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**ATP-Binding Cassette (ABC) Transporter Proteins, Multidrug Resistance and Novel
Flavonoid Dimers as Potent, Nontoxic and Selective Inhibitors**

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ABSTRACT

Multidrug resistance (MDR) is often a major impediment to successful chemotherapy in the treatment of cancer. A common mechanism for MDR is the overexpression of an active ATP-binding cassette (ABC) transporter protein: either the P-glycoprotein (P-gp/ABCB1, also known as MDR1), the multidrug resistance protein 1 (MRP1/ABCC1) or the breast cancer resistant protein (BCRP/ABCG2), on the plasma membrane of cancer cells. These transporters can pump many structurally diverse anticancer drugs out of the cancer cells and render these drugs ineffective at a therapeutic dosage, i.e., multidrug resistance. Coadministration of a potent inhibitor of ABC transporter with an anticancer drug has been evaluated in several clinical trials to overcome MDR but led to a disappointing outcome. By taking advantage of the pseudo-dimeric structure (Figure 1) of ABC transporters, we demonstrated that some flavonoid dimers, using polyvalent interactions, can be potent inhibitors of the ABC transporters. Selective inhibition of the three different transporters with the flavonoid dimers can be achieved by placing the two flavonoid

moieties at an optimal distance apart specific for each of the transporters. In addition to being potent and selective inhibitors of the transporters, the flavonoid dimers are found to be nontoxic to normal cells at their corresponding effective concentrations. The *in vivo* efficacy of the flavonoid dimers has been demonstrated. Further investigation of these flavonoid dimers as clinical candidates to overcome multidrug resistance in cancer chemotherapy is warranted.

Keywords: Click chemistry, CuAAC reaction, multidrug resistance, G-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1), breast cancer resistant protein (BCRP), modulators, flavonoids

1. INTRODUCTION

1.1 ATP-Binding Cassette (ABC) Transporter Proteins

The ATP-binding cassette (ABC) transporters refer to a superfamily of proteins in membranes. They transport a variety of compounds through membranes against concentration gradients, a process driven by ATP hydrolysis.¹ ABC transporters usually consist of multiple subunits, one or more of which are the transmembrane domain (TMD) and one or two of which are membrane-associated ATPases (the nucleotide binding domain, NBD). The ATPase subunit(s) bind with

adenosine triphosphate (ATP) and hydrolyze it to ADP and inorganic phosphate to provide the energy needed for the transport of substrates across membranes. The sequence and architecture of TMDs are variable whereas the NBDs are located in the cytoplasm and have a highly conserved sequence.

ABC transporters are central to many physiological and biomedical functions. There are 48 known ABC transporters present in humans and they constitute one of the largest superfamilies of all protein families. They are classified into seven families by the Human Genome Organization² as ABCA to ABCG. In this review, we focus on three members of the ABC transporters because of their role in multidrug resistance (MDR) in cancer chemotherapy. They are the P-glycoprotein (P-gp/ABCB1, also known as MDR1), the multidrug resistance protein 1 (MRP1/ABCC1) and the breast cancer resistant protein (BCRP/ABCG2) (Figure 1).³

Figure 1: Schematic representation of P-gp, MRP1 and BCRP.³ P-gp has 2 trans-membrane domains (TMD) consisting of 6 α -helices each and 2 ATP bindings sites. MRP1 also contains 2 TMD containing 6 α -helices with 1 additional TMD consisting of 5 α -helices. ABCG2 is a half transporter containing 1 TMD of 6 α -helices and 1 ATP-binding domain.

P-gp (ABCB1) is a 170 KDa transmembrane glycoprotein first characterized in 1976.⁴ The N-terminal half of the molecule contains 6 TMDs and a large cytoplasmic NBD followed by a duplication at the C-terminal half with 6 TMDs and a NBD (Figure 1). The second half shows over

65% of amino acid similarity with the first half of the transporter. Recently, the molecular structure of human P-glycoprotein in the ATP-bound, outward-facing conformation has been determined using electron cryo-microscopy (cryo-EM).⁵

Similar to P-gp, MRP1 (ABCC1) has two transmembrane domains (TMD1 and TMD2) and two cytosolic NBD.⁶ However, MRP1 has an extra N-terminal transmembrane domain (TMD0) containing five membrane-spanning helices (Figure 1). The functional role of this third TMD0 is not clear. The electron cryo-microscopy structure of bovine MRP1 in two different inward-facing conformations and an outward-facing conformation with an ATP-bound at 3.1 Å resolution have been determined recently.^{7,8} Because bovine MRP1 and human MRP1 share a high level of similarity in their function and amino acid sequence identities (91% identical), these cryo-EM structures of bovine MRP1 are good homology models for human MRP1.

BCRP (ABCG2), unlike P-gp and MRP1, is a 72kDa half transporter consisting of only one cytosolic nucleotide-binding domain (NBD) and one transmembrane domain (TMD) (Figure 1).⁹

When it functions as a transporter, it is likely that it may form a dimeric or tetrameric structure.^{10,11}

The structures of dimeric ABCG2 in the unliganded and liganded states and in the presence of chemotherapeutic agents have been characterized by cryo-EM and biochemical studies.^{12,13,14}

These data show how ABCG2 harnesses the energy of ATP binding to extrude substrates^{12,14} and demonstrate that, depending on the size of the compounds, one or two inhibitor molecules are required for full inhibition of ABCG2.¹³

These structures present a unified mechanism of how these transporters function as efflux pump by first binding with its substrate, leading to a conformational change which facilitates binding with ATP. The binding of ATP induces another conformational change to render the transporter as outward facing, thus releasing the substrate out of the cell. Hydrolysis of the ATP leads to another conformational change of the transporter, restoring it to its resting conformation (Figure 2).¹⁵

1.2 Figure 2: mechanistic models of substrate transport and ATP hydrolysis for ABC exporters.¹⁵

The ABC transporter, in the inward open conformation, first binds with its substrate, leading

to a conformational change to the occluded conformation which facilitates binding with ATP.

