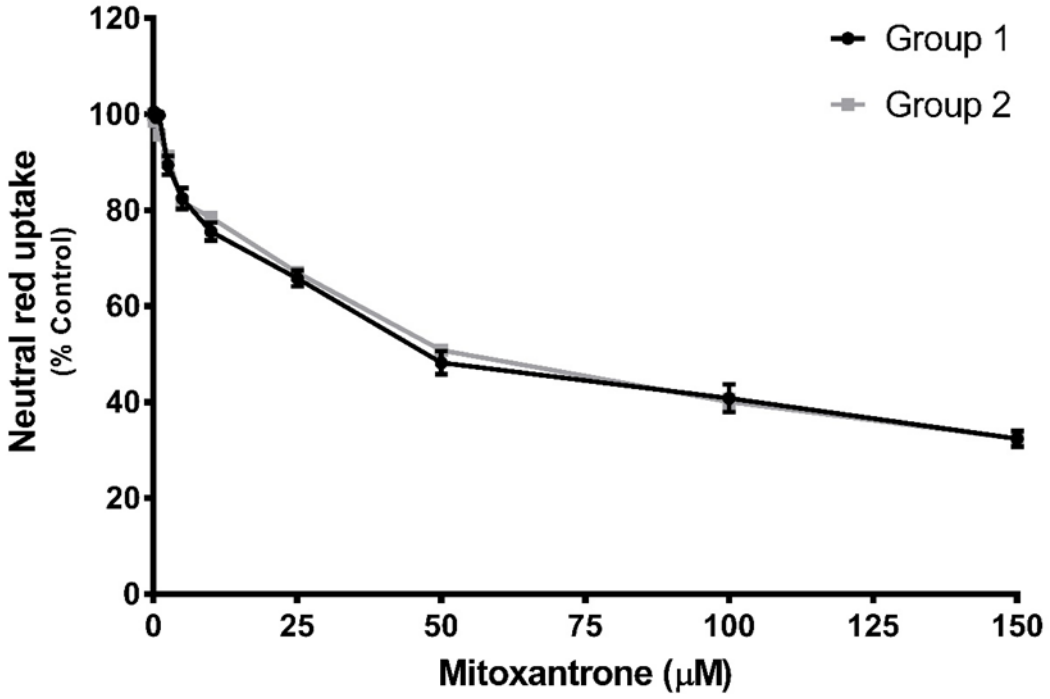

Figure 26. **Mitoxantrone (0 – 150 μM) cytotoxicity in SW480 cells evaluated by the NR uptake assay, 24 h after exposure.** Results are presented as mean ± SEM from 4 independent experiments, performed in triplicate. Statistical comparisons were made using Two-way ANOVA, followed by the Sidak's multiple comparisons test.

As shown in **Figure 27**, after simultaneous incubation of SW480 cells with MTX (0 - 150 μM) and the compounds **X2**, **X5**, **X6** or **X12**, no significant differences were observed in the cytotoxicity of the substrate, when compared to cells incubated with MTX alone. In turn, and unexpectedly, when SW480 cells were simultaneously exposed to MTX (0 - 150 μM) and **X16**, a significant increase in NR uptake was observed for the 1 and 25 μM MTX concentrations, and when compared to cells incubated with MTX alone, demonstrating a small but significant protection of SW480 cells against the cytotoxic effects caused by MTX. Given the lack of positive modulatory effects of X16 on P-gp and BCRP expression and activity, mechanisms other than the modulation of these ABC transporters may be responsible for the observed protection of SW480 against MTX-induced cytotoxicity.

Conversely, **X1**, when simultaneously incubated with MTX, significantly increased the cytotoxicity of the substrate at the 50 and 100 μM concentrations, in comparison with the cells incubated with MTX alone. From this analysis, it can be concluded that, despite the ability of the tested compounds to significantly increase P-gp expression and activity, being considered the most efficient P-gp activators (**Figure 15**), X5, X6 and X12 were not able to protect SW480 cells against the MTX-induced cytotoxicity.

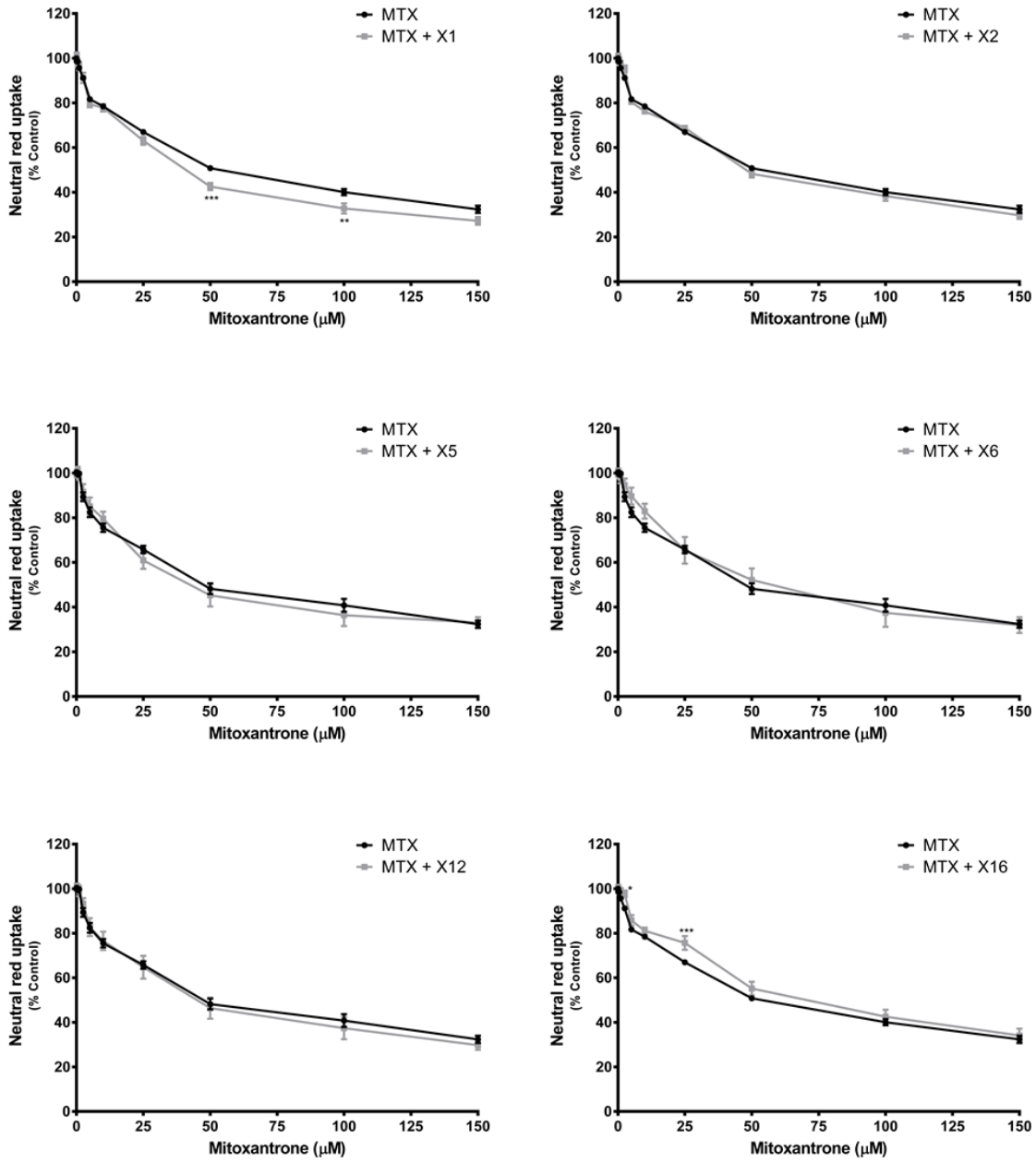

Figure 27. Mitoxantrone (0 – 150 μM) cytotoxicity in SW480 cells evaluated by the NR uptake assay, 24 h after exposure. Results are presented as mean ± SEM from 4 independent experiments, performed in triplicate. Statistical comparisons were made using Two-way ANOVA, followed by the Sidak's multiple comparisons test [*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. MTX alone].

The potential protective effect mediated by the tested compounds against the MTX cytotoxicity was also evaluated in the Caco-2 cell line. For this purpose, MTX (500, 1000 and 1500 μM) cytotoxicity was assessed by the NR uptake assay, with or without simultaneous exposure to the tested xanthones (20 μM), for 24 h. The concentrations of the toxic substrate were selected based on EC50 described in the literature (268). As shown in **Figure 28**, none of the tested compounds, namely **X1**, **X2**, **X5**, **X12** and **X16**, were able to afford protection to Caco-2 cells against MTX (0 - 1500 μM) cytotoxicity,

when compared to cells incubated with MTX alone. In turn, **X6**, when simultaneously incubated with MTX (1000 and 1500 μM), and also in comparison to cells incubated with MTX alone, caused a significant increase in the substrate cytotoxicity.

