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Review Article

Overcoming BCRP-mediated multidrug resistance

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Abstract

Breast cancer resistance protein (BCRP, MXR, ABCG2) is a well-known protein related to acute myeloid leukemia (AML) resistance to chemotherapeutic treatments. This protein is a member of the ATP-binding cassette family which to date comprises 48 members. BCRP is able to efflux mitoxantrone, anthracyclines, taxoids and methotrexate, as well as topoisomerase I inhibitors. BCRP function can easily be evaluated by anticancer drug uptake. This uptake can be directly followed by flow cytometry or indirectly by cytotoxic assays. A variety of BCRP inhibitors have been described which are more or less specific and active. Despite the great variety of BCRP inhibitors available, the use of these agents in therapy has not fulfilled its promise, at least concerning solid tumors. This article reviews the state of the art of BCRP inhibitors and attempts to draw conclusions related to the potential clinical use of these compounds.

Introduction

One of the major challenges faced by physicians, researchers and patients concerning cancer treatment is the development of intrinsic or acquired resistance to a variety of chemotherapeutic agents by cancer cells. This issue is further complicated by the fact that resistance is exerted towards drugs which are not structurally related and which possess different mechanisms of action. Molecular studies have shown that one way by which cancer cells develop multidrug resistance (MDR) is the overexpression of ATP-dependent drug efflux transporter proteins on their plasma membrane (1). These efflux pumps exert their actions mainly by reducing the intracellular levels of cytotoxic agents below concentrations lethal to the cell or by redistributing the cytotoxic agents inside the cell, limiting the exposure of the targeted intracellular organelle to the drug (2).

The most extensively studied of these efflux proteins are the permeability glycoprotein (P-glycoprotein, or P-gp, also known as multidrug resistance protein 1, or MDR1) (3), multidrug resistance-associated protein (MRP1 or ABCC1) (4) and the more recently described breast cancer resistance protein (BCRP), also known as placenta-specific ABC protein (ABCP), mitoxantrone resistance-associated protein 4 (MXR4) or ATP-binding cassette subfamily G member 2 (ABCG2). BCRP was first identified as an overexpressed mRNA in an MDR breast cancer cell subline, MCF7/AdrVp, relative to parental drug-sensitive MCF7 cells (5). Around the same time, two cDNAs nearly identical to BCRP were cloned, one isolated from the placenta and termed ABCP (6) and the other cloned from a mitoxantrone-resistant colon cancer cell line and termed MXR (6, 7). Subsequent studies showed that overexpression of BCRP is a common feature of a number of mitoxantrone-resistant cell lines, suggesting a common mechanism of resistance of these tumor cells involving BCRP (8).

BCRP is a 72-kDa protein encoded by the *ABCG2* gene on chromosome 4 (4q22), which belongs to the ATP-binding cassette family of transporters (9). To date, at least 48 different human ABC transporter genes have been described (10). These transporters are plasma membrane glycoproteins which extrude various substrates from the inside of cells to the outside against a concentration gradient by using the energy yielded by ATP hydrolysis (11). As ABC transporters play a protective role against the penetration of toxic agents into normal cells, they are found predominantly in organs with an important role in metabolism and excretion (liver and kidney), at sites of absorption (lung, intestine) and in blood-tissue barriers (blood-brain, blood-cerebrospinal fluid, blood-testis and placenta) (12). However, in a variety of cancer cells, these transporters are overexpressed and actively extrude therapeutic agents from inside the tumor cells, contributing to their MDR phenotype (10).

BCRP possesses a wide substrate specificity, which in some cases overlaps that of P-gp/MDR1 and MRP1.

BCRP is able to actively transport positively or negatively charged hydrophobic molecules, including cytotoxic compounds (mitoxantrone, flavopiridol, methotrexate and camptothecins [SN-38, topotecan]), fluorescent dyes (Hoechst-33342) and different toxic compounds found in food, such as PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) or pheophorbide A (13-15).

Functional ABC transporters require the presence of two nucleotide-binding domains (NBDs) and two membrane-spanning domains (MSDs) containing six putative transmembrane α helices (16). Therefore, most of these transporters are encoded by genes containing all four domains in their open reading frame and are termed full transporters. However, a subgroup of these transporters, termed half-transporters and belonging to the G subfamily of ABC transporters, contain only one NBD and one MSD, and are believed to function as homodimers (or homo-oligomers) linked by disulfide bonds (17). The MSDs could be responsible for the recognition of BCRP substrates, and conformational changes of these domains are thought to be responsible for the transport of substrates through the plasma membrane. Recent work has suggested that BCRP could contain more than one ligand fixation site (18). The predicted topology of the half-transporters is represented in Figure 1, but still needs confirmation by detailed experimental studies.