The binding of ATP induces another conformational change to render the transporter as outward facing, thus releasing the substrate out of the cell. Hydrolysis of the ATP leads to another conformational change of the transporter, restoring it to its resting inward open conformation. **Multidrug resistance in cancer chemotherapy**

Cancer is a major cause of human deaths in the world.¹⁶ For cancer patients, chemotherapy, the treatment of cancer with anti-cancer drug(s), is usually necessary and critical. However, multidrug resistance (MDR) often becomes a major impediment to successful chemotherapy. It has been well known that cultured cell lines subjected to increasing concentrations of various cytotoxic drugs *in vitro* can develop resistance, which may occur by a variety of mechanisms. A common mechanism is due to the overexpression of an active ABC transporter protein on the plasma membrane of cancer cells. However, the selection of cultured cells using drugs *in vitro* does not imply necessarily the emergence of MDR tumors *in vivo*. In a recent multi-platform analysis¹⁷ the expression of the ABC transporters of over 50000 patients (n=51,939) had been profiled. MRP1 expression was highest at 81%, BCRP at 66% and P-gp the lowest at 23%. About 29% of the patients tested exhibit co-expression of all three transporters. These transporters can pump

many structurally diverse anticancer drugs out of the cancer cells and render these drugs ineffective at a therapeutic dosage, i.e., multidrug resistance.

1.3 Substrate Specificity

A substrate is a molecule that interacts specifically with the transporter protein's binding pocket and is transported by the protein. P-gp, MRP1, and BCRP exhibit broad substrate promiscuity.

Each transporter has the potential to interact with literally hundreds of structurally diverse substrates and the substrate spectrum of each, while not identical, overlaps to some extent.

Substrates that bind to P-gp are generally organic molecules with molecular weight of 200-1600 Da, amphipathic and lipid-soluble in nature. They frequently possess aromatic ring(s). Some may possess a positively charged N atom at physiological pH¹⁸ and some may be linear and cyclic peptides and ionophores.¹⁵ Pgp substrates include anticancer drugs such as anthracyclines, Vinca alkaloids, taxanes, tyrosine kinase inhibitors, immunosuppressant and human immunodeficiency virus (HIV) protease inhibitors. MRP1, on the other hand, transports most of its substrates either as glutathione conjugates or together with free glutathione. It transports a wide variety of endogenous molecules, such as leukotriene C4 (LTC₄), prostaglandins, glucuronide conjugates of steroids and bilirubin, sulfate conjugates, and toxins like aflatoxin B1.¹⁵ Several anticancer drugs,

including anthracyclines, Vinca alkaloids, epipodophyllotoxins, and methotrexate can also be transported by MRP1 and resulting in drug resistance. BCRP shares much of its substrate complement with P-gp and MRP1 but there are also some significant differences. Like MRP1, but unlike P-gp, BCRP appears to transport both positively and negatively charged drugs, including sulfate and glucuronide conjugates.¹⁵ Unlike Pgp or MRP1, BCRP cannot transport anthracyclines or Vinca alkaloids or taxanes and verapamil. However, it can transport epipodophyllotoxins such as etoposide and camptothecins such as topotecan, which are also substrates of both P-gp and MRP1. Many tyrosine kinase inhibitors are substrates of all three transporter proteins. Thus, there seems to be some redundancy in substrate specificity between these three drug transporters.¹⁵

Such redundancy may be beneficial, for example, in the blood brain barrier (BBB) in preventing undesirable xenobiotics in blood capillary from entering vital organs such as the brain thereby keeping the brain as a sanctuary from serious central nervous system (CNS) side-effects. A number of transporter proteins, including P-gp, MRP1 and BCRP, are localized in the brain capillary endothelial cell (Figure 3).¹⁹ This blood brain barrier could result in exclusion of anticancer drugs from reaching its target in the brain, thus negating the drug's efficacy. Furthermore, recent studies suggest that the transporters might work together cooperatively.²⁰ It was found that the area under

the brain and plasma tissue concentration-time curve (AUC) of the anticancer drug topotecan in *Mdr1a/b*^(-/-) or *Bcrp1*^(-/-) knockout mice was only 1.5-fold higher than wild-type mice, implying a modest role of *Mdr1a/b* and *Bcrp1* alone in topotecan efflux in the brain. In contrast, the AUC of topotecan was increased by 12-fold in the double knockout *Mdr1a/b*^(-/-)*Bcrp1*^(-/-) mice.¹⁷ These results suggest that P-gp and BCRP work cooperatively together in limiting the brain penetration of topotecan.

Figure 3. Localization of multidrug resistance transporters on brain capillary endothelial cell¹⁶

In addition to the ABC transporters (P-gp, MRPs and BCRP), several members of the organic anion transporting-polypeptide family (OATPs) and the organic anion transporter-family (OAT-family) are expressed in the brain.

1.4 Inhibitors/modulators of ABC transporter proteins

It has been discovered that some compounds can reverse MDR mediated by the ABC transporter proteins. These compounds are known as inhibitors, modulators, reversers, or chemosensitizers. Inhibitors/modulators reverse MDR in cells *in vitro* by interfering with the ability of the transporter to efflux drugs. They generally do not kill MDR cells directly, but when they are co-administered with a cytotoxic drug, they restore cytotoxicity of the drug because efflux of the drug is blocked. The IC_{50} of the cytotoxic drug shifts to a lower value, sometime to the same level as for the sensitive cells, in the presence of inhibitor/modulator, so the cells are killed. The potency of an inhibitor is usually measured by its EC_{50} which describe the concentration of inhibitors required to reduce the IC_{50} of the cytotoxic drug by half compared to control without the inhibitors. Tsuruo et al.²¹ first reported in 1983 that verapamil (VP), a calcium channel blocker, could reverse resistance by inhibiting P-gp mediated drug efflux. Since then, many inhibitors have been identified for P-gp, including cyclosporine,²² PSC 833 (valsopodar),²³ VX-710 (biricodar);²⁴ zosuquidar (LY335979), tariquidar (XR9576), laniquidar (R101933) and Elacridar (GF120918),²⁵ some with high potencies having low nM range of EC_{50} *in vitro*. Recently it has been reported that carbonic anhydrase XII (CA XII), an enzyme associated with pH regulation, is co-expressed

and co-located with P-gp in drug resistant cancer cells.²⁶ Some CA XII inhibitors, in combination with chemotherapeutics that are substrates for P-gp, appear to restore the efficacy of chemotherapy in resistant cells.²⁶

For MRP1, since its discovery in 1992 by Cole,²⁷ many MRP1 modulators have been extensively studied but with only limited success. Some of the MRP1 modulators include: MK-571,²⁸ probenecid²⁹ and some tricyclic isoxazoles.³⁰ The tricyclic isoxazoles are the most potent and specific MRP1 inhibitors with $EC_{50} = 93$ nM *in vitro* in reversing doxorubicin (DOX) resistance using MRP1 transfected HeLa-T5 cells.²⁶

In the case of BCRP, Ko143, an analogue of fumitremorgin C was identified as a potent inhibitor of BCRP with EC_{50} around 10 nM.³¹ Tariquidar, a third-generation P-gp inhibitor, has also been reported to inhibit BCRP with EC_{50} around 100 nM.³² Most recently, novel derivatives of acrylonitrile,³³ 2,4,6-substituted quinazolines ($EC_{50} = 20-71$ nM),³⁴ 2,4-disubstituted pyridopyrimidines ($EC_{50} = 37$ nM),³⁵ and 4-anilino-2-pyridylquinazolines and -pyrimidines ($EC_{50} = 21$ nM)³⁶ have been reported to be highly potent and nontoxic inhibitors of BCRP.

