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Modulation of Multidrug Resistance Protein 1 (MRP1/ABCC1)-Mediated Multidrug Resistance by Bivalent Apigenin Homodimers and Their Derivatives

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Here we showed that bivalency approach is effective in modulating multidrug resistance protein 1 (MRP1/ABCC1)-mediated doxorubicin (DOX) and etoposide (VP16) resistance in human 2008/MRP1 ovarian carcinoma cells. Flavonoid dimers bearing five or six ethylene glycol (EG) units with 6-methyl (**4e**, **4f**) or 7-methyl (**5e**, **5f**) substitution on the ring A of flavonoid dimers have the highest modulating activity for DOX against MRP1 with an EC_{50} ranging from 73 to 133 nM. At 0.5 μM, the flavonoid dimer **4e** was sufficient to restore DOX accumulation in 2008/MRP1 to parental 2008/P level. Lineweaver−Burk and Dixon plot suggested that it is likely a competitive inhibitor of DOX transport with a $K_i = 0.2\,\mu$M. Our data suggest that flavonoid dimers have a high affinity toward binding to DOX recognition site of MRP1. This results in inhibiting DOX transport, increasing intracellular DOX retention, and finally resensitizing 2008/MRP1 to DOX. The present study demonstrates that flavonoid dimers can be employed as an effective modulator of MRP1-mediated drug resistance in cancer cells.

Introduction

Tumor cell resistance to a wide spectrum of anticancer agents continues to be a major obstacle to successful cancer chemotherapy. This resistance is referred to as multidrug resistance (MDR[a]). One of the mechanisms by which tumors acquire MDR is the overexpression of ATP-binding cassette (ABC) superfamily of membrane transporter such as drug efflux pump P-glycoprotein (P-gp/ABCB1) and multidrug resistance protein 1 (MRP1/ABCC1). The transport protein of ABC superfamily is involved in ATP-driven transmembrane transport of structurally and functionally unrelated compounds. Most ABC transporters share a conserved structure of two hydrophobic membrane spanning domains (MSDs), each with six transmembrane (TM) α-helices and two highly conserved nucleotide-binding domains (NBDs) with the general structure of NH_2-MSD_1-NBD-MSD_2-NBD-CO_2H. In contrast, MRP1 and its related proteins MRP2 (ABCC2), MRP3 (ABCC3), MRP6 (ABCC6), and MRP7 (ABCC10), and the sulfonylurea receptors, SUR1 (ABCC9) and SUR2 (ABCC8), are five domain proteins (NH_2-MSD_0-MSD_1-NBD-MSD_2-NBD-CO_2H) with two NBDs and three MSDs.[1−3] The extra NH_2-terminal MSD, MSD_0, consists of five TMs, and its function in these proteins may be important for transport activity.[4−6]

MRP1 was identified by Cole et al. in 1992 by virtue of its overexpression in a drug-selected human small lung cancer cell line H69AR.[7] When overexpressed in tumor cells, MRP1 confers resistance to anticancer drugs that include *Vinca* alkaloids, epipodophyllotoxins, and anthracyclines, as well as some heavy metal oxyanions.[8−10] MRP1 has been shown to be a primary active transporter of glutathione-, glucuronide-, and sulfate-conjugated organic anions, such as glutathione conjugate cysteinyl leukotriene 4 (LTC_4) and glucuronate conjugate 17β-estradiol 17-β-(D-glucuronide) (E217βG).[11−16] Therefore, MRP1 is also known as multispecific organic anion transporter (MOAT) or GS-X pump.[17,18] In addition, transport of some unmodified chemotherapeutic drugs by MRP1 is GSH-dependent. GSH appears to be cotransported with these compounds[19−21] or that GSH in some way activates MRP1, facilitating substrate binding and/or transport.

P-gp,[22] MRP1,[7] and breast cancer resistance protein (BCRP/ABCG2)[23] are the three major ABC members that confer cancer MDR.[24] There has been a lot of interest in developing modulators and/or inhibitors of these transporters with the ultimate goal of reversing cancer MDR. Since the discovery of MRP1 in 1992 by Cole,[7] many MRP1 modulators have been extensively studied but with only limited success.[25,26] Some of the MRP1 modulators include: (1) nonspecific inhibitors of organic anion transporters like NSAIDs (e.g., indomethacin)[27−30] and probenecid,[28] (2) leukotriene receptor antagonist (e.g., **9** (MK571) and **10** (ONO-1078)) (Figure 1),[31,32] (3) P-gp inhibitors like verapamil and

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[a]Abbreviations: MDR, multidrug resistance; ABC, ATP-binding cassette; MRP1, multidrug resistance protein 1; P-gp, P-glycoprotein; TMD, transmembrane domain; NBD, nucleotide binding domain; DOX, doxorubicin; VP16, etoposide; PEG, polyethylene glycol; RF, relative fold; K_i, inhibition constant.