Amino acid 482 has been shown to play a key role in the substrate and inhibitor specificity of BCRP (19). Studies using stable transfectants of wild-type and mutant BCRP have shown that both types confer resistance to mitoxantrone and camptothecins, but mutants of BCRP with a glycine (G) or threonine (T) residue at position 482 in place of an arginine (R) are also able to extrude anthracyclines and rhodamine 123, in addition to a gain of function for ATP hydrolysis and mitoxantrone (20, 21). However, this gain-of-function phenotype is not common to all BCRP substrates, as it was shown that the R482G and R482GT mutants are unable to transport methotrexate, which is a substrate for wild-type BCRP (22). Further studies showed that mutation of amino acid residue 482 to any other type of amino acid also affects the activity of BCRP inhibitors (for review see Ref. 23). Unfortunately, regarding the potential clinical relevance of these mutant phenotypes, it is important to point out that, until now, no clinical sample has been shown to harbor any mutation at amino acid 482. Three glycosylation sites have also been described, but glycosylation does not appear to have a role in BCRP's transport function (24).

Methods of evaluation of BCRP activity

Several methods have been developed to evaluate BCRP substrates, inhibitors and transport activity. In some cases, an emphasis has been made on the distinction between the activity of wild-type BCRP and of amino acid 482 mutants, as these mutants show an altered substrate specificity and in some cases increased ATP hydrolytic activity relative to their wild-type counterparts (20-22). The literature survey indicates that there are two major *in vitro* methods to evaluate the activity of BCRP inhibitors: flow cytometry-based fluorescent assays and cytotoxicity assays.

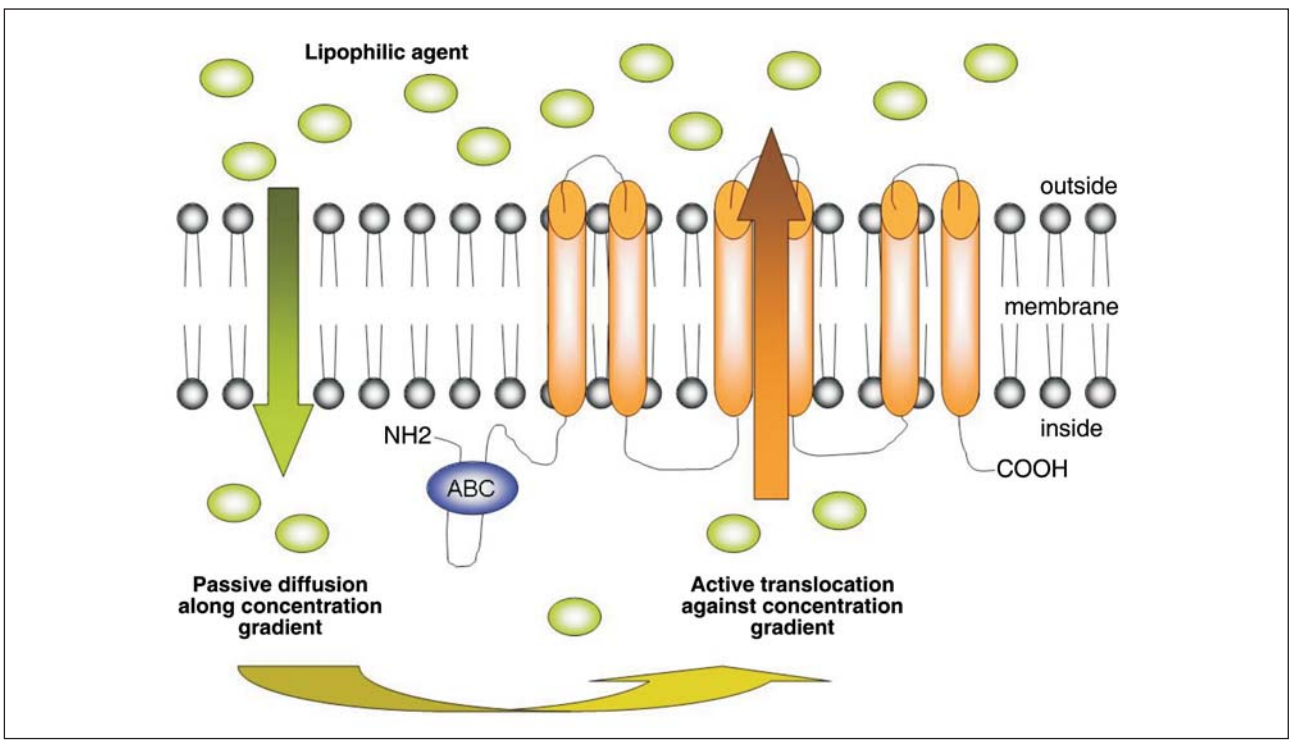

Fig. 1. The predicted topology of the G subfamily of ABC half-transporters.

The first method is based on fluorescent dye efflux measurement and has been widely used to quantitate the expression of drug efflux transporters. In the case of BCRP, the first assays to be designed used fluorescent substrates, such as mitoxantrone, rhodamine 123, BODIPY-prazosin, LysoTracker and topotecan. Wild-type BCRP is not able to transport rhodamine 123 or doxorubicin, whereas R482G and R482T mutants are able to efflux these two compounds (19). Inhibitors can be identified by their ability to increase the intracellular accumulation of the fluorescent substrate.

The second method is a cytotoxicity determination and is indirectly correlated to the accumulation of BCRP substrates in the cells. Most BCRP substrates have antiproliferative activity and were identified by their IC_{50} (drug concentration sufficient to inhibit 50% of colony formation) in cells overexpressing BCRP compared to wild-type cells (14). Inhibitors can be identified by their ability to increase cytotoxicity when co-incubated with anticancer drugs which are BCRP substrates.