1.5 Clinical trials

Coadministration of a potent inhibitor of ABC transporter with an anticancer drug has been evaluated in several clinical trials to overcome MDR but led to a disappointing outcome.³⁷ Phase III clinical trials of P-gp inhibitors including PSC833,³⁸ Tariquidar³⁹ and Zosuquidar⁴⁰ have all ended with negative results.³³ An important factor for the lack of success may be due to the fact that in none of the clinical trials was patient selection based on prospective evaluation of the expression of the drug transporters. For example, in the trials that evaluated P-gp inhibitors in non-small cell lung cancer, transporters other than P-gp, such as MRP1 or BCRP might have accounted for the drug resistance.^{41,42} The factors pertaining to the inhibitors which have been suggested for the lack of success in this strategy of overcoming MDR are: (1) enhanced toxicity at non-target site due to the inhibitor, (2) drug-drug interaction, namely, the effect of the inhibitor on the pharmacokinetics of the anticancer drug leading to enhanced toxicity and (3) the inhibitor may adversely modify drug distribution in solid tumours.³³ It is suggested that further improvement of inhibitors of ABC transporters is necessary and should focus on potency, specificity and safety.⁴³ This review describes our research effort on novel flavonoid dimers as potent, non-toxic and specific inhibitors of ABC transporters.

2. Novel flavonoid dimers as potent, non-toxic and specific inhibitors of ABC transporters.

2.1 Flavonoids

Flavonoids are polyphenolic compounds that are naturally abundant in fruits, vegetables, nuts, flowers, wine, and tea.⁴⁴ They are common components of human food and are generally considered to be safe. Flavonoids have been associated with a range of beneficial properties for human health, such as antioxidizing, anti-inflammatory, antiviral, and anticancer properties.⁴⁵ Flavonoids have also been implicated in the modulation of ABC transporters.^{46,47} For example, apigenin was reported to inhibit MRP1-mediated LTC₄ transport in membrane vesicles at μM concentrations.⁴⁸ Even with their low toxicity, the flavonoid modulators have limitations.⁴⁹ The first is that their activity potency tend to be moderate, in the μM range. Second, they have a broad spectrum of biological activities including anti-estrogen and inhibition of other ATPases which may lead to undesirable side effects at high dosage.

2.2 Bivalent interactions in biological systems

An approach which may improve the potency of the flavonoids is to take advantage of the pseudo-dimeric structure (Figure 1) and the multiple binding sites of ABC transporters by using polyvalent interactions. Polyvalent interactions in biological systems refer to the simultaneous binding of multiple ligands on one biological entity.⁵⁰ Successful bivalent,⁵¹ trivalent⁵² and

pentavalent⁵³ ligands to different receptor systems with a level of potency 103-105 times higher than that of the corresponding monovalent ligands have been reported. Since flavonoids have moderate activity in inhibiting ABC transporters,^{42,43} there is presumably a binding site in the transporter for flavonoids. Because the transporter is pseudo-dimeric in structure (Figure 2), there may be an additional binding site for flavonoids. The additional binding site may be similar but not necessarily identical in structure to the first binding site. The concept may be illustrated in Figure 4. The hypothesis is that modulators with a linker joining two flavonoid moieties will bind to the ABC transporter (Figure 4 A) at one of the two binding sites of the ABC transporter (Figure 4 B). What happen next depends on the linker length. If the linker length is either too long or too short for the second flavonoid ligand to bind with the second binding site of the transporter, the modulator would behave as if it was a monovalent modulator (Figure 4 C), leading to a moderate potency. If the linker is of the appropriate length, the moderator binds to the transporter strongly (Figure 4 D) leading to higher potency. We proceeded to test this hypothesis.

Figure 4: Schematic illustration of bivalent interaction between ligands and biological receptor.

A: ABC transporter with pseudo-dimeric structure and two similar binding sites; B: ABC transporter binding with a bivalent modulator having two ligands joined by a linker; C: when the linker distance between the two ligands is not optimal, the ABC transporter binding with the modulator as if it was monovalent; D: when the linker distance between the two ligands is optimal, the ABC transporter binds strongly with the bivalent modulator.

2.3 Synthetic apigenin dimers with defined-length linkers as inhibitors of P-gp

We synthesized a series of apigenin-based flavonoid dimers **9a-9k**, linked by poly(ethylene glycol) chains of various lengths, and the flavonoid monomers **10a** and **10b**, (Figure 5A) and tested their chemo-sensitizing activities on P-gp.⁵⁴ We used the MDA435/LCC6 cell, an estrogen-independent

human breast cancer cell line with a classical MDR1 resistance pattern due to overexpression of P-gp. Paclitaxel is the anti-cancer drug of choice for treating breast cancer and a known substrate of P-gp. The IC_{50} of paclitaxel to the resistant MDA435/LCC6 cell was 115 nM (Figure 5B, DMSO, the solvent control). Verapamil (VP), a well-established P-gp inhibitor,²⁰ at 5 μ M was used as a positive control and the IC_{50} of paclitaxel was lower to 5.2 nM. Apigenin monomers **10a** and **10b** at 10 μ M were used as the negative controls. As shown in Figure 5B, different dimers potentiated the toxicity of paclitaxel to different extents. Compound **9d**, with a linker length of four ethylene glycol units, exhibited the most dramatic reversal activity and lower the IC_{50} of paclitaxel to 4.4 nM. The reversal activity is expressed in terms of the relative fold (RF), defined as the ratio of (IC_{50} without modulator) to (IC_{50} with modulator). The RF change of **9d** for paclitaxel was about 26, comparable to that of VP (RF=22). Compounds **9b** and **9c**, with linker lengths of two and three ethylene glycol units, also reversed paclitaxel resistance: their relative folds (RF) of IC_{50} were about 5.8 and 5.4 respectively. However, dimers with linkers shorter than two ethylene glycol units (**9a**) or longer than five ethylene glycol units (**9e**, **9f**, **9g**, **9i**) showed little or no reversing effect at 5 μ M. The apigenin monomers with three and four ethylene glycol units (**10a** and **10b**) as the negative controls had little reversing effect even when used at double the concentration (10 μ M).