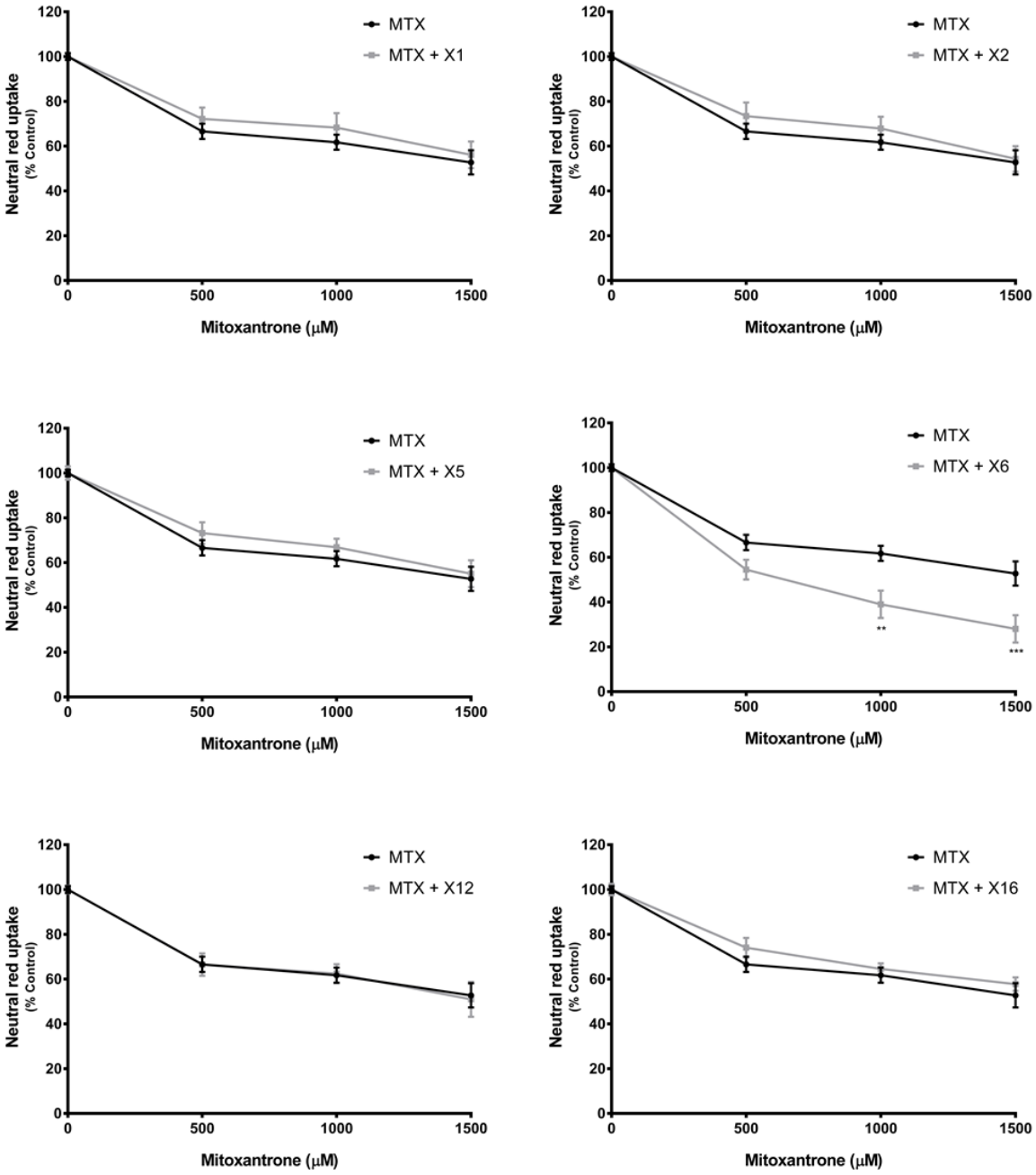

Figure 28. Mitoxantrone (0 – 1500 μM) cytotoxicity in Caco-2 cells evaluated by the NR uptake assay, 24 h after exposure. Results are presented as mean ± SEM from 2 independent experiments, performed in duplicate. Statistical comparisons were made using Two-way ANOVA, followed by the Sidak's multiple comparisons test [$^{**}p < 0.01$; $^{***}p < 0.001$ vs. MTX alone].

Therefore, although the observed increases in P-gp and BCRP activity, the xanthonic derivatives failed to afford protection of both cell lines against the cytotoxicity induced by the harmful substrate, MTX. However, previous studies performed in the Laboratory of

Toxicology demonstrated that some of the tested xanthones significantly protected Caco-2 cells against PQ-induced cytotoxicity, an effect prevented upon incubation with the P-gp inhibitor, elacridar (unpublished data). For that reason, and given the existence of multiple drug-binding sites at the P-gp drug-binding pocket, further studies are needed to assess whether the expected protection is dependent on the binding sites of both the toxic substrate and the potential activator. For that purpose, the evaluation of the potential protective effects of the tested xanthones against other P-gp and BCRP substrates is of utmost importance.

V. CONCLUSIONS

5. CONCLUSIONS

P-gp and BCRP are two ABC transporters important both for the maintenance of cellular homeostasis and for the detoxification of substances with toxic potential, being an integral part of the pharmacokinetic process known as ADMET. Given their polarized expression in many excretory and barrier tissues, such as the intestinal barrier, their broad substrate specificity and their great efflux capacity, P-gp and BCRP should be highlighted as an important mechanism of cellular protection against the toxic effects of their harmful substrates, contributing to the reduction in their intracellular accumulation. Indeed, both transporters, by promoting the extrusion of toxic substances from inside the cells, thus decreasing their intracellular concentration, are able to limit the absorption of their substrates, limiting the amount of xenobiotics that reaches the target organ/tissue and, consequently, reducing their toxicity.

This important physiological role played by ABC transporters has led, in recent years, to an increasingly interest in studying the mechanism(s) underlying the induction and activation of these proteins. For this reason, several compounds, including xanthonic derivatives, have been extensively studied for their ability to increase the expression and/or activity of these transporters, and thus afford protection in cases of exposure to their toxic substrates. Therefore, the main objective of this work was to study new chemical entities in relation to their potential to increase the expression and/or activity of P-gp and BCRP at the intestinal barrier, and thus be further investigated and proposed as potential antidotes in intoxications caused by toxic substrates of these efflux transporters.

The results included in this dissertation demonstrate that the synthetic xanthonic derivatives **X1**, **X2**, **X5**, **X6**, **X12** and **X16** did not show significant toxicity towards SW480 cells, after 24 h of exposure, and for concentrations up to 20 μM. Some of the tested compounds (**X1**, **X2**, **X5**, **X6** and **X12**) showed the capacity to **immediately increase P-gp activity** in SW480 cells, thus acting as **P-gp activators**. ***Ex vivo*** studies demonstrated that **X12, the most potent P-gp activator** found in the *in vitro* studies, significantly increased the efflux of RHO 123 in rat intestinal everted sacs, thus reflecting an efficient and rapid increase in the efflux pump activity, and further corroborating the *in vitro* results. Furthermore, this increase in the pump activity was blocked by ZOS, a specific P-gp inhibitor, which proved the involvement of the protein in the efflux of the substrate.

Additionally, **X1, X2, X6** and **X12** significantly increased cell-surface **P-gp expression** in SW480 cells, 24 h after exposure, thus demonstrating to be **P-gp inducers**. For **X1**, **X6** and **X12**, the observed increases in the protein expression were accompanied by significant increases in P-gp transport activity, although to a lower extent in cells pre-exposed to **X6**. Furthermore, for **X2**, the observed increase in the cell-surface

protein expression did not result in a significant increase in protein transport activity, thus reinforcing the need to simultaneously evaluate both expression and activity in the screening of inducers and/or activators of these efflux pumps, since an increased protein expression does not always translates into an increased transport activity, and increases in the pump activity can be observed regardless the level of expression. Furthermore, **X5** significantly increased P-gp activity in SW480 cells pre-exposed to the xanthonic derivative for 24 h, although no significant effects were observed in the P-gp cell-surface expression. Since **X5** was confirmed as a P-gp activator, the significant increase in P-gp activity observed in cells pre-exposed to **X5** for 24 h may result from a direct pump activation mediated by the compound that remained intracellularly, instead of resulting from an increased P-gp expression.

Concerning **BCRP**, xanthones **X1**, **X5** and **X6** showed the capacity to immediately increase **BCRP activity**, therefore acting as **BCRP activators**. However, the ability of the tested compounds to positively modulate BCRP activity was only observed in the Caco-2 cell line.

In addition, none of the tested compounds that positively modulated P-gp and/or BCRP protected SW480 or Caco-2 cells against the cytotoxicity induced by the toxic substrate, MTX. Moreover, and unexpectedly, xanthones **X1** and **X6** exacerbated MTX cytotoxicity in SW480 and Caco-2 cells, respectively.

In conclusion, the work included in this dissertation demonstrated the ability of xanthonic derivatives for **P-gp and/or BCRP modulation** at the intestinal level, opening new perspectives to mechanistically explore the physiological role of these transporters. Furthermore, knowing that the induction and/or activation of these ABC transporters may represent a potential antidotal pathway in cases of intoxication by their toxic substrates (reducing their intracellular accumulation and, consequently, their toxicity), and given the *in vitro* potential of these xanthonic derivatives as P-gp and/or BCRP activators, this important class of compounds deserves particular attention at the toxicological level, as they can constitute a promising source of novel derivatives that worth to be further studied.

VI. FUTURE PERSPECTIVES

6. FUTURE PERSPECTIVES

In this dissertation, some of the tested xanthones demonstrated to significantly and immediately increase BCRP activity, therefore acting as BCPR activators, although the effects were only observed in the Caco-2 cell line. In the continuity of this project, the evaluation of BCRP expression and activity, 24 h after exposure to these compounds, would be useful to verify whether the tested compounds are BCRP inducers, increasing the protein expression levels, and also if this induction effect translates into an increased activity. In fact, an increased protein expression does not always translates into an increased transport activity, as well as increases in pump activity can be observed independently of the level of expression, which reinforces the need to simultaneously assess both expression and activity in the screening for inducers and/or activators of this efflux pump.

In addition, according to the obtained results, none of the tested compounds that positively modulated P-gp and/or BCRP protected SW480 or Caco-2 cells against the cytotoxicity induced by the toxic substrate, MTX. Thus, it becomes useful to elucidate whether the protection against toxic substrates of these efflux pumps is dependent on the binding site of both substrate and potential activator. Therefore, in the future, the evaluation of the potential protective effects of these xanthonic derivatives against other P-gp and/or BCRP substrates will be of utmost importance.