BCRP inhibitors as potential modulators of MDR

Many efforts have been undertaken to identify agents able to reverse the MDR phenotype mediated by P-gp, MRP1 and BCRP, taking into account their clinical relevance. Due to its role in the efflux of a large number of anticancer drugs, BCRP is an interesting target for the design of MDR-reversing agents (15). Known BCRP inhibitors are structurally unrelated compounds belonging to different chemical classes and are being studied using at least two different biological assays in different cell lines, which makes any global structure-activity relationship (SAR) studies among BCRP inhibitors potentially risky. The literature survey indicates that BCRP inhibitors can be classified into three distinct groups: 1) drugs unrelated to cancer treatment (such as ciclosporin, used as an immunosuppressive agent in kidney transplantation); 2) derivatives of known P-gp inhibitors; and 3) compounds identified from natural sources or designed to act as BCRP inhibitors. In this article, we focus mainly on selective or specific BCRP inhibitors regarding ABC transporters.

Antiviral agents

The treatment of HIV-1-infected patients is hampered by the emergence of resistant forms of virus. Besides viral mutations, ABC transporters may also have a role in this resistance. Indeed, it has been reported that P-gp is a key determinant of the oral bioavailability of HIV-1 protease inhibitors (25). More recently, it was found that the expression of BCRP in a $CD4^+$ T-cell line confers cellular resistance to nucleoside reverse transcriptase inhibitors (NRTIs) (26). Thus, BCRP is considered to be an interesting target to improve the treatment of HIV with antiretroviral agents. Ideally, the use of an antiretroviral agent possessing intrinsic inhibitory activity on BCRP could allow the administration of lower doses of the drug, and therefore reduce its side effects.

In this context, considerable efforts have been made to investigate the effects of clinically used anti-HIV agents on BCRP. In a recent study (27), the effects of important anti-HIV drugs on BCRP activity as assessed by pheophorbide A accumulation in MDCKII-BCRP cells were compared with the corresponding parental MDCKII cell line lacking human BCRP. According to the IC_{50} estimations, the protease inhibitors are the most active compounds on BCRP function (Fig. 2). The rank order for

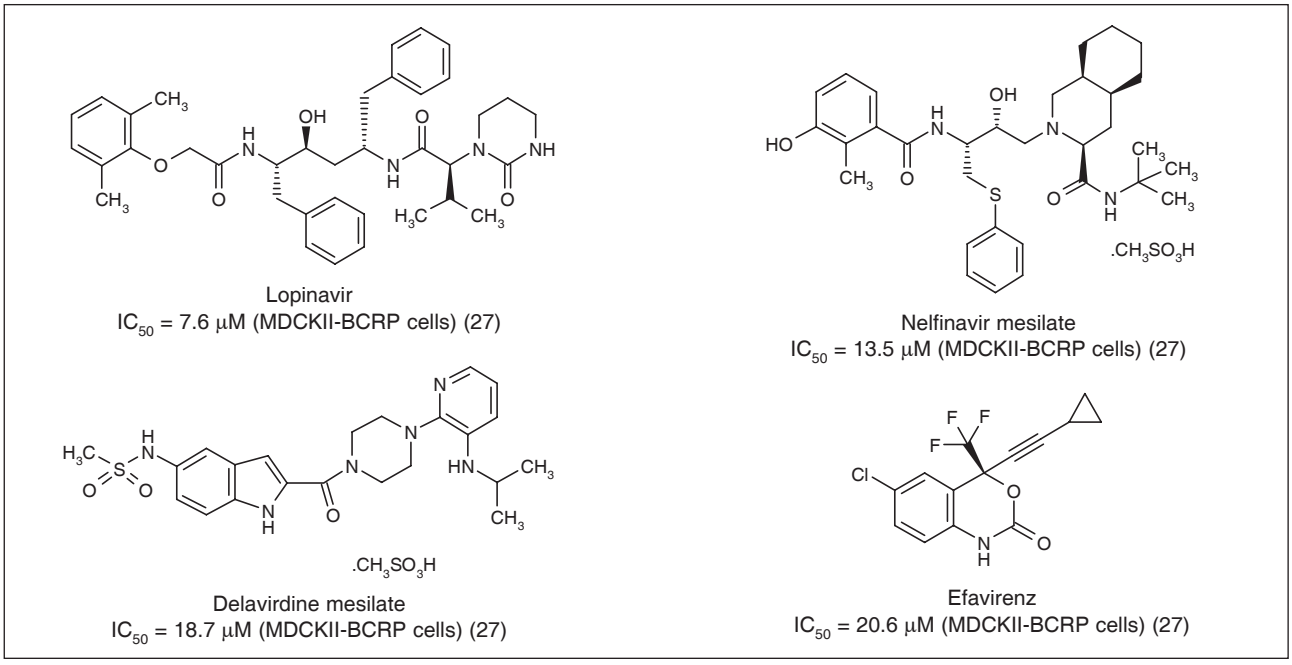

Fig. 2. Structures of selected anti-HIV drugs.