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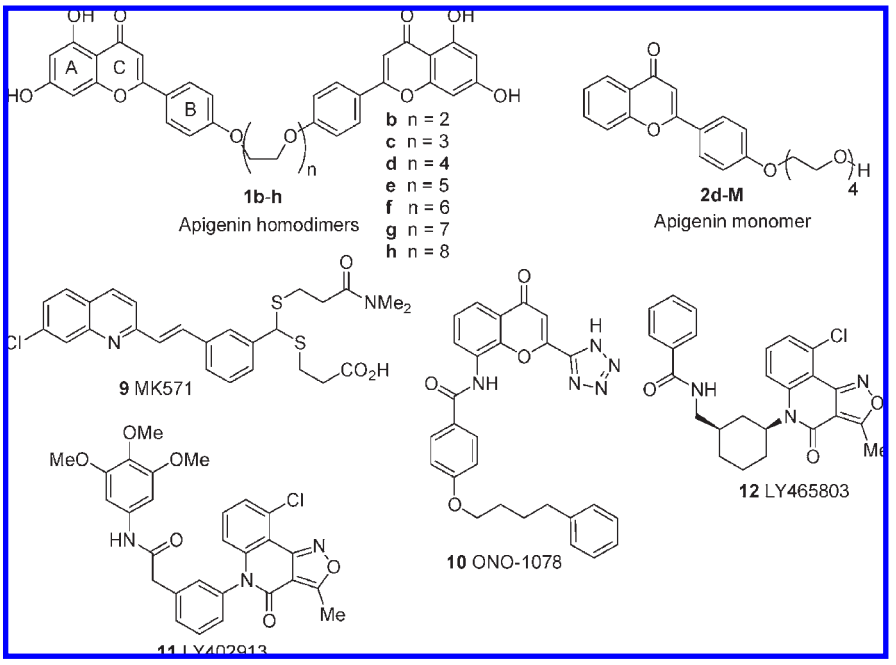


Figure 1. Structures of apigenin homodimers **1**, apigenin monomer **2d-M**, and known MRP1 inhibitors.

cyclosporine A,[33] (4) tricyclic isoxazoles (e.g., **11** (LY402913) and **12** (LY465803)) (Figure 1),[34−36] and (5) different kinds of naturally occurring flavonoids.[37−44] The tricyclic isoxazoles are the most potent and specific MRP1 inhibitors with EC_{50} = 93 nM (EC_{50} = effective concentration that can lower the IC_{50} of resistant cancer cells by 50%) in vitro in reversing doxorubicin (DOX; $C_{27}H_{29}NO_{11}$) resistance using MRP1-transfected HeLa-T5 cells and in vivo (improvement in vincristine efficacy in nude mice model with 63% regression in tumor volume when used at 8 mg/kg dose).[34−36]

Flavonoids are polyphenolic compounds commonly found in fruits, vegetables, and plant-derived products of the human diet. They have long been associated with many beneficial properties, such as antioxidant, anti-inflammatory, anticancer, and antiviral activities.[45−48] Because humans consume large amounts of flavonoids daily, it is generally accepted that flavonoids are not toxic. In the past decades, some flavonoids have been implicated in the modulation of P-gp type MDR in cancers and shown to inhibit a variety of ATP-binding proteins such as plasma membrane ATPase.[49,50] Therefore, flavonoids that are consumed daily and without any detrimental side effects are attractive candidates for development of novel modulators of MDR.