of the dimers **9c** and **9d** (5 μ M) with the same number of ethylene glycol units. These results suggest that the modulating activity of **9d**, **9c**, and **9b** is due to their bivalent structures and is consistent with the concept illustrated in Figure 4. Compound **9d** also enhanced cytotoxicity of doxorubicin, daunomycin, vincristine, and vinblastine, all substrates of P-gp in MDA435/LCC6 cell as well as in P388/ADR (a drug resistant murine leukemia cell line), resulting in reduction of IC_{50} of the anticancer drugs by 5-50 times.⁵²

Figure 5: A: Structures of apigenin, flavonoid dimers **9a – 9i** and flavonoid monomers **10a** and **10b**, B: Modulation of paclitaxel resistance in LCC6MDR cells by flavonoid dimers **9a – 9i**, monomers **10a** and **10b**. DMSO was solvent control where no modulator was added. VP (verapamil) was positive control.

To further improve the potency of these dimers, a small library of flavonoid homodimers and heterodimers was synthesized and their *in vitro* activities in reversing cellular resistance to paclitaxel were evaluated.⁵⁵ Structure–activity relationships (SAR) study, using the cell line MDA435/LCC6MDR, revealed that replacement of the OH group in the A ring with H led to more potent P-gp modulators. Furthermore, nonpolar and hydrophobic substituents (e.g., methyl, ethyl, or fluoro groups) were better than polar and hydrophilic substituents (e.g., hydroxyl groups). Two

compounds (Table 1), **11** and **12**, showed promising reversing activity at sub-micromolar concentrations with no cytotoxic effects.⁵³

Compound	RF with DOX ^a	RF with vincristine ^a	RF with vinblastine ^a
11	4.4	16.8	7.7
12	9.8	44.8	14.1
VP	3.1	2.4	2.8

^a RF values were obtained by dividing IC₅₀ of drug without modulator by IC₅₀ of drug with 1 μ M modulator.

Table 1. Relative fold (RF) for VP and flavonoid dimers **11** and **12** modulating P-gp-mediated resistance to DOX, vincristine, and vinblastine in MDA435/LCC6MDR cells.

The flavonoid dimers **11** and **12** are, however, barely soluble in water because of their hydrophobic property. Attempts to use them for *in vivo* animal experiments proved to be difficult. In order to

improve their physicochemical properties as potential drug candidates, hydrophilic groups need to be introduced into the structure. Since it is known that introduction of hydrophilic groups into the flavonoid moiety reduced potency, the linker is left as the only possible site for structural modification. A series of new flavonoid dimers with nitrogen-containing PEG linker, were synthesized.⁵⁶ Their P-gp-modulating activity, cytotoxicity against cancer cells and normal cells were determined. Several of them are active. Compound **13b** which contains a N-benzyl group at the linker has the highest potencies in reversing P-glycoprotein (P-gp) mediated resistance in LCC6MDR cells to various anticancer drugs with EC_{50} in the nanomolar range (Figure 6).⁵⁷ Furthermore, it has low cytotoxicity towards normal fibroblast L929 cell which has been used as an indication of the safety of the compound. The efficacy of **13b** in reversing drug resistance in cancer chemotherapy has been evaluated *in vivo* in an animal model.⁵⁵

Figure 6. Structure activity relationship of flavonoid dimers in modulating P-gp. EC₅₀ is defined as the concentration of flavonoid dimers needed to reduce the IC₅₀ of PTX of LCC6MDR by 50%.

2.4 *In vivo* efficacy of flavonoid dimer **13b** on modulating paclitaxel (PTX) resistance in LCC6MDR xenograft.

The flavonoid dimer **13b** can modulate multidrug resistance toward paclitaxel (PTX) and other anti-cancer drugs such as vinblastine, vincristine, doxorubicin, daunorubicin, and mitoxantrone in cancer cell LCC6MDR *in vitro*. Pharmacokinetics studies indicated that intraperitoneal administration of 45 mg/kg **13b** was enough to maintain a plasma level above 148 nM, the EC₅₀ for reversing PTX resistance in LCC6MDR cells, for more than 600 min.⁵⁷ Toxicity studies with **13b** (90 mg/kg, i.p. for 12 times in 22 days) with PTX (12 mg/kg, i.v. for 12 times in 22 days) revealed no obvious toxicity or death in mice.⁵⁷ An *in vivo* PTX-resistant human breast cancer xenograft model in female Balb/c nu nu mice was established. Without any PTX treatment, parental LCC6 xenograft can grow to $611 \pm 8 \text{ mm}^3$ in 27 days (Fig. 7A), whereas PTX treatment (12 mg/kg; i.v. for 8 times in 17 days) reduced LCC6 xenograft volume to $248 \pm 78 \text{ mm}^3$ on 27th day (Fig. 7A), suggesting that LCC6 xenograft was sensitive to PTX treatment. In contrast, the resistant LCC6MDR xenograft did not respond to PTX treatment. The tumour grew to 1029 ± 82 and $879 \pm 127 \text{ mm}^3$ on 27th day for solvent control or upon PTX treatment (12 mg/kg; i.v. for 8 times in 17 days), respectively (Fig. 7A). We tested if **13b** can modulate PTX resistance using the

above LCC6MDR xenograft animal model. **Compound 13b** (solvent or 45 mg/kg; i.p.) was administered 1 hour before PTX (solvent or 12 mg/kg; i.v.), for a total of 12 times in a period of 22 days (arrows in Fig. 7B). There was no significant difference in LCC6MDR xenograft volume on 32nd day for controls including [PTX solvent + **13b** solvent] ($1422 \pm 174 \text{ mm}^3$), [PTX solvent + **13b** (45 mg/kg)] ($1225 \pm 84 \text{ mm}^3$) and [PTX (12 mg/kg) + **13b** solvent] ($1201 \pm 118 \text{ mm}^3$) (Fig. 7B). Tumour doubling time (9 days) was almost identical for these 3 controls. In contrast, treatment with [PTX (12 mg/kg) + **13b** (45 mg/kg)] reduced LCC6MDR tumor volume to $648 \pm 83 \text{ mm}^3$ after 30 days of treatment (Fig. 7B). Tumor doubling time was increased to 13.0 ± 1.1 days. No animal deaths occurred in the treatment group and there was no significant difference in wet weight of vital organs (liver, kidneys, spleen, heart and brain) between the 3 control groups and the treatment group [PTX (12 mg/kg) + **13b** (45 mg/kg)]. These results suggested that **13b** (45 mg/kg) was effective in modulating PTX resistance in *in vivo* LCC6MDR xenografted Balb/c nude mice model without any significant toxicity. In parallel, we measured the ability of PSC833, a well-established P-gp inhibitor,²³ in the same animal model. PSC833 (2.5 mg/kg; i.p.) was administered 1 h before PTX (8 mg/kg; i.v.) with the treatment protocol of eight times in a period of 17 days (Figure 7B). The treatment resulted in a reduction of LCC6MDR tumor volume to 430