BCRP inhibition was lopinavir > nelfinavir > delavirdine > efavirenz > saquinavir > atazanavir > amprenavir > abacavir. The well-known anti-HIV drug AZT (zidovudine) exerted weak inhibition. By comparing the results of this study with the structure of the different compounds tested, it appears that the superior hydrophobicity of protease inhibitors compared to the NRTIs and the non-nucleoside reverse transcriptase inhibitors (NNRTIs) might be responsible for their greater activity.

Tyrosine kinase inhibitors (TKIs)

Protein kinases play a major role in cellular signaling and are major targets for anticancer drugs. In patients, the expression of BCRP on intestinal epithelial cells could modify the oral absorption of TKIs, as well as their plasma concentration.

TKIs are one of the most extensively studied classes of BCRP inhibitors (28-34). In 2001, Erlichman *et al*. (30) described the action of the HER family TKI CI-1033 (PD-183805, canertinib hydrochloride) on BCRP (Fig. 3). This compound was studied in combination with the active metabolite of irinotecan (7-ethyl-10-hydroxycamptothecin, SN-38) or the related agent topotecan in cell lines expressing endogenous BCRP and in a BCRP-transfected cell line. The latter study concluded that CI-1033 was able to increase the uptake and cytotoxicity of SN-38 and topotecan. However, it was also demonstrated that the accumulation of CI-1033 alone was reduced in cells expressing BCRP, which indicates that the compound was also a substrate.

Imatinib mesilate (STI-571, Gleevec™/Glivec®) (Fig. 3) is a well-known TKI that is widely used for the treatment of chronic myelogenous leukemia (CML) by inhibiting BCR-ABL and for gastrointestinal stromal tumors (GISTs) by inhibiting c-Kit. Various clinical trials in other types of cancers, such as glioblastoma, prostate and lung carcinoma, are in progress. Imatinib, similar to many other TKIs, including members of the 4-anilinoquinazoline class, competes for ATP binding. However, in patients, limited penetration of imatinib into the brain has been reported. Imatinib is transported *in vitro* and *in vivo* by P-gp. Overexpression of BCRP has been shown to be involved in resistance to imatinib, but the effect of imatinib on BCRP has been the subject of controversy. First, it was reported that imatinib potently reverses BCRP-mediated resistance, but that it is not a BCRP substrate (31). In the same year, an independent study challenged the latter assumption, concluding that imatinib is a substrate for BCRP (29). Shortly thereafter, a third study determined that imatinib is a competitive substrate for BCRP (28). At concentrations of 0.1-1.0 μM, ZD-1839 (gefitinib, Iressa®) and its analogue EKI-785 (Fig. 3) stimulated ATPase activity, whereas imatinib strongly inhibited this activity. At higher concentrations, all three TKIs inhibited ATPase activity. This discrepancy at low concentrations could be explained by the fact that ZD-1839 and EKI-785 are BCRP substrates (33).

Flavonoid derivatives

Flavonoids constitute a large class of naturally occurring polyphenols that have been extensively studied on P-gp and more recently on BCRP. Several naturally occurring flavonoids (Fig. 4) have been studied using mitoxantrone accumulation and cytotoxicity assays (35, 36). Among all the compounds evaluated, only two (naringenin and phloretin) were tested in their aglycone and glycosylated forms. As expected, the more hydrophobic aglycone analogues were more active. Most active

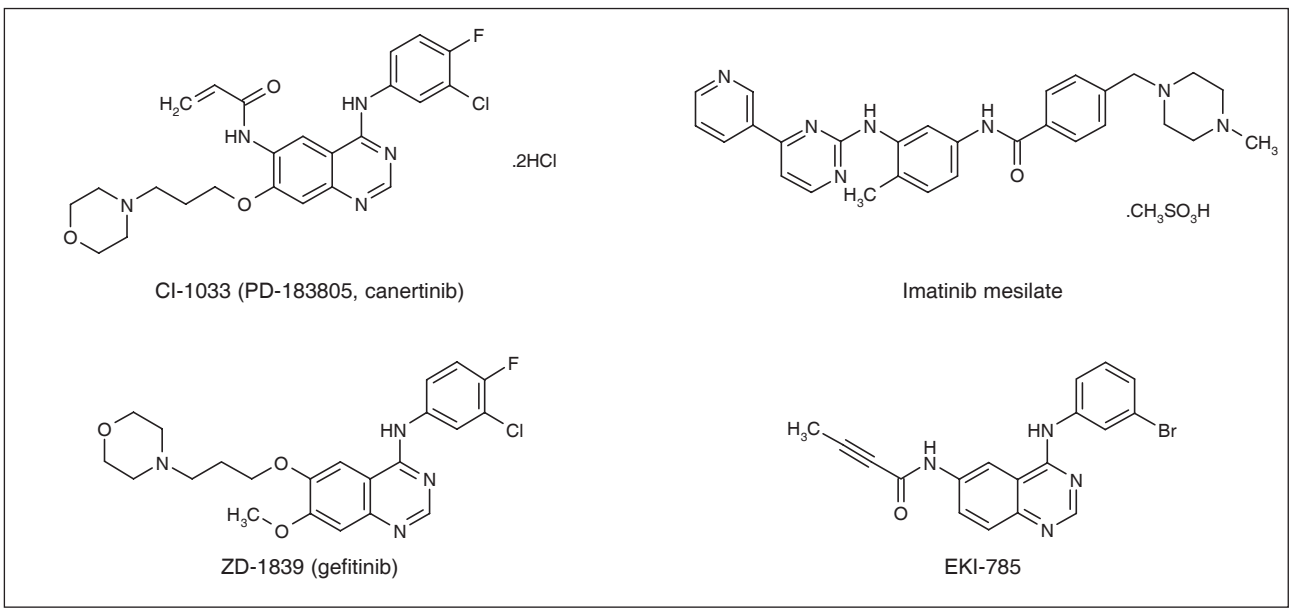

Fig. 3. Structures of selected tyrosine kinase inhibitors.