We have previously reported that, by using a bivalent approach, synthetic apigenin homodimers **1** with polyethylene glycol (PEG) linker (Figure 1) can modulate the P-gp activity in human cancer[51,52] and parasitic protozoan *Leishmania*[53,54] much more potently than the monomeric apigenin. The highest efficacy was found for apigenin homodimer **1d** having four ethylene glycol (EG) units as a linker.[51,53] This result indicates that the bivalent approach is successful in enhancing the reversal activity of apigenin on Pgp-mediated resistance. Moreover, the P-gp modulating activity of the flavonoid dimers in human MDR cancer cells has recently been optimized by structural modification of the dimers.[52] Because P-gp is structurally and functionally similar to MRP1,[24] it is reasonable to suggest that the same bivalent approach can also be applied to modulate MRP1 transport activity. Here, we describe that apigenin homodimers can reverse MRP1-mediated drug resistance. Their modulating activity has been further improved by structure activity studies. Their inhibition mechanism for MRP1 transporter has also been characterized by biochemical methods.

Results

Chemistry. The apigenin homodimers **1** were synthesized as previously reported[51] and were tested for modulating activity at 0.5 μM concentration against MRP1. Apigenin homodimers **1e**−**1h** with 5−8 EG units showed encouraging modulating activity relative to the positive controls including verapamil, **9**, and probenecid (Table 1). The optimal linker length seems to be longer in the case of MRP1 (n = 5−8) than that of P-gp case (n = 4).[51] These results prompted us to carry out further optimization. Our structural modifications were aimed at (1) confirmation of the optimal linker length for MRP1 modulation using a new series of flavonoid dimer analogue **2**, (2) replacement of the hydrophilic OH groups on ring A with more hydrophobic substituents to see the effect on activity, and (3) modification of other positions of the flavonoid moiety. Thus, flavonoid dimers **2**−**7** with various linker lengths were synthesized as shown in Scheme 1. Compounds **2d**, **2d-M**, **3d**, **4d**, and **5d** were prepared as previously reported.[52] The new compounds were prepared in moderate yields by treating substituted 4′-hydroxyflavones **8**[52] with corresponding PEG dimesylate under basic condition in dimethylformamide at refluxing temperature. Their dimeric nature was evident from the high-resolution mass spectrum. It should be mentioned that polyethylene glycol 600 with average number of ethylene glycol units of 13 was used to prepare compound **2j**.

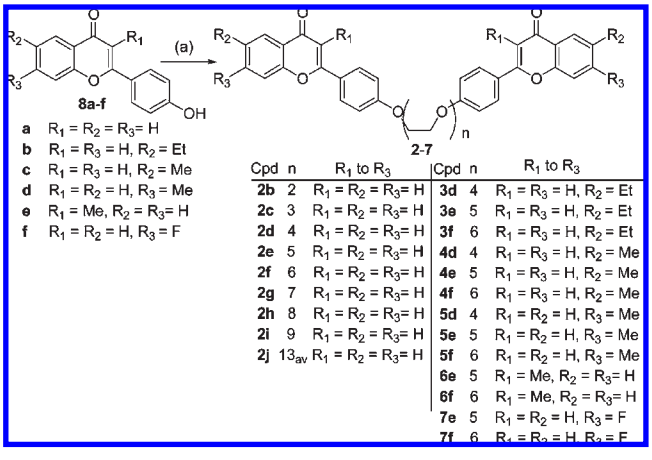
Biological Assay Results. 1. Effect of Apigenin Homodimers 1 on Reversing DOX Resistance in 2008/MRP1 Cells. We used two cell lines in this study, namely the 2008/P cell line (a human ovarian carcinoma) and its MRP1-transfected derivative (2008/MRP1).[55] 2008/P cells (IC_{50} = 63 ± 5 nM) are 9.2-fold more sensitive to DOX than 2008/MRP1 cells (IC_{50} = 577 ± 39 nM) (Table 1). DOX is an anthracycline-type anticancer

Table 1. DOX Cytotoxicity and RF Using 0.5 μM Modulator in 2008/MRP1 Cells[a]