$\pm 60 \text{ mm}^3$ after 30 days of treatment (Figure 7B). However, significant toxicity (five deaths out of seven animals) was observed. These results suggested that PSC833 although effective in modulating P-gp-mediated PTX resistance in this xenograft model, can induce severe toxicity and animal death.⁵⁷

Figure 7: *In vivo* efficacy of **13b** on modulating P-gp-mediated PTX resistance.⁵⁷ Balb/c nu nu mice were xenografted with either (A) LCC6 or (B) LCC6MDR cells and used as an *in vivo* PTX-sensitive or PTX-resistant cancer model, respectively. PSC833, a well-known inhibitor of P-gp,²³ was used as positive control.

2.5 Synthetic flavonoid dimers as inhibitors of MRP1

Because MRP1 is structurally and functionally similar to P-gp (Figure 1), it is reasonable to expect that the same bivalent approach can be applied to discover potent inhibitor of MRP1 transport activity. The apigenin dimers **9b** to **9h** were similarly tested for modulating activity at 0.5 μM concentration against MRP1. Two cell lines were used in this study, namely the 2008/P cell line (a human ovarian carcinoma) and its MRP1-transfected derivative (2008/MRP1). 2008/MRP1 cells ($\text{IC}_{50}=577\text{ nM}$) are more resistant to DOX than the parental 2008 cells ($\text{IC}_{50}=6.3\text{ nM}$) (Table 2). Apigenin dimers showed encouraging modulating activity relative to the positive controls including verapamil, and probenecid (Table 2).⁵⁸ The dimers **9e** and **9f**, with 5 and 6 EG units, showed the highest modulating activity whereas dimers with shorter (**9b**, **9c** and **9d**) or longer (**9g**) linker lengths showed a relatively lower MRP1-modulating activity (Table 2). A series of flavonoid dimers with the OH groups removed from C-5 and C-7 and replaced with different substituents were synthesized and evaluated for activity.⁵⁵ The biological data showed that removal of OH groups from **9** increased the modulating activity in 2008/MRP1 cells. Furthermore, the monomer **14d-M** showed no modulating activity ($\text{RF}=1.0$), even when it was added at a double concentration, compared to its dimer **14d** ($\text{RF}=3.8$). The result suggests that the modulating activity of flavonoid dimers is due to their bivalent interaction with MRP1. Among those

flavonoid dimers with optimal PEG linker lengths of 5 or 6, addition of methyl group at either C-6 (**15e** and **15f**) or C-7 position of A-ring (**16e** and **16f**) resulted in the highest MRP1-modulating activity (RF ranging from 10.7 to 13.7) (Table 2). Among different flavonoid dimers, **15e** has the lowest EC₅₀ (73 nM) in reversing DOX resistance in 2008/MRP1 cells (Table 2). In contrast, the standard MRP1 inhibitors verapamil, MK571, and probenecid have higher EC₅₀ values of 2.6, 18, and >500 μ M respectively. These flavonoid dimers can thus be considered as effective modulators of MRP1-mediated drug resistance in cancer cells. However, they are similar in their physicochemical properties to the P-gp inhibitors **11** and **12** of being hydrophobic with relatively low aqueous solubility which render them poor candidate for further *in vivo* animal study.

Compound 14d: n=4, R₁=R₂=R₃=H;
 15e: n=5, R₁=R₃=H, R₂=Me;
 15f: n=6, R₁=R₃=H, R₂=Me;
 16e: n=5, R₁=R₂=H, R₃=Me;
 16f: n=6, R₁=R₂=H, R₃=Me;

Compound 14d-M: n=4, R₁=R₂=R₃=H;

Modulators	EC ₅₀ for lowering DOX resistance of in 2008/MRP1
15e	73±13 nM
15f	105±20 nM
16e	133±16 nM
16f	86±23 nM
Verapamil	2.6±1.0 µM
MK571	18 µM
Probenecid	>500 µM

Table 2: EC₅₀ values of 15e, 15f, 16e, 16f, verapamil, MK571 and probenecid.

3. Triazole-linked flavonoid dimers via Click chemistry

1,4-Disubstituted 1,2,3-triazoles can be synthesized readily by the copper(I) catalyzed Huisgen 1,3-dipolar cycloaddition reaction between azides and alkynes (commonly known as CuAAC reaction)⁵⁹ according to Scheme 1. The CuAAC reaction, belonging to a class of reactions known as “click chemistry”,⁶⁰ features high efficiency (usually ~100% reaction yield) and chemoselectivity. They offer a rapid method to assemble compound libraries and permit direct *in vitro* screening of the triazole products without the need of purification. Because the triazoles are basic, their salts are usually soluble in aqueous medium and may have better physiochemical properties as potential drug candidates. High throughput screening platform based on “click chemistry” has been developed in cases where the target enzyme is available.⁶¹ Click chemistry has also been reported in the study of P-gp mediated cellular efflux.⁶² However, because whole cells have to be used for the bioassay of ABC transporter-mediated cellular efflux, high throughput screening platform analogous to the enzyme inhibition had not been developed. Only a small library of pure click products was prepared and studied.⁶³ With a view to rapidly generate a large number of dimeric MRP1 modulators which may have better physiochemical properties as potential drug candidates, we have developed a Click-chemistry-based high throughput screening.

Scheme 1.