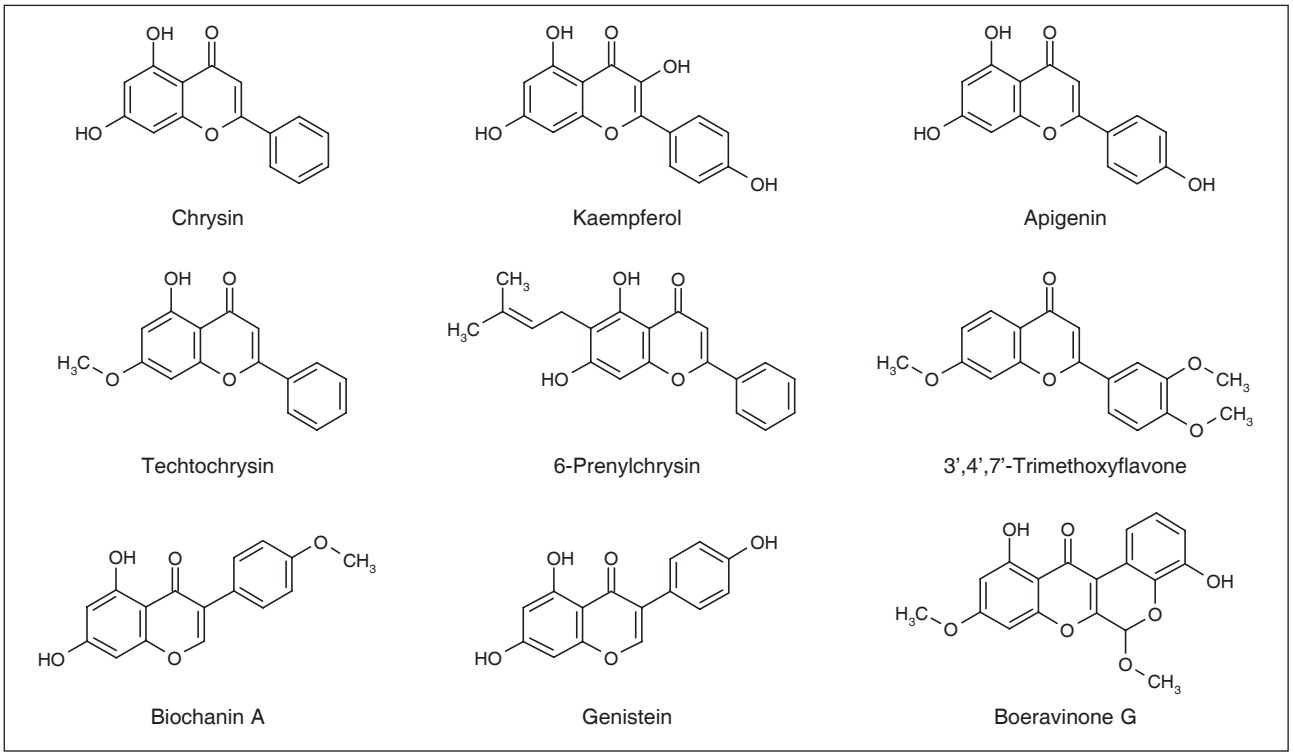

Fig. 4. Structures of selected flavonoids and related compounds.

flavonoids tested at 50 μM were able to reduce the IC_{50} value of mitoxantrone in BCRP-overexpressing MCF7 cells from 199 μM to < 4 μM.

Recently, Katayama *et al*. (37) reported a comparative study of a panel of 32 natural flavonoids. Using topotecan accumulation and cytotoxicity assays, they identified 3',4',7-trimethoxyflavone and apigenin (Fig. 4) as the strongest BCRP inhibitors. In addition, these compounds appeared to have good specificity for BCRP. What is surprising is that glycosylated flavonoids, such as diosmin, are active on BCRP. This is the first time that these highly hydrophilic molecules have been reported to be inhibitors of BCRP. Knowing that the flavonoids studied by Katayama *et al*. are widely present in fruits and vegetables, such molecules might have positive effects on the pharmacokinetics of anticancer agents.

Techtochrysin (5-hydroxy-7-methoxyflavone) and 6-prenylchrysin (Fig. 4) were also reported to be potent inhibitors of wild-type BCRP (38). The substitution pattern among these molecules is critical. For example, the presence of the methoxy group at the 7-position of techtochrysin is necessary for higher activity. When this group is moved to other positions, the activity of the compound is dramatically decreased. In the same way, the presence of the prenyl group at the 6- or 8-position is also important.