Furthermore, and based on the obtained results, the tested compounds can be faced as a source of new xanthonic derivatives that can be synthetized and tested for their potential P-gp and BCRP modulation, disclosing new perspectives in the treatment of intoxications induced by toxic ABC transporters substrates.

VII. REFERENCES

7. REFERENCES

1. Doring B, Petzinger E. Phase 0 and phase III transport in various organs: combined concept of phases in xenobiotic transport and metabolism. Drug Metab Rev. 2014;46(3):261-82.

2. Williams JA, Bauman J, Cai H, Conlon K, Hansel S, Hurst S, et al. In vitro ADME phenotyping in drug discovery: current challenges and future solutions. Curr Opin Drug Discov Devel. 2005;8(1):78-88.

3. Flynn E. Pharmacokinetic Parameters. 2007:1-3.

4. Silva R, Vilas-Boas V, Carmo H, Dinis-Oliveira RJ, Carvalho F, de Lourdes Bastos M, et al. Modulation of P-glycoprotein efflux pump: induction and activation as a therapeutic strategy. Pharmacol Ther. 2015;149:1-123.

5. Gameiro M, Silva R, Rocha-Pereira C, Carmo H, Carvalho F, Bastos ML, et al. Cellular Models and In Vitro Assays for the Screening of modulators of P-gp, MRP1 and BCRP. Molecules. 2017;22(4).

6. Jani M, Ambrus C, Magnan R, Jakab KT, Beery E, Zolnerciks JK, et al. Structure and function of BCRP, a broad specificity transporter of xenobiotics and endobiotics. Arch Toxicol. 2014;88(6):1205-48.

7. Szakacs G, Varadi A, Ozvegy-Laczka C, Sarkadi B. The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME-Tox). Drug Discov Today. 2008;13(9-10):379-93.

8. Vasiliou V, Vasiliou K, Nebert DW. Human ATP-binding cassette (ABC) transporter family. Hum Genomics. 2009;3(3):281-90.

9. Higgins CF. ABC transporters: from microorganisms to man. Annu Rev Cell Biol. 1992;8:67-113.

10. Choudhuri S, Klaassen CD. Structure, function, expression, genomic organization, and single nucleotide polymorphisms of human ABCB1 (MDR1), ABCC (MRP), and ABCG2 (BCRP) efflux transporters. Int J Toxicol. 2006;25(4):231-59.

11. Dean M, Allikmets R. Complete characterization of the human ABC gene family. J Bioenerg Biomembr. 2001;33(6):475-9.

12. Colabufo NA, Berardi F, Contino M, Niso M, Perrone R. ABC pumps and their role in active drug transport. Curr Top Med Chem. 2009;9(2):119-29.

13. Mo W, Zhang JT. Human ABCG2: structure, function, and its role in multidrug resistance. Int J Biochem Mol Biol. 2012;3(1):1-27.

14. Sharom FJ. ABC multidrug transporters: structure, function and role in chemoresistance. Pharmacogenomics. 2008;9(1):105-27.

15. Marcellino T, Srinivasan V. Biophysical methods toolbox to study ABC exporter structure and function. Biol Chem. 2017;398(2):229-35.

16. Zhao C, Tampe R, Abele R. TAP and TAP-like--brothers in arms? Naunyn Schmiedebergs Arch Pharmacol. 2006;372(6):444-50.

17. Linton KJ, Higgins CF. Structure and function of ABC transporters: the ATP switch provides flexible control. Pflugers Arch. 2007;453(5):555-67.

18. Theodoulou FL, Kerr ID. ABC transporter research: going strong 40 years on. Biochem Soc Trans. 2015;43(5):1033-40.

19. Couture L, Nash JA, Turgeon J. The ATP-binding cassette transporters and their implication in drug disposition: a special look at the heart. Pharmacol Rev. 2006;58(2):244-58.

20. Gottesman MM, Ambudkar SV. Overview: ABC transporters and human disease. J Bioenerg Biomembr. 2001;33(6):453-8.

21. Stefkova J, Poledne R, Hubacek JA. ATP-binding cassette (ABC) transporters in human metabolism and diseases. Physiol Res. 2004;53(3):235-43.

22. Leslie EM, Deeley RG, Cole SP. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicol Appl Pharmacol. 2005;204(3):216-37.

23. Hennessy M, Spiers JP. A primer on the mechanics of P-glycoprotein the multidrug transporter. Pharmacol Res. 2007;55(1):1-15.

24. Juliano RL, Ling V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys Acta. 1976;455(1):152-62.

25. Zhou SF. Structure, function and regulation of P-glycoprotein and its clinical relevance in drug disposition. Xenobiotica. 2008;38(7-8):802-32.

26. Sharom FJ. The P-glycoprotein multidrug transporter. Essays Biochem. 2011;50(1):161-78.

27. Chang G. Multidrug resistance ABC transporters. FEBS Lett. 2003;555(1):102-5.

28. Urbatsch IL, Julien M, Carrier I, Rousseau ME, Cayrol R, Gros P. Mutational analysis of conserved carboxylate residues in the nucleotide binding sites of P-glycoprotein. Biochemistry. 2000;39(46):14138-49.

29. Sharom FJ, Liu R, Romsicki Y, Lu P. Insights into the structure and substrate interactions of the P-glycoprotein multidrug transporter from spectroscopic studies. Biochim Biophys Acta. 1999;1461(2):327-45.

30. Ambudkar SV, Kimchi-Sarfaty C, Sauna ZE, Gottesman MM. P-glycoprotein: from genomics to mechanism. Oncogene. 2003;22(47):7468-85.

31. Ni Z, Bikadi Z, Rosenberg MF, Mao Q. Structure and function of the human breast cancer resistance protein (BCRP/ABCG2). Curr Drug Metab. 2010;11(7):603-17.

32. Mao Q, Unadkat JD. Role of the breast cancer resistance protein (BCRP/ABCG2) in drug transport--an update. AAPS J. 2015;17(1):65-82.

33. Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, et al. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci U S A. 1998;95(26):15665-70.

34. Miyake K, Mickley L, Litman T, Zhan Z, Robey R, Cristensen B, et al. Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. Cancer Res. 1999;59(1):8-13.

35. Xu J, Liu Y, Yang Y, Bates S, Zhang JT. Characterization of oligomeric human half-ABC transporter ATP-binding cassette G2. J Biol Chem. 2004;279(19):19781-9.

36. Graf GA, Li WP, Gerard RD, Gelissen I, White A, Cohen JC, et al. Coexpression of ATP-binding cassette proteins ABCG5 and ABCG8 permits their transport to the apical surface. J Clin Invest. 2002;110(5):659-69.

37. Li YF, Polgar O, Okada M, Esser L, Bates SE, Xia D. Towards understanding the mechanism of action of the multidrug resistance-linked half-ABC transporter ABCG2: a molecular modeling study. J Mol Graph Model. 2007;25(6):837-51.

38. Oostendorp RL, Beijnen JH, Schellens JH. The biological and clinical role of drug transporters at the intestinal barrier. Cancer Treat Rev. 2009;35(2):137-47.

39. Sai Y. Biochemical and molecular pharmacological aspects of transporters as determinants of drug disposition. Drug Metab Pharmacokinet. 2005;20(2):91-9.

40. Xia CQ, Milton MN, Gan LS. Evaluation of drug-transporter interactions using in vitro and in vivo models. Curr Drug Metab. 2007;8(4):341-63.

41. Montanari F, Ecker GF. Prediction of drug-ABC-transporter interaction--Recent advances and future challenges. Adv Drug Deliv Rev. 2015;86:17-26.

42. Lee W, Kim RB. Transporters and renal drug elimination. Annu Rev Pharmacol Toxicol. 2004;44:137-66.

43. Morrissey KM, Stocker SL, Wittwer MB, Xu L, Giacomini KM. Renal transporters in drug development. Annu Rev Pharmacol Toxicol. 2013;53:503-29.

44. Begley DJ. ABC transporters and the blood-brain barrier. Curr Pharm Des. 2004;10(12):1295-312.

45. Sun H, Dai H, Shaik N, Elmquist WF. Drug efflux transporters in the CNS. Adv Drug Deliv Rev. 2003;55(1):83-105.

46. Girardin F. Membrane transporter proteins: a challenge for CNS drug development. Dialogues Clin Neurosci. 2006;8(3):311-21.

47. de Lange EC. Potential role of ABC transporters as a detoxification system at the blood-CSF barrier. Adv Drug Deliv Rev. 2004;56(12):1793-809.

48. Bart J, Hollema H, Groen HJ, de Vries EG, Hendrikse NH, Sleijfer DT, et al. The distribution of drug-efflux pumps, P-gp, BCRP, MRP1 and MRP2, in the normal blood-testis barrier and in primary testicular tumours. Eur J Cancer. 2004;40(14):2064-70.

49. Behravan J, Piquette-Miller M. Drug transport across the placenta, role of the ABC drug efflux transporters. Expert Opin Drug Metab Toxicol. 2007;3(6):819-30.

50. Joshi AA, Vaidya SS, St-Pierre MV, Mikheev AM, Desino KE, Nyandege AN, et al. Placental ABC Transporters: Biological Impact and Pharmaceutical Significance. Pharm Res. 2016;33(12):2847-78.

51. Fromm MF. Importance of P-glycoprotein at blood-tissue barriers. Trends Pharmacol Sci. 2004;25(8):423-9.

52. Mizutani T, Nakamura T, Morikawa R, Fukuda M, Mochizuki W, Yamauchi Y, et al. Real-time analysis of P-glycoprotein-mediated drug transport across primary intestinal epithelium three-dimensionally cultured in vitro. Biochem Biophys Res Commun. 2012;419(2):238-43.

53. Fetsch PA, Abati A, Litman T, Morisaki K, Honjo Y, Mittal K, et al. Localization of the ABCG2 mitoxantrone resistance-associated protein in normal tissues. Cancer Lett. 2006;235(1):84-92.