3.1. Discovery of Novel Flavonoid Dimers to Reverse Multidrug Resistance Protein 1 (MRP1, ABCC1) Mediated Drug Resistance in Cancers Using a High Throughput Platform with “Click Chemistry”

A library of 300 triazole bridged flavonoid bearing molecules was constructed according to Scheme 1 from twenty-five flavonoids bearing terminal alkynes (**AcN**), and twelve flavonoids bearing azido groups (**AzM**). The crude library was prepared in microtiter plates and in millimolar scale by placing 1 mM of each acetylene (**AcN**), 1 mM azides (**AzM**) in 100 μ L of THF solvent, followed by addition of catalytic amount of bromotris(triphenylphosphine)copper(I) to yield about 1 mM of triazole bridged products (**AcNAzM**) without purification, recognizing that each of the unpurified 300-member library contained at least four components: (1) the alkyne **AcN**, (2) the azide **AzM**, (3) the catalyst bromotris(triphenylphosphine)copper(I) and (4) the product **AcNAzM**. The assembled products were directly screened for MRP1 modulating activity in 96-well plates

containing 2008/MRP1 cells and the anticancer drug, doxorubicin, at 100 nM concentration, a concentration at which it showed no toxicity toward 2008/MRP1 cells. The relative potency of MRP1-modulating activity of the clicked dimers was determined by MTS proliferation assay and compared to those with DOX but without the unpurified clicked dimer.⁶⁴ Controlled experiments with the pure alkyne, with the pure azide, or with the catalyst were also performed and they showed no or low MRP1-modulating activity. In this primary screening, out of the 300-member library, 53 members were considered as “hit” compounds with % of survival ranging from 20% to 49%. Among these 53 “hits”, 18 of them exhibited cytotoxicity toward 2008/MRP1 cells at 2 μ M. After exclusion of these 18 cytotoxic flavonoid dimers, the remaining 35 “hit” compounds were further screened at a lower concentration of 1 μ M. At this lower concentration, 21 compounds consistently maintained promising modulating activity. These top 21 “hit” compounds (% survival of <51% at 1 μ M) were resynthesized, purified, and their MRP1-modulating activities were determined.⁶⁴ It is interesting to note that of the 21 active compounds discovered by this process, their EC₅₀ values ranged from 53 nM to 298 nM and did not show cytotoxicity toward L929 cells with IC₅₀ > 50 μ M. Furthermore, they all have linkers with 13-17 atoms between the two flavonoid moieties. This is consistent with the previous conclusion in section 2.4 that flavonoid dimers with optimal

PEG linker lengths of 5 or 6, i.e., 15-18 atoms, between the two flavonoid moieties, resulted in the highest MRP1-modulating activity. Among the triazole bridged flavonoid dimers, **Ac3Az11** has the lowest EC_{50} in 2008/MRP1 cells ($EC_{50} = 53$ nM).⁶⁴ Under similar protocol, it is more potent than the well-established MRP1 inhibitors verapamil, MK571 and compound **15e** (Table 2). These results indicate that the CuAAC reaction coupled with high throughput screening is a reliable platform for the rapid discovery of MRP1 modulators.

Figure 8: Chemical structure of **Ac3Az11**

3.2. *In silico* docking studies of bovine MRP1 with flavonoid dimers **Ac3Az11**, **15e** and **DOX**

The electron cryo-microscopy structures of bovine MRP1 in the apo form, the substrate bound form, and an ATP-bound outward facing form have been reported recently.^{7,8} Due to the high similarities in amino acid sequences and functional properties between bovine and human MRP1,

it is reasonable to assume that the structure of bovine MRP1 would be a reasonable starting point for structural studies of human MRP1. By comparing the two inward facing structures, the substrate LTC₄ is found to bind to two pockets (H and P) of bMRP1, with one pocket in TMD1 and the other pocket in TMD2.⁷ In addition, conformational change is induced by substrate binding, bringing the two halves of the transporter together. The flavonoid dimer modulators in our study may bind to the same bipartite substrate binding site.

In the *in silico* study, the bovine MRP1 was used to perform the docking studies with flavonoid dimers **Ac3Az11**, **15e** and DOX using CLC Drug Discovery Workbench software.⁶⁴ The substrate LTC₄ was used as the reference compound. Compounds **Ac3Az11**, **15e**, LTC₄ and DOX were found by the *in silico* docking study to interact with bovine MRP1 in the same binding site with a docking score of -114, -110, -89 and -82 respectively (Figure 9).⁶⁴ The scores suggest that both flavonoid dimers **Ac3Az11** and **15e** are expected to bind more strongly than LTC₄ or DOX and function as competitive inhibitor. We demonstrated experimentally that **15e** is a competitive inhibitor of DOX transport by MRP1, presumably by binding to the same binding site as the substrate.⁵⁶

Figure 9. *In silico* docking. (A1) Representation of bovine MRP1 with labeled TMD0, TMD1, TMD2, NBD1 and NBD2 (PDB ID: 5UJA). The red square indicates the bipartite binding site with zoomed in view of (A2) **Ac3Az11** (violet color), (A3) **15e** (blue color) and (A4) DOX (brown color) in the same binding site with important amino acids highlighted in black and hydrogen bonding interactions indicated as dotted blue lines.⁶⁴

3.3. Triazole Bridged Flavonoid Dimers as Potent, Nontoxic, and Highly Selective Breast Cancer Resistance Protein (BCRP/ABCG2) Inhibitors

Out of the 300 member synthetic library of triazole linked flavonoid dimers in the above study, we also found that some of the compounds showed inhibitory activities against BCRP.⁶⁴ To further investigate, additional 74 pure triazole and *bis*-triazole bridged homo- and hetero-flavonoid dimers were synthesized and tested with biological assays for inhibitory activity against BCRP, MRP1, and P-gp.⁶⁵ Four different cell lines were employed in the biological assays: (i) BCRP-transfected human embryonic kidney cell line HEK293/R2; (ii) mitoxantrone selected breast cancer cell line MCF7-MX100 in which the BCRP transporter protein was found to be overexpressed; (iii) MRP1-transfected ovarian cancer cell line 2008/MRP1; and (iv) P-gp-transfected human breast cancer cell line LCC6MDR. Among the 74 compounds tested, 11 compounds, **Ac2Az9**, **Ac3Az4**, **Ac3Az9**, **Ac3Az11**, **Ac5Az8**, **Ac5Az9**, **Ac12Az8**, **Ac12Az9**, **Ac13Az9**, **Ac15(Az8)₂**, and **Ac15(Az9)₂**, displayed a more potent inhibitory activity against BCRP than Ko143 in the two cell lines HEK293/R2 and MCF7-MX100 in restoring topotecan cytotoxicity with EC₅₀ in the range of 0.9–7.9 nM. Importantly, these potent compounds were also nontoxic toward L929 with IC₅₀ > 100 µM whereas Ko143 had an IC₅₀ = 31 µM. The inhibitory activities of these 74 compounds