Flavonoids behaving as weak estrogens, termed "phytoestrogens", have been studied as reversers of BCRP-mediated MDR. Since few sulfated estrogens have been identified as BCRP substrates, Imai *et al*. conducted a study with the aim of evaluating the activity of phytoestrogens on BCRP (39). Genistein, naringenin, acacetin and kaempferol (Fig. 4) potentiated the cytotoxicity of mitoxantrone and SN-38 in BCRP-transduced K-562 cells. In addition, these molecules are selective inhibitors, as no effect was observed on P-gp or BCRP. This finding was recently reinforced by an independent study showing that two analogues of genistein, boeravinones G and H, are potent BCRP inhibitors (40).

Estrogens and related compounds

Steroidal and nonsteroidal estrogen receptor mixed agonists/antagonists were used by Lilly as a pharmacophore model to develop selective modulators of MRP1-mediated MDR (41). Imai and co-workers reported that estrone and 17β-estradiol were able to potentiate the cytotoxicity of mitoxantrone and topotecan in BCRP-transduced K-562 cells (42). The same authors also reported that metabolites of estrogen, and especially sulfated estrogens, are transported by BCRP (43).

Following this study, estrogen antagonists and agonists have been evaluated as BCRP reversal agents. The pharmacophore of tamoxifen was used as a basis for the synthesis of analogues and diversity was achieved by introducing small substituents at the A and/or B cycles (Fig. 5). Diethylstilbestrol showed the strongest BCRP-reversing activity by increasing the intracellular accumulation of topotecan and reversing drug resistance in K-562/BCRP cells. Tamoxifen and toremifene were less active in enhancing topotecan uptake in the same cell line. The higher activity of diethylstilbestrol is expected to be correlated with its higher hydrophobicity (44).

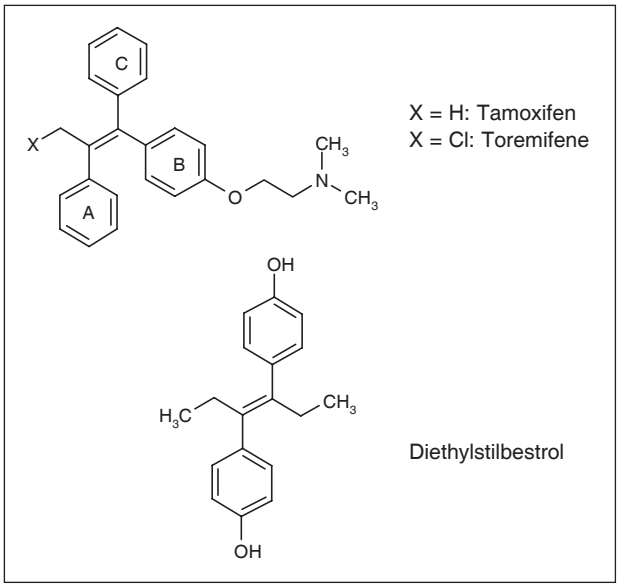

Fig. 5. Structures of selected estrogens and related compounds.

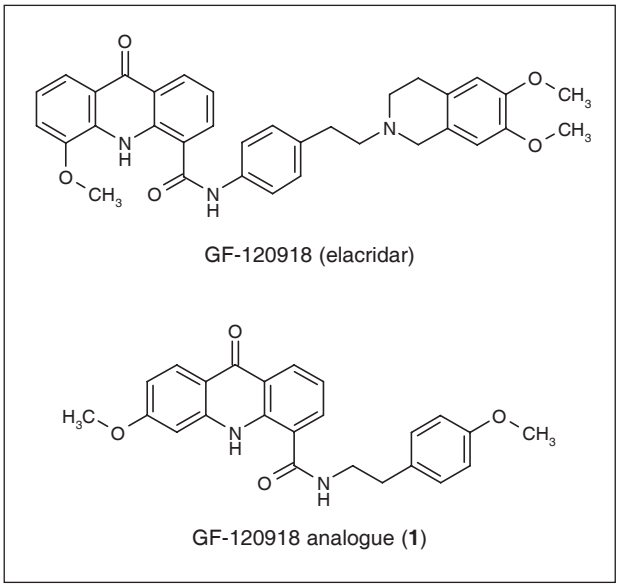

Fig. 6. Structures of acridones.

Acridone derivatives

GF-120918 (elacridar) (Fig. 6) is the first of the series to be studied as a BCRP inhibitor. This compound was initially developed as a P-gp inhibitor (45) and was later demonstrated to be an efficient inhibitor of BCRP. Various studies have shown that GF-120918 can be tolerated in humans and animals at concentrations sufficient to inhibit BCRP (46-48).

Studies on GF-120918 have covered almost all types of cancers both in human and murine systems. Using flow cytometry and confocal microscopy, scientists from the National Institutes of Health (NIH) were among the first to show the effects of GF-120918 in human cell lines expressing BCRP (49). The acridone was able to block both rhodamine and mitoxantrone efflux. Later studies examined the effect of GF-120918 on resistance to the topoisomerase I inhibitor camptothecin. The compound was found to be a potent inhibitor, with almost complete reversal at 100 nM (50). Following these important results, the structure of GF-120918 was used as a basis to identify new BCRP inhibitors. Analogues such as compound **1** (Fig. 6) were prepared by shortening the arylic side-chain and were found to be slightly more active than GF-120918, as shown by mitoxantrone efflux from human wild-type (R482) *ABCG2*-transfected cells (51).