54. Kock K, Brouwer KL. A perspective on efflux transport proteins in the liver. Clin Pharmacol Ther. 2012;92(5):599-612.

55. Jonker JW, Stedman CA, Liddle C, Downes M. Hepatobiliary ABC transporters: physiology, regulation and implications for disease. Front Biosci (Landmark Ed). 2009;14:4904-20.

56. Inui KI, Masuda S, Saito H. Cellular and molecular aspects of drug transport in the kidney. Kidney Int. 2000;58(3):944-58.

57. Mottino AD, Catania VA. Hepatic drug transporters and nuclear receptors: regulation by therapeutic agents. World J Gastroenterol. 2008;14(46):7068-74.

58. DeGorter MK, Xia CQ, Yang JJ, Kim RB. Drug transporters in drug efficacy and toxicity. Annu Rev Pharmacol Toxicol. 2012;52:249-73.

59. Meyer zu Schwabedissen HE, Kroemer HK. In vitro and in vivo evidence for the importance of breast cancer resistance protein transporters (BCRP/MXR/ABCP/ABCG2). Handb Exp Pharmacol. 2011(201):325-71.

60. Zamek-Gliszczynski MJ, Hoffmaster KA, Tweedie DJ, Giacomini KM, Hillgren KM. Highlights from the International Transporter Consortium second workshop. Clin Pharmacol Ther. 2012;92(5):553-6.

61. Delou JM, Lopes AG, Capella MA. Unveiling the role of multidrug resistance proteins in hypertension. Hypertension. 2009;54(2):210-6.

62. Bodo A, Bakos E, Szeri F, Varadi A, Sarkadi B. The role of multidrug transporters in drug availability, metabolism and toxicity. Toxicol Lett. 2003;140-141:133-43.

63. Loscher W, Potschka H. Blood-brain barrier active efflux transporters: ATP-binding cassette gene family. NeuroRx. 2005;2(1):86-98.

64. Chaves C, Shawahna R, Jacob A, Scherrmann JM, Decleves X. Human ABC transporters at blood-CNS interfaces as determinants of CNS drug penetration. Curr Pharm Des. 2014;20(10):1450-62.

65. Pang KS. Modeling of intestinal drug absorption: roles of transporters and metabolic enzymes (for the Gillette Review Series). Drug Metab Dispos. 2003;31(12):1507-19.

66. Varma MV, Ambler CM, Ullah M, Rotter CJ, Sun H, Litchfield J, et al. Targeting intestinal transporters for optimizing oral drug absorption. Curr Drug Metab. 2010;11(9):730-42.

67. Estudante M, Morais JG, Soveral G, Benet LZ. Intestinal drug transporters: an overview. Adv Drug Deliv Rev. 2013;65(10):1340-56.

68. Tucker TG, Milne AM, Fournel-Gigleux S, Fenner KS, Coughtrie MW. Absolute immunoquantification of the expression of ABC transporters P-glycoprotein, breast cancer resistance protein and multidrug resistance-associated protein 2 in human liver and duodenum. Biochem Pharmacol. 2012;83(2):279-85.

69. Doherty MM, Charman WN. The mucosa of the small intestine: how clinically relevant as an organ of drug metabolism? Clin Pharmacokinet. 2002;41(4):235-53.

70. Bruyere A, Decleves X, Bouzom F, Ball K, Marques C, Treton X, et al. Effect of variations in the amounts of P-glycoprotein (ABCB1), BCRP (ABCG2) and CYP3A4 along the human small intestine on PBPK models for predicting intestinal first pass. Mol Pharm. 2010;7(5):1596-607.

71. Taipalensuu J, Tornblom H, Lindberg G, Einarsson C, Sjoqvist F, Melhus H, et al. Correlation of gene expression of ten drug efflux proteins of the ATP-binding cassette transporter family in normal human jejunum and in human intestinal epithelial Caco-2 cell monolayers. J Pharmacol Exp Ther. 2001;299(1):164-70.

72. Ueda K, Taguchi Y, Morishima M. How does P-glycoprotein recognize its substrates? Semin Cancer Biol. 1997;8(3):151-9.

73. Wang RB, Kuo CL, Lien LL, Lien EJ. Structure-activity relationship: analyses of p-glycoprotein substrates and inhibitors. J Clin Pharm Ther. 2003;28(3):203-28.

74. Seelig A. A general pattern for substrate recognition by P-glycoprotein. Eur J Biochem. 1998;251(1-2):252-61.

75. Seelig A, Landwojtowicz E. Structure-activity relationship of P-glycoprotein substrates and modifiers. Eur J Pharm Sci. 2000;12(1):31-40.

76. Romsicki Y, Sharom FJ. Phospholipid flippase activity of the reconstituted P-glycoprotein multidrug transporter. Biochemistry. 2001;40(23):6937-47.

77. Eckford PD, Sharom FJ. P-glycoprotein (ABCB1) interacts directly with lipid-based anti-cancer drugs and platelet-activating factors. Biochem Cell Biol. 2006;84(6):1022-33.

78. Ernest S, Bello-Reuss E. Secretion of platelet-activating factor is mediated by MDR1 P-glycoprotein in cultured human mesangial cells. J Am Soc Nephrol. 1999;10(11):2306-13.

79. Bello-Reuss E, Ernest S, Holland OB, Hellmich MR. Role of multidrug resistance P-glycoprotein in the secretion of aldosterone by human adrenal NCI-H295 cells. Am J Physiol Cell Physiol. 2000;278(6):C1256-65.

80. Liu Y, Huang L, Hoffman T, Gosland M, Vore M. MDR1 substrates/modulators protect against beta-estradiol-17beta-D-glucuronide cholestasis in rat liver. Cancer Res. 1996;56(21):4992-7.

81. Lam FC, Liu R, Lu P, Shapiro AB, Renoir JM, Sharom FJ, et al. beta-Amyloid efflux mediated by p-glycoprotein. J Neurochem. 2001;76(4):1121-8.

82. Pawlik A, Baskiewicz-Masiuk M, Machalinski B, Safranow K, Gawronska-Szklarz B. Involvement of P-glycoprotein in the release of cytokines from peripheral blood mononuclear cells treated with methotrexate and dexamethasone. J Pharm Pharmacol. 2005;57(11):1421-5.

83. Matheny CJ, Lamb MW, Brouwer KR, Pollack GM. Pharmacokinetic and pharmacodynamic implications of P-glycoprotein modulation. Pharmacotherapy. 2001;21(7):778-96.

84. Sun Z, Shen B, Zhang J, Su T, Dong P. [The effect of MDR1 (P-gp) and ABCG2 on the drug resistance in Hep 2 cells]. Lin Chung Er Bi Yan Hou Tou Jing Wai Ke Za Zhi. 2015;29(11):1016-9.

85. Ishikawa T, Tamura A, Saito H, Wakabayashi K, Nakagawa H. Pharmacogenomics of the human ABC transporter ABCG2: from functional evaluation to drug molecular design. Naturwissenschaften. 2005;92(10):451-63.

86. Robert J, Jarry C. Multidrug resistance reversal agents. J Med Chem. 2003;46(23):4805-17.

87. Litman T, Brangi M, Hudson E, Fetsch P, Abati A, Ross DD, et al. The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2). J Cell Sci. 2000;113 (Pt 11):2011-21.

88. Nakatomi K, Yoshikawa M, Oka M, Ikegami Y, Hayasaka S, Sano K, et al. Transport of 7-ethyl-10-hydroxycamptothecin (SN-38) by breast cancer resistance protein ABCG2 in human lung cancer cells. Biochem Biophys Res Commun. 2001;288(4):827-32.

89. Chen ZS, Robey RW, Belinsky MG, Shchaveleva I, Ren XQ, Sugimoto Y, et al. Transport of methotrexate, methotrexate polyglutamates, and 17beta-estradiol 17-(beta-D-glucuronide) by ABCG2: effects of acquired mutations at R482 on methotrexate transport. Cancer Res. 2003;63(14):4048-54.

90. Volk EL, Farley KM, Wu Y, Li F, Robey RW, Schneider E. Overexpression of wild-type breast cancer resistance protein mediates methotrexate resistance. Cancer Res. 2002;62(17):5035-40.

91. Elkind NB, Szentpetery Z, Apati A, Ozvegy-Laczka C, Varady G, Ujhelly O, et al. Multidrug transporter ABCG2 prevents tumor cell death induced by the epidermal growth factor receptor inhibitor Iressa (ZD1839, Gefitinib). Cancer Res. 2005;65(5):1770-7.

92. Burger H, van Tol H, Boersma AW, Brok M, Wiemer EA, Stoter G, et al. Imatinib mesylate (STI571) is a substrate for the breast cancer resistance protein (BCRP)/ABCG2 drug pump. Blood. 2004;104(9):2940-2.

93. Kitamura S, Maeda K, Wang Y, Sugiyama Y. Involvement of multiple transporters in the hepatobiliary transport of rosuvastatin. Drug Metab Dispos. 2008;36(10):2014-23.

94. van der Heijden J, de Jong MC, Dijkmans BA, Lems WF, Oerlemans R, Kathmann I, et al. Development of sulfasalazine resistance in human T cells induces expression of the multidrug resistance transporter ABCG2 (BCRP) and augmented production of TNFalpha. Ann Rheum Dis. 2004;63(2):138-43.

95. Pavek P, Merino G, Wagenaar E, Bolscher E, Novotna M, Jonker JW, et al. Human breast cancer resistance protein: interactions with steroid drugs, hormones, the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, and transport of cimetidine. J Pharmacol Exp Ther. 2005;312(1):144-52.

96. Nakagawa R, Hara Y, Arakawa H, Nishimura S, Komatani H. ABCG2 confers resistance to indolocarbazole compounds by ATP-dependent transport. Biochem Biophys Res Commun. 2002;299(4):669-75.