were also tested against MRP1 and P-gp. Compounds **Ac2Az4**, **Ac3Az11**, **Ac5Az1**, **Ac12Az1**, **Ac16Az1**, and **Ac16Az2** displayed high MRP1 inhibitory activities, more potent than the positive control **FD-4e**. In contrast, the most active P-gp inhibitors **Ac3Az11** and **Ac3Az12** were less potent in their P-gp inhibitory activity than the positive control, cyclosporine A. In this study, all the potent MRP1 inhibitors have linkers with 13–17 atoms between the two flavonoid moieties, similar in linker length to **15e** or **15f** with $n = 5-6$ ethylene glycol units, equivalent to 15–18 atoms between the two flavonoid moieties. For the most active P-gp inhibitors, **Ac3Az11** and **Ac3Az12** contain 13–16 atoms between the two flavonoids, slightly longer than the optimal length of **11** or **12** with $n = 4$ ethylene glycol units or 12 atoms in between the two flavonoids. In this context, the 11 most potent compounds against BCRP contain 13–27 atoms between the two flavonoids. The linker length requirement for inhibitory activity against BCRP thus appears to be less stringent and can be quite variable. Specifically, the two *bis*-triazole bridged dimers, **Ac15(Az8)₂** and **Ac15(Az9)₂**, have the longest linker length with 21 and 27 atoms, respectively, and are beyond the linker length requirement for P-gp or MRP1 inhibition. Indeed, as expected from their longer than optimal linker length for MRP1 and P-gp inhibition, **Ac15(Az8)₂** and **Ac15(Az9)₂**, show only

modest activity toward MRP1 ($EC_{50} = 442\text{--}458\text{ nM}$) and essentially no activity toward P-gp ($EC_{50} > 1000\text{ nM}$).

To further improve the BCRP selectivity, analogues of **Ac15(Az8)₂** and **Ac15(Az9)₂** had been synthesized by replacing **Ac15** with various diacetylenes. Five dimers, **Ac22(Az8)₂**, **Ac24(Az8)₂**, **Ac25(Az8)₂**, **Ac26(Az8)₂**, and **Ac26(Az9)₂**, displayed a more potent activity than Ko143 in inhibiting BCRP and were essentially devoid of inhibitory activity against MRP1 and P-gp ($EC_{50} > 1000\text{ nM}$). All five compounds were nontoxic toward normal L929, 3T3, and HFF-1 cells with $IC_{50} > 100\text{ }\mu\text{M}$. (Table 3). They are therefore potent ($EC_{50} = 3\text{--}5\text{ nM}$), nontoxic and selective inhibitors of BCRP.⁶⁵

Compounds		IC ₅₀ (μM) towards			EC ₅₀ (nM) needed for reversing MDR				BCRP-selectivity			
					BCRP-mediated topotecan resistance	BCRP-mediated topotecan resistance	MRP1-mediated DOX resistance	P-gp mediated paclitaxel resistance	Relative to MRP1-inhibitory activity		Relative to P-gp-inhibitory activity	
		L929	3T3	HFF-1	HEK293/R2	MCF7-MX100	2008/MRP1	LCC6MDR	HEK293/R2	MCF7-MX100	HEK293/R2	MCF7-MX100
Ac15(Az8) ₂		>100	>100	>100	5.3 ± 2.7	3.3 ± 4.1	457.5 ± 10.6	>1000	86	139	189	303
Ac15(Az9) ₂		>100	>100	>100	3.3 ± 2.1	4.3 ± 4.6	441.7 ± 14.4	>1000	134	103	303	233
Ac22(Az8) ₂		>100	>100	>100	2.2 ± 1.5	1.1 ± 0.4	>1000	>1000	455	909	455	909
Ac22(Az9) ₂		>100	>100	>100	2.8 ± 1.7	12.8 ± 1.1	>1000	>1000	357	78	357	78
Ac23(Az8) ₂		>100	ND	ND	13.5 ± 0.1	26.5 ± 9.2	>1000	>1000	74	38	74	38
Ac23(Az9) ₂		>100	>100	>100	2.4 ± 1.1	13.7 ± 4.0	>1000	>1000	417	73	417	73
Ac24(Az8) ₂		>100	>100	>100	6.1 ± 2.5	2.4 ± 1.0	>1000	>1000	164	417	164	417
Ac25(Az8) ₂		>100	>100	>100	4.2 ± 1.6	2.1 ± 1.6	>1000	>1000	238	476	238	476
Ac26(Az8) ₂		>100	>100	>100	6.8 ± 2.2	2.2 ± 2.3	>1000	>1000	147	455	147	455
Ac26(Az9) ₂		>100	>100	>100	5.8 ± 2.0	3.9 ± 1.6	>1000	>1000	172	256	172	256
Ac28(Az8) ₂		>50	ND	ND	48.8 ± 6.8	24.5 ± 7.6	>1000	>1000	20	41	20	41
Ac29(Az8) ₂		>100	ND	ND	17.8 ± 11.4	18.7 ± 11.6	>1000	683.2 ± 301.4	56	53	38	37
Ac29(Az9) ₂		>100	ND	ND	30.7 ± 8.3	39.5 ± 20.5	>1000	>1000	33	25	33	25
Ac30(Az8) ₂		>100	ND	ND	108.3 ± 28.4	55.4 ± 23.5	>1000	573.3 ± 23.1	9	18	5	10
Ac31(Az8) ₂		>100	ND	ND	133.3 ± 20.8	84.0 ± 27.5	>1000	>1000	8	12	8	12
Ko143		31.4 ± 5.0	>100	56.5 ± 5.1	8.7 ± 4.9	9.0 ± 2.1	1950.0 ± 354	1060.0 ± 207.8	224	217	122	118

	BCRP-selectivity > 400
	BCRP-selectivity = 301 - 400
	BCRP-selectivity = 201 - 300
	BCRP-selectivity = 101 - 200
	BCRP-selectivity < 100

Table 3. EC₅₀ (nM) of Ac15(Az8)₂ and Ac15(Az9)₂ analogues for reversing MDR and their BCRP-selectivity.

3.4. *In Silico* Docking Studies of human BCRP with Ac22(Az8)₂, mitoxantrone and topotecan.