Fumitremorgin C (FTC) and analogues

Fumitremorgin C (FTC) (Fig. 7) is a fungal toxin isolated from *Aspergillus fumigatus*, discovered by Rabindran *et al*. in 1998 (52), the same year as the description of BCRP by Doyle *et al*. (5). This compound was isolated from a large library of microbial extracts and tested on a colon carcinoma cell line resistant to mitoxantrone (S1-M1-3.2). FTC and the related molecules FTA and FTB (Fig. 7) belong to a class of diketopiperazines that are mycotoxins. FTA and FTB, which differ from FTC at two positions, are less effective on BCRP, with an increase in side effects *in vivo*, especially on the brainstem and spinal cord. When taken orally at 25 mg/kg, FTC causes tremors in cockerels (52). FTC was demonstrated to be a specific inhibitor of BCRP by its lack of modulation of P-gp-positive cells (S1-B1-20) or MRP1-positive cells (HL-60/AR). FTC was able to increase the amount of mitoxantrone and increase the cytotoxicity of mitoxantrone, doxorubicin and topotecan in cell lines expressing BCRP.

Using cells transfected with the *ABCG2* gene, Rabindran *et al*. demonstrated that FTC could reverse resistance mediated by this transporter (53). Using a cytotoxicity assay based on a sulforhodamine B probe, FTC was shown to restore the accumulation and the sensitivity of *ABCG2*-transfected MCF7 cells to mitoxantrone, topotecan and doxorubicin. However, the inhibitory effect of FTC is compromised by its neurotoxic effects, thus excluding its clinical use (52). In order to provide less toxic and specific BCRP inhibitors, FTC analogues have therefore become attractive targets.

Shinkel's group screened a variety of libraries derived from indolyldiketopiperazines (corresponding to A/B/C/D-rings of FTC) and found three compounds, namely KO-132, KO-134 and KO-143 (Fig. 7), as the most promising leads. The three compounds are potent and specific inhibitors of BCRP and less toxic than FTC (54). KO-143 significantly increased the oral availability of topotecan in mice.

Tryprostatin A (TPS-A) (Fig. 7) is another fungal secondary metabolite and an analogue of FTC. TPS-A was reported to be an inhibitor of microtubule-associated protein (MAP)-dependent microtubule assembly (55). Using classical mitoxantrone uptake and cytotoxicity assays, TPS-A was demonstrated to be an inhibitor of wild-type BCRP (56). At relatively high concentrations (up to