97. Breedveld P, Zelcer N, Pluim D, Sonmezer O, Tibben MM, Beijnen JH, et al. Mechanism of the pharmacokinetic interaction between methotrexate and benzimidazoles: potential role for breast cancer resistance protein in clinical drug-drug interactions. Cancer Res. 2004;64(16):5804-11.

98. Robey RW, Medina-Perez WY, Nishiyama K, Lahusen T, Miyake K, Litman T, et al. Overexpression of the ATP-binding cassette half-transporter, ABCG2 (Mxr/BCrp/ABCP1), in flavopiridol-resistant human breast cancer cells. Clin Cancer Res. 2001;7(1):145-52.

99. Merino G, Jonker JW, Wagenaar E, van Herwaarden AE, Schinkel AH. The breast cancer resistance protein (BCRP/ABCG2) affects pharmacokinetics, hepatobiliary excretion, and milk secretion of the antibiotic nitrofurantoin. Mol Pharmacol. 2005;67(5):1758-64.

100. Brendel C, Scharenberg C, Dohse M, Robey RW, Bates SE, Shukla S, et al. Imatinib mesylate and nilotinib (AMN107) exhibit high-affinity interaction with ABCG2 on primitive hematopoietic stem cells. Leukemia. 2007;21(6):1267-75.

101. Erlichman C, Boerner SA, Hallgren CG, Spieker R, Wang XY, James CD, et al. The HER tyrosine kinase inhibitor CI1033 enhances cytotoxicity of 7-ethyl-10-hydroxycamptothecin and topotecan by inhibiting breast cancer resistance protein-mediated drug efflux. Cancer Res. 2001;61(2):739-48.

102. Doyle L, Ross DD. Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). Oncogene. 2003;22(47):7340-58.

103. Rigalli JP, Scholz PN, Tocchetti GN, Ruiz ML, Weiss J. The phytoestrogens daidzein and equol inhibit the drug transporter BCRP/ABCG2 in breast cancer cells: potential chemosensitizing effect. Eur J Nutr. 2017.

104. Linton KJ. Structure and function of ABC transporters. Physiology (Bethesda). 2007;22:122-30.

105. Hopfner KP, Karcher A, Shin DS, Craig L, Arthur LM, Carney JP, et al. Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. Cell. 2000;101(7):789-800.

106. Chang G. Structure of MsbA from Vibrio cholera: a multidrug resistance ABC transporter homolog in a closed conformation. J Mol Biol. 2003;330(2):419-30.

107. Locher KP, Lee AT, Rees DC. The E. coli BtuCD structure: a framework for ABC transporter architecture and mechanism. Science. 2002;296(5570):1091-8.

108. Deeley RG, Westlake C, Cole SP. Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. Physiol Rev. 2006;86(3):849-99.

109. Higgins CF, Linton KJ. The ATP switch model for ABC transporters. Nat Struct Mol Biol. 2004;11(10):918-26.

110. Sharom FJ, Yu X, Chu JW, Doige CA. Characterization of the ATPase activity of P-glycoprotein from multidrug-resistant Chinese hamster ovary cells. Biochem J. 1995;308 (Pt 2):381-90.

111. Al-Shawi MK, Polar MK, Omote H, Figler RA. Transition state analysis of the coupling of drug transport to ATP hydrolysis by P-glycoprotein. J Biol Chem. 2003;278(52):52629-40.

112. Senior AE, al-Shawi MK, Urbatsch IL. The catalytic cycle of P-glycoprotein. FEBS Lett. 1995;377(3):285-9.

113. Ambudkar SV, Lelong IH, Zhang J, Cardarelli CO, Gottesman MM, Pastan I. Partial purification and reconstitution of the human multidrug-resistance pump: characterization of the drug-stimulatable ATP hydrolysis. Proc Natl Acad Sci U S A. 1992;89(18):8472-6.

114. Litman T, Zeuthen T, Skovsgaard T, Stein WD. Structure-activity relationships of P-glycoprotein interacting drugs: kinetic characterization of their effects on ATPase activity. Biochim Biophys Acta. 1997;1361(2):159-68.

115. Frelet A, Klein M. Insight in eukaryotic ABC transporter function by mutation analysis. FEBS Lett. 2006;580(4):1064-84.

116. Nobili S, Landini I, Giglioni B, Mini E. Pharmacological strategies for overcoming multidrug resistance. Curr Drug Targets. 2006;7(7):861-79.

117. Perez-Tomas R. Multidrug resistance: retrospect and prospects in anti-cancer drug treatment. Curr Med Chem. 2006;13(16):1859-76.

118. Sauna ZE, Ambudkar SV. Characterization of the catalytic cycle of ATP hydrolysis by human P-glycoprotein. The two ATP hydrolysis events in a single catalytic cycle are kinetically similar but affect different functional outcomes. J Biol Chem. 2001;276(15):11653-61.

119. Martin C, Higgins CF, Callaghan R. The vinblastine binding site adopts high- and low-affinity conformations during a transport cycle of P-glycoprotein. Biochemistry. 2001;40(51):15733-42.

120. Rosenberg MF, Velarde G, Ford RC, Martin C, Berridge G, Kerr ID, et al. Repacking of the transmembrane domains of P-glycoprotein during the transport ATPase cycle. EMBO J. 2001;20(20):5615-25.

121. Kim Y, Chen J. Molecular structure of human P-glycoprotein in the ATP-bound, outward-facing conformation. Science. 2018;359(6378):915-9.

122. Mao Q, Conseil G, Gupta A, Cole SP, Unadkat JD. Functional expression of the human breast cancer resistance protein in Pichia pastoris. Biochem Biophys Res Commun. 2004;320(3):730-7.

123. Ozvegy C, Varadi A, Sarkadi B. Characterization of drug transport, ATP hydrolysis, and nucleotide trapping by the human ABCG2 multidrug transporter. Modulation of substrate specificity by a point mutation. J Biol Chem. 2002;277(50):47980-90.

124. McDevitt CA, Crowley E, Hobbs G, Starr KJ, Kerr ID, Callaghan R. Is ATP binding responsible for initiating drug translocation by the multidrug transporter ABCG2? FEBS J. 2008;275(17):4354-62.

125. Raggers RJ, Vogels I, van Meer G. Multidrug-resistance P-glycoprotein (MDR1) secretes platelet-activating factor. Biochem J. 2001;357(Pt 3):859-65.

126. Chen Z, Shi T, Zhang L, Zhu P, Deng M, Huang C, et al. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family in multidrug resistance: A review of the past decade. Cancer Lett. 2016;370(1):153-64.

127. Pauwels EK, Erba P, Mariani G, Gomes CM. Multidrug resistance in cancer: its mechanism and its modulation. Drug News Perspect. 2007;20(6):371-7.

128. Lehnert M. Clinical multidrug resistance in cancer: a multifactorial problem. Eur J Cancer. 1996;32A(6):912-20.

129. Wu CP, Calcagno AM, Ambudkar SV. Reversal of ABC drug transporter-mediated multidrug resistance in cancer cells: evaluation of current strategies. Curr Mol Pharmacol. 2008;1(2):93-105.

130. Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. Nat Rev Drug Discov. 2006;5(3):219-34.

131. Merino V, Jimenez-Torres NV, Merino-Sanjuan M. Relevance of multidrug resistance proteins on the clinical efficacy of cancer therapy. Curr Drug Deliv. 2004;1(3):203-12.

132. Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y. Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Res. 1981;41(5):1967-72.

133. Palmeira A, Sousa E, Vasconcelos MH, Pinto MM. Three decades of P-gp inhibitors: skimming through several generations and scaffolds. Curr Med Chem. 2012;19(13):1946-2025.

134. Silva R, Palmeira A, Carmo H, Barbosa DJ, Gameiro M, Gomes A, et al. P-glycoprotein induction in Caco-2 cells by newly synthetized thioxanthones prevents paraquat cytotoxicity. Arch Toxicol. 2015;89(10):1783-800.

135. Silva R, Carmo H, Dinis-Oliveira R, Cordeiro-da-Silva A, Lima SC, Carvalho F, et al. In vitro study of P-glycoprotein induction as an antidotal pathway to prevent cytotoxicity in Caco-2 cells. Arch Toxicol. 2011;85(4):315-26.

136. Silva R, Sousa E, Carmo H, Palmeira A, Barbosa DJ, Gameiro M, et al. Induction and activation of P-glycoprotein by dihydroxylated xanthones protect against the cytotoxicity of the P-glycoprotein substrate paraquat. Arch Toxicol. 2014;88(4):937-51.

137. Wessler JD, Grip LT, Mendell J, Giugliano RP. The P-glycoprotein transport system and cardiovascular drugs. J Am Coll Cardiol. 2013;61(25):2495-502.

138. Varma MV, Ashokraj Y, Dey CS, Panchagnula R. P-glycoprotein inhibitors and their screening: a perspective from bioavailability enhancement. Pharmacol Res. 2003;48(4):347-59.

139. Shapiro AB, Ling V. Effect of quercetin on Hoechst 33342 transport by purified and reconstituted P-glycoprotein. Biochem Pharmacol. 1997;53(4):587-96.

140. Drori S, Eytan GD, Assaraf YG. Potentiation of anticancer-drug cytotoxicity by multidrug-resistance chemosensitizers involves alterations in membrane fluidity leading to increased membrane permeability. Eur J Biochem. 1995;228(3):1020-9.

141. Ruetz S, Gros P. Phosphatidylcholine translocase: a physiological role for the mdr2 gene. Cell. 1994;77(7):1071-81.

142. Lage H. An overview of cancer multidrug resistance: a still unsolved problem. Cell Mol Life Sci. 2008;65(20):3145-67.

143. Callaghan R, Crowley E, Potter S, Kerr ID. P-glycoprotein: so many ways to turn it on. J Clin Pharmacol. 2008;48(3):365-78.