High-resolution cryo-electron microscopy (cryo-EM) structures of human BCRP in complex with its substrate estrone-3-sulfate (PDB ID: 6HCO) and with potent inhibitors (PDB ID: 6ETI and 6FEQ) have been reported recently.^{12,13} Based on these structures, the drug-binding site of inward-facing BCRP was observed to preferentially bind flat, hydrophobic and polyphenolic molecules of either BCRP substrates or inhibitors. We have interrogated the interactions of the flavonoid dimer **Ac22(Az8)₂**, the substrates topotecan and mitoxantrone, with BCRP using *in silico* molecular docking studies. Using the BCRP model (6ETI)¹³, the binding poses with the top-ranked scores positioned the inhibitor **Ac22(Az8)₂** and the two substrates into the same drug-binding site located at the central trans location pathway of BCRP (Figure 10). For other BCRP models (6HCO and 6FEQ), top-ranked binding poses were found to be similar.⁶⁵ With all three BCRP models, the top-ranked scores of flavonoid dimer **Ac22(Az8)₂** were generally lower than that of mitoxantrone and topotecan (Table 4), suggesting that **Ac22(Az8)₂** acted as inhibitor by binding preferentially to BCRP and blocking the access for the substrates topotecan or mitoxantrone.⁶⁵ Detailed analysis revealed that strong π - π interactions were formed between the linker part 1,3-*bis*-(triazol-4-yl)benzene of **Ac22(Az8)₂** and Phe439 of helix 2' as well as between one of the benzyl groups of **Ac22(Az8)₂** and Phe547 of helix 5a (Figure 10B). In addition, extensive networks of van der Waals

contacts with many amino acid residues existed inside the drug binding site of BCRP, thus providing strong interaction with BCRP. It had been previously proposed that a “leucine plug” (Leu554 and Leu555), located in a flexible loop between helix 5a and 5b, acted as a gate to allow BCRP substrate moving outside (Figure 10A).¹³ In the docking studies, one of the flavonoid moieties of **Ac22(Az8)₂** was predicted to insert into a cavity between helix 5a and 5b and form van der Waals contacts with amino acid residues of helix 5a and 5b (Leu568, Leu565). We suggested that by occupying the cavity, this flavonoid moiety might reduce the flexibility of this loop and allow only very limited movement of the “leucine plug” and account for its inhibitory activity.⁶⁵ Structural comparison between transporters P-gp, MRP1, and BCRP⁵⁻⁷ revealed that such cavity exists only in BCRP but not in P-gp or MRP1. We believe this structural difference may account for the high BCRP specificity of **Ac22(Az8)₂** over P-gp and MRP1.

Figure 10. *In silico* docking study using cryo-EM structure of BCRP (PDB ID: 6ETI). (A) Ribbon diagram of the overall human BCRP homodimer with labeled TMD, NBD and “leucine plug”. The red square indicates the location of binding pocket of estrone-3-sulfate, MZ29, MB136, mitoxantrone, topotecan and Ac22(Az8)₂; Top-ranked binding poses of (B) inhibitor Ac22(Az8)₂ (blue sticks), (C) substrate mitoxantrone (deep green sticks) and (D) substrate topotecan (brown sticks). Transmembrane helices 1b, 1b', 2, 2', 5a and 5a' were labeled in red. Important amino

acid residues (grey sticks) are highlighted in black and hydrogen bonding interactions are indicated as dotted blue lines.⁶⁵

Table 4. *In silico* docking study. The binding scores of the top-ranked binding poses of **Ac22(Az8)₂**, mitoxantrone and topotecan using different cryo-EM structures of BCRP.

PDB ID	Ligands docked		
	Ac22(Az8) ₂	Topotecan	Mitoxantrone
6HCO	-126	-80	-82
6ETI	-137	-88	-92
6FEQ	-122	-81	-91

4. Discussion and Conclusion

Overexpression of ABC transporters such as P-gp, MRP1 and BCRP has been associated with tumor MDR and poor patient outcome. To circumvent MDR, combination of inhibitors and anticancer drugs has been considered as a potential treatment. By taking advantage of the pseudo-dimeric structure (Figure 1) of ABC transporters, we demonstrated that some flavonoid dimers,

using polyvalent interactions, can be potent inhibitors of the ABC transporters.⁵⁴⁻⁵⁸ These flavonoid dimers can be synthesized readily by using the copper(I) catalyzed Huisgen 1,3-dipolar cycloaddition reaction (commonly known as CuAAC reaction) between flavonoid monomers bearing azides and flavonoid monomers bearing alkynes to form triazole-linked flavonoid dimers (Scheme 1).^{64,65} Through biochemical and *in silico* docking studies, it is suggested that these flavonoid dimers bind strongly to the substrate binding sites of the ABC transporters and function as competitive inhibitors. As revealed by their cryo-EMs, the three transporters P-gp, MRP1 and BCRP, have different sizes for their substrate binding cavities.^{5,8,12-14} Selective inhibition of the three different transporters with the flavonoid dimers can be achieved by placing the two flavonoid moieties at an optimal distance apart specific for each of the transporters. For the most potent P-gp inhibitors, a distance of 12 atoms between the two flavonoids moieties proved to be optimal (Figure 5).⁵⁴ In the case of MRP1 inhibitors, a distance of 13-17 atoms between the two flavonoid moieties resulted in the highest MRP1-inhibiting potency.⁶⁴ The linker length between the two flavonoid moieties for inhibitory activity against BCRP appeared to be less stringent and could be quite variable. The *bis*-triazole bridged flavonoid dimers, such as **Ac15(Az8)₂** and **Ac15(Az9)₂**, having the linker length with 21 and 27 atoms between the two flavonoids, are potent BCRP inhibitors but are essentially devoid of inhibitory activity against MRP1 and P-gp (Table 3).⁶⁵ These flavonoid dimers, in addition to being potent and selective inhibitors of the transporters, are found to be nontoxic to normal cells at the effective concentrations. For examples, the flavonoid dimers which are potent inhibitors for BCRP with EC₅₀ in the nmol range are nontoxic toward normal L929, 3T3, and HFF-1 cells with IC₅₀ > 100 µM. (Table 3). On the other hand, poor bioavailability is a hurdle that often confronts most flavonoids. Furthermore, because these flavonoid dimers have high molecular weights, there is concern about the druggability of these

compounds. We demonstrated by pharmacokinetics studies that intraperitoneal administration of 45 mg/kg **13b** was sufficient to maintain a plasma level above the EC_{50} for reversing PTX resistance in LCC6MDR cells, for more than 600 min.⁵⁷ The *in vivo* efficacy of the flavonoid dimers has been demonstrated. Compound **13b** (45 mg/kg) was effective in modulating PTX resistance in *in vivo* LCC6MDR xenografted Balb/c nude mice model without any significant toxicity (Figure 7). Further investigation of these flavonoid dimers as clinical candidates to overcome multidrug resistance in cancer chemotherapy is warranted.

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5. Conflict of Interest

The authors declare no competing financial interest. The patents WO 2007/135592, WO 2011/137516, WO 2013/127361 associated with this manuscript have been licensed to Athenex Inc.

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