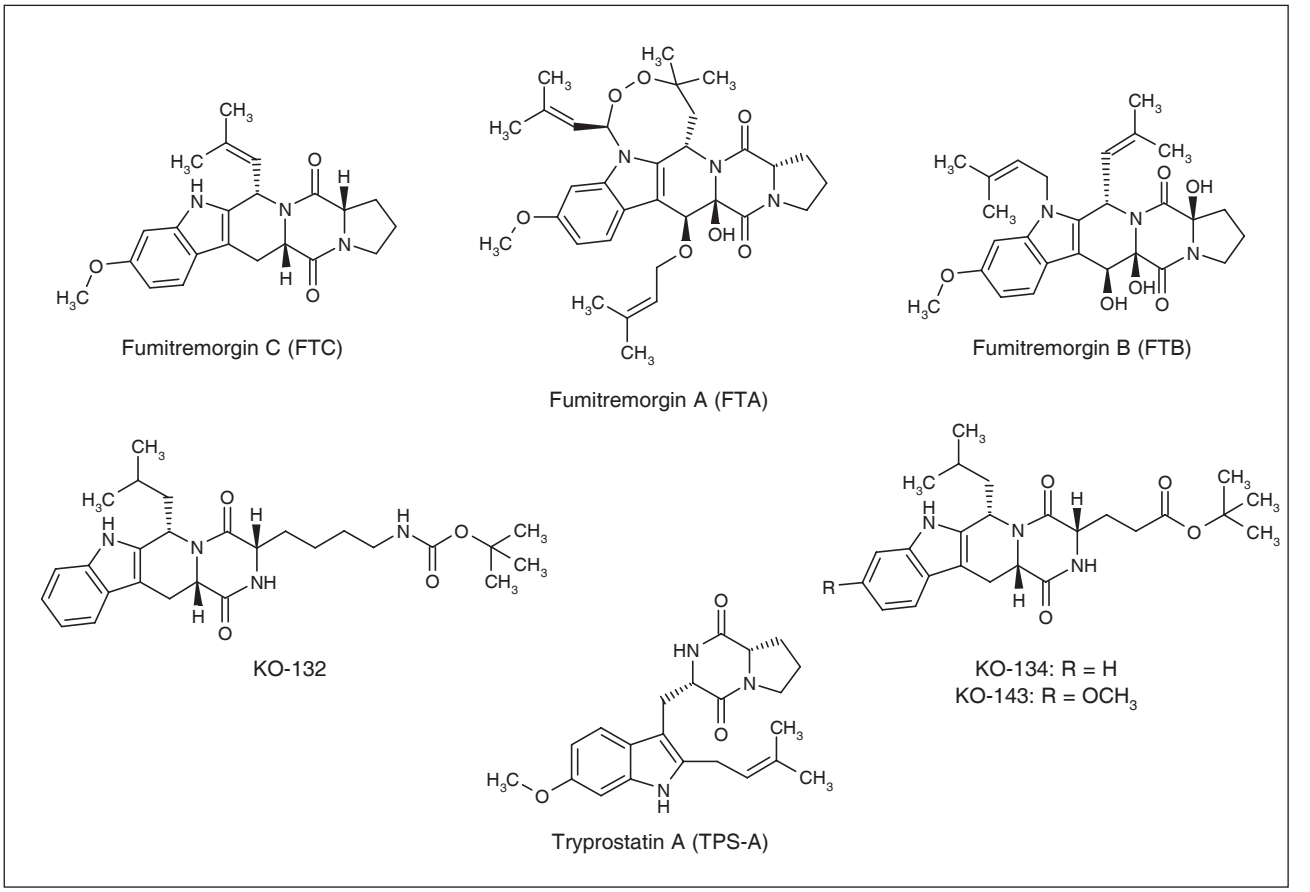

Fig. 7. Structures of fumitremorgin C (FTC) and derivatives.

50 μM), TPS-A was able to restore cell sensitivity toward mitoxantrone without noticeable toxicity. The structural difference between FTC and TPS-A resides essentially in the lack of the diketopiperazine noncore C-ring in TPS-A, allowing alternative conformations.

Miscellaneous compounds

Several promising compounds acting as selective BCRP inhibitors have been reported recently, for example, noncytotoxic taxoids. These compounds were prepared by eliminating the side-chain found in the two anticancer drugs paclitaxel and docetaxel, and introducing a hydrophobic diaryl in the taxane nucleus. Plant-derived cannabinoids, metabolites of ginsenosides and clinically used antifungal azoles have also been investigated as BCRP inhibitors (57-60).

Clinical use of BCRP inhibitors

Depending on the type of anticancer agent used to target a specific tumor, several possibilities can be envisaged. The first case concerns anticancer agents that are not substrates for ABC transporters, or anticancer agents that also possess intrinsic inhibitory activity on ABC transporters, in which case they can be administered alone. However, in the case of anticancer agents that are substrates for or substrates for and weak inhibitors of ABC transporters, the coadministration of an inhibitor of ABC transporters is required, and the type of inhibitor used will depend on the tumor targeted. If several ABC transporters responsible for the MDR phenotype need to be targeted, the inhibitor should exhibit a broad spectrum of activity against different transporters, whereas in the case of tumor cells expressing only one type of efflux transporter, the inhibitor should be specific, or at least selective, for the targeted transporter.

On the other hand, the coadministration of an efflux pump inhibitor with a cytostatic can cause unknown and unpredictable side effects due to the potential diffusion of the drug through a specific blood-tissue barrier. In the case of efflux pump inhibitors that also possess intrinsic cytostatic activity, the dual action of these molecules could be particularly useful in the case of a tumor located on the other side of a blood-tissue barrier.

In summary, the development of efficient methods of evaluation of the potential interactions of a cytostatic drug with one or several efflux pumps is essential in order to predict the clinical response to the drug. There is a need for standardization of the methods of evaluation in order to determine if a molecule is considered as an inhibitor, a substrate, or both, the latter being the case for most of them.

Conclusions and perspectives

It is obvious that establishing SAR among a single, structurally close class of molecules can be easily done. Unfortunately, this is not the case when dealing with diverse classes of molecules which interact with the same target. In this regard, P-gp inhibitors are illustrative and BCRP is expected to share this complexity, representative of the known "polyspecificity" of multidrug transporters. In addition, the multiplicity of the biological screening tests used to evaluate BCRP inhibitors, the types of cells used and their origin complicate SAR studies even further.

One way to gain insight into the physicochemical properties governing a given activity is to perform quantitative structure-activity relationships (QSAR). Performance tools are available to map molecular interaction fields, pharmacokinetic properties, hydrophobicity, polarizability and hydrogen-bonding capacity and correlate them with affinity towards BCRP. Beyond information related to the structural elements responsible for the inhibitory activity, QSAR studies can be very helpful in the design of new specific inhibitors. The design of specific inhibitors can be achieved by performing and cross-linking QSAR studies on the three major ABC transporters, P-gp, MRP1 and BCRP.

In a recent elegant study, Matsson and coworkers reported interesting results for drugs and drug-like molecules (61). The inhibitory effect of this series of compounds on mitoxantrone efflux was studied in Saos-2 cells transfected with human wild-type (Arg482) BCRP. A model capable of discriminating inhibitors from noninhibitors using only 2D QSAR (two molecular descriptors, octanol-water partition coefficient and molecular polarizability) was obtained.

Another unresolved question concerns the binding site(s) and mechanism(s) of action of BCRP inhibitors. These questions have been raised for P-gp-mediated MDR modulators and no precise answer is yet available. This is mainly due to the absence of X-ray structures for these transporters. The polyspecificity of MDR proteins may also contribute to the misunderstanding of their mechanism of action.

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