144. Higgins CF, Callaghan R, Linton KJ, Rosenberg MF, Ford RC. Structure of the multidrug resistance P-glycoprotein. Semin Cancer Biol. 1997;8(3):135-42.

145. Krishna R, Mayer LD. Multidrug resistance (MDR) in cancer. Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. Eur J Pharm Sci. 2000;11(4):265-83.

146. Wilson WH, Jamis-Dow C, Bryant G, Balis FM, Klecker RW, Bates SE, et al. Phase I and pharmacokinetic study of the multidrug resistance modulator dexverapamil with EPOCH chemotherapy. J Clin Oncol. 1995;13(8):1985-94.

147. Yanagisawa T, Newman A, Coley H, Renshaw J, Pinkerton CR, Pritchard-Jones K. BIRICODAR (VX-710; Incel): an effective chemosensitizer in neuroblastoma. Br J Cancer. 1999;80(8):1190-6.

148. Calcagno AM, Kim IW, Wu CP, Shukla S, Ambudkar SV. ABC drug transporters as molecular targets for the prevention of multidrug resistance and drug-drug interactions. Curr Drug Deliv. 2007;4(4):324-33.

149. Sharma R, Awasthi YC, Yang Y, Sharma A, Singhal SS, Awasthi S. Energy dependent transport of xenobiotics and its relevance to multidrug resistance. Curr Cancer Drug Targets. 2003;3(2):89-107.

150. Thomas H, Coley HM. Overcoming multidrug resistance in cancer: an update on the clinical strategy of inhibiting p-glycoprotein. Cancer Control. 2003;10(2):159-65.

151. Coley HM. Overcoming multidrug resistance in cancer: clinical studies of p-glycoprotein inhibitors. Methods Mol Biol. 2010;596:341-58.

152. Hugger ED, Novak BL, Burton PS, Audus KL, Borchardt RT. A comparison of commonly used polyethoxylated pharmaceutical excipients on their ability to inhibit P-glycoprotein activity in vitro. J Pharm Sci. 2002;91(9):1991-2002.

153. Vilas-Boas V, Silva R, Nunes C, Reis S, Ferreira L, Vieira C, et al. Mechanisms of P-gp inhibition and effects on membrane fluidity of a new rifampicin derivative, 1,8-dibenzoyl-rifampicin. Toxicol Lett. 2013;220(3):259-66.

154. Pick A, Klinkhammer W, Wiese M. Specific inhibitors of the breast cancer resistance protein (BCRP). ChemMedChem. 2010;5(9):1498-505.

155. Allen JD, van Loevezijn A, Lakhai JM, van der Valk M, van Tellingen O, Reid G, et al. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. Mol Cancer Ther. 2002;1(6):417-25.

156. Shiozawa K, Oka M, Soda H, Yoshikawa M, Ikegami Y, Tsurutani J, et al. Reversal of breast cancer resistance protein (BCRP/ABCG2)-mediated drug resistance by novobiocin, a coumermycin antibiotic. Int J Cancer. 2004;108(1):146-51.

157. Maliepaard M, van Gastelen MA, Tohgo A, Hausheer FH, van Waardenburg RC, de Jong LA, et al. Circumvention of breast cancer resistance protein (BCRP)-mediated resistance to camptothecins in vitro using non-substrate drugs or the BCRP inhibitor GF120918. Clin Cancer Res. 2001;7(4):935-41.

158. de Bruin M, Miyake K, Litman T, Robey R, Bates SE. Reversal of resistance by GF120918 in cell lines expressing the ABC half-transporter, MXR. Cancer Lett. 1999;146(2):117-26.

159. Mao Q, Unadkat JD. Role of the breast cancer resistance protein (ABCG2) in drug transport. AAPS J. 2005;7(1):E118-33.

160. Cooray HC, Janvilisri T, van Veen HW, Hladky SB, Barrand MA. Interaction of the breast cancer resistance protein with plant polyphenols. Biochem Biophys Res Commun. 2004;317(1):269-75.

161. Ahmed-Belkacem A, Pozza A, Munoz-Martinez F, Bates SE, Castanys S, Gamarro F, et al. Flavonoid structure-activity studies identify 6-prenylchrysin and tectochrysin as potent and specific inhibitors of breast cancer resistance protein ABCG2. Cancer Res. 2005;65(11):4852-60.

162. Yanase K, Tsukahara S, Asada S, Ishikawa E, Imai Y, Sugimoto Y. Gefitinib reverses breast cancer resistance protein-mediated drug resistance. Mol Cancer Ther. 2004;3(9):1119-25.

163. Ozvegy-Laczka C, Cserepes J, Elkind NB, Sarkadi B. Tyrosine kinase inhibitor resistance in cancer: role of ABC multidrug transporters. Drug Resist Updat. 2005;8(1-2):15-26.

164. Hegedus T, Orfi L, Seprodi A, Varadi A, Sarkadi B, Keri G. Interaction of tyrosine kinase inhibitors with the human multidrug transporter proteins, MDR1 and MRP1. Biochim Biophys Acta. 2002;1587(2-3):318-25.

165. Shukla S, Sauna ZE, Ambudkar SV. Evidence for the interaction of imatinib at the transport-substrate site(s) of the multidrug-resistance-linked ABC drug transporters ABCB1 (P-glycoprotein) and ABCG2. Leukemia. 2008;22(2):445-7.

166. Allen JD, Van Dort SC, Buitelaar M, van Tellingen O, Schinkel AH. Mouse breast cancer resistance protein (Bcrp1/Abcg2) mediates etoposide resistance and transport, but etoposide oral availability is limited primarily by P-glycoprotein. Cancer Res. 2003;63(6):1339-44.

167. Mahringer A, Fricker G. ABC transporters at the blood-brain barrier. Expert Opin Drug Metab Toxicol. 2016;12(5):499-508.

168. Farabegoli F, Papi A, Bartolini G, Ostan R, Orlandi M. (-)-Epigallocatechin-3-gallate downregulates Pg-P and BCRP in a tamoxifen resistant MCF-7 cell line. Phytomedicine. 2010;17(5):356-62.

169. Klaassen CD, Slitt AL. Regulation of hepatic transporters by xenobiotic receptors. Curr Drug Metab. 2005;6(4):309-28.

170. Urquhart BL, Tirona RG, Kim RB. Nuclear receptors and the regulation of drug-metabolizing enzymes and drug transporters: implications for interindividual variability in response to drugs. J Clin Pharmacol. 2007;47(5):566-78.

171. Nagy L, Schule R, Gronemeyer H. Twenty years of nuclear receptors: Conference on Nuclear Receptors: from Chromatin to Disease. EMBO Rep. 2006;7(6):579-84.

172. Huang P, Chandra V, Rastinejad F. Structural overview of the nuclear receptor superfamily: insights into physiology and therapeutics. Annu Rev Physiol. 2010;72:247-72.

173. Borst P, Elferink RO. Mammalian ABC transporters in health and disease. Annu Rev Biochem. 2002;71:537-92.

174. Nawa A, Fujita-Hamabe W, Tokuyama S. Altered intestinal P-glycoprotein expression levels in a monosodium glutamate-induced obese mouse model. Life Sci. 2011;89(23-24):834-8.

175. Tada Y, Wada M, Kuroiwa K, Kinugawa N, Harada T, Nagayama J, et al. MDR1 gene overexpression and altered degree of methylation at the promoter region in bladder cancer during chemotherapeutic treatment. Clin Cancer Res. 2000;6(12):4618-27.

176. Hartz AM, Miller DS, Bauer B. Restoring blood-brain barrier P-glycoprotein reduces brain amyloid-beta in a mouse model of Alzheimer's disease. Mol Pharmacol. 2010;77(5):715-23.

177. Fojo AT, Whang-Peng J, Gottesman MM, Pastan I. Amplification of DNA sequences in human multidrug-resistant KB carcinoma cells. Proc Natl Acad Sci U S A. 1985;82(22):7661-5.

178. Roninson IB. From amplification to function: the case of the MDR1 gene. Mutat Res. 1992;276(3):151-61.

179. Scotto KW. Transcriptional regulation of ABC drug transporters. Oncogene. 2003;22(47):7496-511.

180. Labialle S, Gayet L, Marthinet E, Rigal D, Baggetto LG. Transcriptional regulators of the human multidrug resistance 1 gene: recent views. Biochem Pharmacol. 2002;64(5-6):943-8.

181. Haslam IS, Jones K, Coleman T, Simmons NL. Induction of P-glycoprotein expression and function in human intestinal epithelial cells (T84). Biochem Pharmacol. 2008;76(7):850-61.

182. Miller DS. Regulation of P-glycoprotein and other ABC drug transporters at the blood-brain barrier. Trends Pharmacol Sci. 2010;31(6):246-54.

183. Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, et al. An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. Cell. 1998;92(1):73-82.

184. Geick A, Eichelbaum M, Burk O. Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. J Biol Chem. 2001;276(18):14581-7.

185. Greiner B, Eichelbaum M, Fritz P, Kreichgauer HP, von Richter O, Zundler J, et al. The role of intestinal P-glycoprotein in the interaction of digoxin and rifampin. J Clin Invest. 1999;104(2):147-53.

186. Owen A, Goldring C, Morgan P, Park BK, Pirmohamed M. Induction of P-glycoprotein in lymphocytes by carbamazepine and rifampicin: the role of nuclear hormone response elements. Br J Clin Pharmacol. 2006;62(2):237-42.

187. Magnarin M, Morelli M, Rosati A, Bartoli F, Candussio L, Giraldi T, et al. Induction of proteins involved in multidrug resistance (P-glycoprotein, MRP1, MRP2, LRP) and of CYP 3A4 by rifampicin in LLC-PK1 cells. Eur J Pharmacol. 2004;483(1):19-28.

188. Martin P, Riley R, Thompson P, Williams D, Back D, Owen A. Effect of prototypical inducers on ligand activated nuclear receptor regulated drug disposition genes in rodent hepatic and intestinal cells. Acta Pharmacol Sin. 2010;31(1):51-65.

189. Vilas-Boas V, Silva R, Palmeira A, Sousa E, Ferreira LM, Branco PS, et al. Development of novel rifampicin-derived P-glycoprotein activators/inducers. synthesis, in silico analysis and application in the RBE4 cell model, using paraquat as substrate. PLoS One. 2013;8(8):e74425.

190. Salje K, Lederer K, Oswald S, Dazert E, Warzok R, Siegmund W. Effects of rifampicin, dexamethasone, St. John's Wort and Thyroxine on maternal and foetal expression of Abcb1 and organ distribution of talinolol in pregnant rats. Basic Clin Pharmacol Toxicol. 2012;111(2):99-105.

191. Arnold KA, Eichelbaum M, Burk O. Alternative splicing affects the function and tissue-specific expression of the human constitutive androstane receptor. Nucl Recept. 2004;2(1):1.

192. Burk O, Arnold KA, Geick A, Tegude H, Eichelbaum M. A role for constitutive androstane receptor in the regulation of human intestinal MDR1 expression. Biol Chem. 2005;386(6):503-13.

193. Natarajan K, Xie Y, Baer MR, Ross DD. Role of breast cancer resistance protein (BCRP/ABCG2) in cancer drug resistance. Biochem Pharmacol. 2012;83(8):1084-103.

194. sBailey-Dell KJ, Hassel B, Doyle LA, Ross DD. Promoter characterization and genomic organization of the human breast cancer resistance protein (ATP-binding cassette transporter G2) gene. Biochim Biophys Acta. 2001;1520(3):234-41.

195. Krishnamurthy P, Ross DD, Nakanishi T, Bailey-Dell K, Zhou S, Mercer KE, et al. The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. J Biol Chem. 2004;279(23):24218-25.

196. Ee PL, Kamalakaran S, Tonetti D, He X, Ross DD, Beck WT. Identification of a novel estrogen response element in the breast cancer resistance protein (ABCG2) gene. Cancer Res. 2004;64(4):1247-51.

197. Wang H, Lee EW, Zhou L, Leung PC, Ross DD, Unadkat JD, et al. Progesterone receptor (PR) isoforms PRA and PRB differentially regulate expression of the breast cancer resistance protein in human placental choriocarcinoma BeWo cells. Mol Pharmacol. 2008;73(3):845-54.

198. To KK, Robey R, Zhan Z, Bangiolo L, Bates SE. Upregulation of ABCG2 by romidepsin via the aryl hydrocarbon receptor pathway. Mol Cancer Res. 2011;9(4):516-27.

199. Szatmari I, Vamosi G, Brazda P, Balint BL, Benko S, Szeles L, et al. Peroxisome proliferator-activated receptor gamma-regulated ABCG2 expression confers cytoprotection to human dendritic cells. J Biol Chem. 2006;281(33):23812-23.

200. Singh A, Wu H, Zhang P, Happel C, Ma J, Biswal S. Expression of ABCG2 (BCRP) is regulated by Nrf2 in cancer cells that confers side population and chemoresistance phenotype. Mol Cancer Ther. 2010;9(8):2365-76.

201. Badolo L, Jensen B, Sall C, Norinder U, Kallunki P, Montanari D. Evaluation of 309 molecules as inducers of CYP3A4, CYP2B6, CYP1A2, OATP1B1, OCT1, MDR1, MRP2, MRP3 and BCRP in cryopreserved human hepatocytes in sandwich culture. Xenobiotica. 2015;45(2):177-87.

202. Jigorel E, Le Vee M, Boursier-Neyret C, Parmentier Y, Fardel O. Differential regulation of sinusoidal and canalicular hepatic drug transporter expression by xenobiotics activating drug-sensing receptors in primary human hepatocytes. Drug Metab Dispos. 2006;34(10):1756-63.

203. Kaur M, Badhan RK. Phytochemical mediated-modulation of the expression and transporter function of breast cancer resistance protein at the blood-brain barrier: An in-vitro study. Brain Res. 2017;1654(Pt A):9-23.

204. Ebert B, Seidel A, Lampen A. Identification of BCRP as transporter of benzo[a]pyrene conjugates metabolically formed in Caco-2 cells and its induction by Ah-receptor agonists. Carcinogenesis. 2005;26(10):1754-63.

205. Burger H, van Tol H, Brok M, Wiemer EA, de Bruijn EA, Guetens G, et al. Chronic imatinib mesylate exposure leads to reduced intracellular drug accumulation by induction of the ABCG2 (BCRP) and ABCB1 (MDR1) drug transport pumps. Cancer Biol Ther. 2005;4(7):747-52.

206. Tompkins LM, Li H, Li L, Lynch C, Xie Y, Nakanishi T, et al. A novel xenobiotic responsive element regulated by aryl hydrocarbon receptor is involved in the induction of BCRP/ABCG2 in LS174T cells. Biochem Pharmacol. 2010;80(11):1754-61.

207. Ebert B, Seidel A, Lampen A. Phytochemicals induce breast cancer resistance protein in Caco-2 cells and enhance the transport of benzo[a]pyrene-3-sulfate. Toxicol Sci. 2007;96(2):227-36.

208. Sterz K, Mollmann L, Jacobs A, Baumert D, Wiese M. Activators of P-glycoprotein: Structure-activity relationships and investigation of their mode of action. ChemMedChem. 2009;4(11):1897-911.

209. Silva R, Carmo H, Vilas-Boas V, Barbosa DJ, Palmeira A, Sousa E, et al. Hypericin-mediated P-glycoprotein induction protects caco-2 cells against paraquat toxicity: In vitro and in silico studies. Toxicology Letters. 2015;238S S317.

210. Silva R, Carmo H, Vilas-Boas V, de Pinho PG, Dinis-Oliveira RJ, Carvalho F, et al. Doxorubicin decreases paraquat accumulation and toxicity in Caco-2 cells. Toxicol Lett. 2013;217(1):34-41.

211. Shapiro AB, Ling V. Positively cooperative sites for drug transport by P-glycoprotein with distinct drug specificities. Eur J Biochem. 1997;250(1):130-7.

212. Shapiro AB, Ling V. The mechanism of ATP-dependent multidrug transport by P-glycoprotein. Acta Physiol Scand Suppl. 1998;643:227-34.

213. Shapiro AB, Fox K, Lam P, Ling V. Stimulation of P-glycoprotein-mediated drug transport by prazosin and progesterone. Evidence for a third drug-binding site. Eur J Biochem. 1999;259(3):841-50.

214. Kondratov RV, Komarov PG, Becker Y, Ewenson A, Gudkov AV. Small molecules that dramatically alter multidrug resistance phenotype by modulating the substrate specificity of P-glycoprotein. Proc Natl Acad Sci U S A. 2001;98(24):14078-83.

215. Lopes A, Martins E, Silva R, Pinto MMM, Remiao F, Sousa E, et al. Chiral Thioxanthones as Modulators of P-glycoprotein: Synthesis and Enantioselectivity Studies. Molecules. 2018;23(3).

216. Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I, Willingham MC. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. Proc Natl Acad Sci U S A. 1987;84(21):7735-8.

217. Kerb R. Implications of genetic polymorphisms in drug transporters for pharmacotherapy. Cancer Lett. 2006;234(1):4-33.

218. Chen P, Chen H, Zang X, Chen M, Jiang H, Han S, et al. Expression of efflux transporters in human ocular tissues. Drug Metab Dispos. 2013;41(11):1934-48.

219. Murakami T, Takano M. Intestinal efflux transporters and drug absorption. Expert Opin Drug Metab Toxicol. 2008;4(7):923-39.

220. Dinis-Oliveira RJ, Remiao F, Duarte JA, Ferreira R, Sanchez Navarro A, Bastos ML, et al. P-glycoprotein induction: an antidotal pathway for paraquat-induced lung toxicity. Free Radic Biol Med. 2006;41(8):1213-24.

221. Dinis-Oliveira RJ, Duarte JA, Remiao F, Sanchez-Navarro A, Bastos ML, Carvalho F. Single high dose dexamethasone treatment decreases the pathological score and increases the survival rate of paraquat-intoxicated rats. Toxicology. 2006;227(1-2):73-85.

222. Arias A, Rigalli JP, Villanueva SS, Ruiz ML, Luquita MG, Perdomo VG, et al. Regulation of expression and activity of multidrug resistance proteins MRP2 and MDR1 by estrogenic compounds in Caco-2 cells. Role in prevention of xenobiotic-induced cytotoxicity. Toxicology. 2014;320:46-55.

223. Zerin T, Kim YS, Hong SY, Song HY. Protective effect of methylprednisolone on paraquat-induced A549 cell cytotoxicity via induction of efflux transporter, P-glycoprotein expression. Toxicol Lett. 2012;208(2):101-7.

224. Masters KS, Brase S. Xanthones from fungi, lichens, and bacteria: the natural products and their synthesis. Chem Rev. 2012;112(7):3717-76.

225. Pinto MM, Sousa ME, Nascimento MS. Xanthone derivatives: new insights in biological activities. Curr Med Chem. 2005;12(21):2517-38.

226. Vieira LM, Kijjoa A. Naturally-occurring xanthones: recent developments. Curr Med Chem. 2005;12(21):2413-46.

227. Lesch B, Brase S. A short, atom-economical entry to tetrahydroxanthenones. Angew Chem Int Ed Engl. 2004;43(1):115-8.

228. Blanco-Ayala T, Lugo-Huitron R, Serrano-Lopez EM, Reyes-Chilpa R, Rangel-Lopez E, Pineda B, et al. Antioxidant properties of xanthones from Calophyllum brasiliense: prevention of oxidative damage induced by FeSO(4). BMC Complement Altern Med. 2013;13:262.

229. Paiva AM, Pinto MM, Sousa E. A century of thioxanthones: through synthesis and biological applications. Curr Med Chem. 2013;20(19):2438-57.

230. Matsumoto K, Akao Y, Kobayashi E, Ohguchi K, Ito T, Tanaka T, et al. Induction of apoptosis by xanthones from mangosteen in human leukemia cell lines. J Nat Prod. 2003;66(8):1124-7.

231. Palmeira A, Vasconcelos MH, Paiva A, Fernandes MX, Pinto M, Sousa E. Dual inhibitors of P-glycoprotein and tumor cell growth: (re)discovering thioxanthones. Biochem Pharmacol. 2012;83(1):57-68.

232. Takara K, Hayashi R, Kokufu M, Yamamoto K, Kitada N, Ohnishi N, et al. Effects of nonsteroidal anti-inflammatory drugs on the expression and function of P-glycoprotein/MDR1 in Caco-2 cells. Drug Chem Toxicol. 2009;32(4):332-7.

233. Hidalgo IJ. Cultured intestinal epithelial cell models. Pharm Biotechnol. 1996;8:35-50.

234. Tomita N, Jiang W, Hibshoosh H, Warburton D, Kahn SM, Weinstein IB. Isolation and characterization of a highly malignant variant of the SW480 human colon cancer cell line. Cancer Res. 1992;52(24):6840-7.

235. Leibovitz A, Stinson JC, McCombs WB, 3rd, McCoy CE, Mazur KC, Mabry ND. Classification of human colorectal adenocarcinoma cell lines. Cancer Res. 1976;36(12):4562-9.

236. Ding Z, Yang L, Xie X, Xie F, Pan F, Li J, et al. Expression and significance of hypoxia-inducible factor-1 alpha and MDR1/P-glycoprotein in human colon carcinoma tissue and cells. J Cancer Res Clin Oncol. 2010;136(11):1697-707.

237. Montazami N, Kheir Andish M, Majidi J, Yousefi M, Yousefi B, Mohamadnejad L, et al. siRNA-mediated silencing of MDR1 reverses the resistance to oxaliplatin in SW480/OxR colon cancer cells. Cell Mol Biol (Noisy-le-grand). 2015;61(2):98-103.

238. Liu KP, Luo F, Xie SM, Tang LJ, Chen MX, Wu XF, et al. Glycogen Synthase Kinase 3beta Inhibitor (2'Z,3'E)-6-Bromo-indirubin- 3'-Oxime Enhances Drug Resistance to 5-Fluorouracil Chemotherapy in Colon Cancer Cells. Chin J Cancer Res. 2012;24(2):116-23.

239. Mishra J, Zhang Q, Rosson JL, Moran J, Dopp JM, Neudeck BL. Lipopolysaccharide increases cell surface P-glycoprotein that exhibits diminished activity in intestinal epithelial cells. Drug Metab Dispos. 2008;36(10):2145-9.

240. Yan X, Zhao J, Zhang R. Visfatin mediates doxorubicin resistance in human colorectal cancer cells via up regulation of multidrug resistance 1 (MDR1). Cancer Chemother Pharmacol. 2017;80(2):395-403.

241. Chikazawa N, Tanaka H, Tasaka T, Nakamura M, Tanaka M, Onishi H, et al. Inhibition of Wnt signaling pathway decreases chemotherapy-resistant side-population colon cancer cells. Anticancer Res. 2010;30(6):2041-8.

242. Cai Y, Xu C, Chen P, Hu J, Hu R, Huang M, et al. Development, validation, and application of a novel 7-day Caco-2 cell culture system. J Pharmacol Toxicol Methods. 2014;70(2):175-81.

243. Artursson P, Palm K, Luthman K. Caco-2 monolayers in experimental and theoretical predictions of drug transport. Adv Drug Deliv Rev. 2001;46(1-3):27-43.

244. Kublbeck J, Hakkarainen JJ, Petsalo A, Vellonen KS, Tolonen A, Reponen P, et al. Genetically Modified Caco-2 Cells With Improved Cytochrome P450 Metabolic Capacity. J Pharm Sci. 2016;105(2):941-9.

245. Meunier V, Bourrie M, Berger Y, Fabre G. The human intestinal epithelial cell line Caco-2; pharmacological and pharmacokinetic applications. Cell Biol Toxicol. 1995;11(3-4):187-94.

246. Barta CA, Sachs-Barrable K, Feng F, Wasan KM. Effects of monoglycerides on P-glycoprotein: modulation of the activity and expression in Caco-2 cell monolayers. Mol Pharm. 2008;5(5):863-75.

247. Hunter J, Jepson MA, Tsuruo T, Simmons NL, Hirst BH. Functional expression of P-glycoprotein in apical membranes of human intestinal Caco-2 cells. Kinetics of vinblastine secretion and interaction with modulators. J Biol Chem. 1993;268(20):14991-7.

248. Watanabe T, Onuki R, Yamashita S, Taira K, Sugiyama Y. Construction of a functional transporter analysis system using MDR1 knockdown Caco-2 cells. Pharm Res. 2005;22(8):1287-93.

249. Xia CQ, Liu N, Yang D, Miwa G, Gan LS. Expression, localization, and functional characteristics of breast cancer resistance protein in Caco-2 cells. Drug Metab Dispos. 2005;33(5):637-43.

250. Chimezie C, Ewing A, Schexnayder C, Bratton M, Glotser E, Skripnikova E, et al. Glyceollin Effects on MRP2 and BCRP in Caco-2 Cells, and Implications for Metabolic and Transport Interactions. J Pharm Sci. 2016;105(2):972-81.

251. Englund G, Rorsman F, Ronnblom A, Karlbom U, Lazorova L, Grasjo J, et al. Regional levels of drug transporters along the human intestinal tract: co-expression of ABC and SLC transporters and comparison with Caco-2 cells. Eur J Pharm Sci. 2006;29(3-4):269-77.

252. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65(1-2):55-63.

253. Borenfreund E, Babich H, Martin-Alguacil N. Comparisons of two in vitro cytotoxicity assays-The neutral red (NR) and tetrazolium MTT tests. Toxicol In Vitro. 1988;2(1):1-6.

254. Lowik CW, Alblas MJ, van de Ruit M, Papapoulos SE, van der Pluijm G. Quantification of adherent and nonadherent cells cultured in 96-well plates using the supravital stain neutral red. Anal Biochem. 1993;213(2):426-33.

255. Repetto G, del Peso A, Zurita JL. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat Protoc. 2008;3(7):1125-31.

256. Vajrabhaya LO, Korsuwannawong S. Cytotoxicity evaluation of Clinacanthus nutans through dimethylthiazol diphenyltetrazolium bromide and neutral red uptake assays. Eur J Dent. 2016;10(1):134-8.

257. Vilas-Boas V, Silva R, Gaio AR, Martins AM, Lima SC, Cordeiro-da-Silva A, et al. P-glycoprotein activity in human Caucasian male lymphocytes does not follow its increased expression during aging. Cytometry A. 2011;79(11):912-9.

258. Mahringer A, Delzer J, Fricker G. A fluorescence-based in vitro assay for drug interactions with breast cancer resistance protein (BCRP, ABCG2). Eur J Pharm Biopharm. 2009;72(3):605-13.

259. Shen F, Bailey BJ, Chu S, Bence AK, Xue X, Erickson P, et al. Dynamic assessment of mitoxantrone resistance and modulation of multidrug resistance by valspodar (PSC833) in multidrug resistance human cancer cells. J Pharmacol Exp Ther. 2009;330(2):423-9.

260. Silva R, Carmo H, Vilas-Boas V, Barbosa DJ, Palmeira A, Sousa E, et al. Colchicine effect on P-glycoprotein expression and activity: in silico and in vitro studies. Chem Biol Interact. 2014;218:50-62.

261. Ghanem CI, Arias A, Novak A, Carpini GD, Villanueva S, Blazquez AG, et al. Acetaminophen-induced stimulation of MDR1 expression and activity in rat intestine and in LS 174T human intestinal cell line. Biochemical Pharmacology. 2011;81(2):244-50.

262. Novak A, Godoy YC, Martinez SA, Ghanem CI, Celuch SM. Fructose-induced metabolic syndrome decreases protein expression and activity of intestinal P-glycoprotein. Nutrition. 2015;31(6):871-6.

263. Novak A, Carpini GD, Ruiz ML, Luquita MG, Rubio MC, Mottino AD, et al. Acetaminophen Inhibits Intestinal P-Glycoprotein Transport Activity. Journal of Pharmaceutical Sciences. 2013;102(10):3830-7.

264. Kim JH, Park JM, Roh YJ, Kim IW, Hasan T, Choi MG. Enhanced efficacy of photodynamic therapy by inhibiting ABCG2 in colon cancers. BMC Cancer. 2015;15:504.

265. Isshiki M, Umezawa K, Tamura H. Coffee induces breast cancer resistance protein expression in Caco-2 cells. Biol Pharm Bull. 2011;34(10):1624-7.

266. Schexnayder C, Stratford RE. Genistein and Glyceollin Effects on ABCC2 (MRP2) and ABCG2 (BCRP) in Caco-2 Cells. Int J Environ Res Public Health. 2015;13(1):ijerph13010017.

267. Lemos C, Kathmann I, Giovannetti E, Dekker H, Scheffer GL, Calhau C, et al. Folate deprivation induces BCRP (ABCG2) expression and mitoxantrone resistance in Caco-2 cells. Int J Cancer. 2008;123(7):1712-20.

268. Peters WHM, Roelofs HMJ. Biochemical Characterization of Resistance to Mitoxantrone and Adriamycin in Caco-2 Human Colon Adenocarcinoma Cells: A Possible Role for Glutathione S-Transferases. Cancer Research. 1992;52(7):1886-90.