compd	IC_{50} (nM)	RF	compd	IC_{50} (nM)	RF
1b	601 ± 107	1.0	**4d**	81 ± 30	7.1
1c	407 ± 97	1.4	**4e**	42 ± 10	13.7
1d	540 ± 137	1.1	**4f**	54 ± 21	10.7
1e	200 ± 45	2.9	**5d**	120 ± 54	4.8
1f	218 ± 63	2.6	**5e**	47 ± 17	12.3
1g	380 ± 135	1.5	**5f**	46 ± 23	12.5
1h	219 ± 53	2.6	**6e**	257 ± 37	2.2
2b	503 ± 53	1.1	**6f**	233 ± 57	2.5
2c	315 ± 51	1.8	**7e**	50 ± 15	11.5
2d	153 ± 36	3.8	**7f**	61 ± 20	9.5
2d-M[d]	565 ± 161	1.0	2008/P[b]	63 ± 5	9.2
2e	80 ± 4	7.2	2008/MRP1[c]	577 ± 39	1.0
2f	74 ± 6	7.8	0.5 μM verapamil	410 ± 89	1.4
2g	114 ± 11	5.1	10 μM verapamil	130 ± 5	4.4
2h	144 ± 31	4.0	20 μM verapamil	77 ± 3	7.5
2i	137 ± 21	4.2	0.5 μM **9**	401 ± 20	1.4
2j	172 ± 40	3.4	25 μM **9**	162 ± 4	3.6
3d	194 ± 48	3.0	50 μM **9**	146 ± 17	3.9
3e	132 ± 33	4.4	0.5 μM probenecid	426 ± 25	1.4
3f	178 ± 48	3.2	50 μM probenecid	342 ± 27	1.7
			500 μM probenecid	311 ± 42	1.9

[a] The IC_{50} value was determined after exposure to a series of DOX concentrations with different flavonoid dimers at 0.5 μM using 2008/MRP1 cells, as described in the Experimental Section. At such low modulator concentration, no cytotoxic effect was observed in all compounds in either 2008/P or 2008/MRP1 cell (see Supporting Information); Relative fold (RF) represents fold-change in drug sensitivity. RF = (IC_{50} without modulator)/(IC_{50} with 0.5 μM modulator). Known MRP1 inhibitors including verapamil, **9**, and probenecid were included for comparison. Compound **9** at 100 μM and probenecid at 1000 μM were found to be cytotoxic to cells and were therefore not tested for their MRP1 reversing activity. N=2−8 independent experiments and the values were presented as mean ± standard error mean. [b] 2008/P cells were used without addition of modulator. [c] 2008/MRP1 cells were used without addition of modulator. [d] 1.0 μM concentration of modulator was used.

Scheme 1. Synthesis of Flavonoid Homodimers **2**−**7** with Various Linker Lengths[a]



[a] Reagents and conditions: (a) $MsO(CH_2CH_2O)_nMs$, K_2CO_3, DMF, reflux, 3 h.

drug and is a known substrate of MRP1.[56] Because all flavonoid dimers tested showed no observable cytotoxic effect in both 2008/MRP1 and 2008/P cells at a concentration of 0.5 μM (see Supporting Information), we used this concentration (0.5 μM) to test their modulating activity against MRP1-mediated DOX resistance in 2008/MRP1 cells. Among the apigenin homodimers (series **1**), **1e** and **1f** with 5 and 6 EG units, showed the highest modulating activity with a relative fold (RF) of 2.9 and 2.6, respectively (Table 1). Apigenin homodimers with shorter (**1b**, **1c**, and **1d**) or longer (**1g**) linker lengths showed a relatively lower MRP1-modulating activity (Table 1). Compound **1h** with 8 EG units showed a comparable MRP1-modulating activity as **1e** and **1f** (Table 1). The optimal linker length for flavonoid dimers **1** in modulating MRP1-mediated DOX resistance seems to be 5 or 6 EG units. This is reminiscent of what we previously observed in P-gp where the optimal length is 4 EG units for flavonoid dimers **1**.[51] These results also suggest that the binding sites on MRP1 may be differently located compared to those of P-gp.

2. Removal of OH Groups on A-Ring of Flavonoid Dimers Increases MRP1-Modulating Activity. We have previously found that removal of the hydroxyl groups from the C-5 and C-7 positions on ring A of **1** can increase P-gp modulating activity.[52] We are interested in determining if similar modification can also increase the MRP1-modulating activity in 2008/MRP1 cells. A series of flavonoid dimers (**2b**−**2j**) were synthesized, all with OH groups removed from C-5 and C-7 but with different PEG linker lengths (Table 1). Comparing series **1** and **2** reveals that removal of OH groups from C-5 and C-7 positions of **1** increases the modulating activity in 2008/MRP1 cells, irrespective of the linker lengths used.

The highest MRP1-modulating activity was observed for flavonoid dimer **2e** and **2f** with a RF of 7.2 and 7.8, respectively (Table 1). Flavonoid dimers with shorter (**2b**, **2c**, and **2d**) or longer linker lengths (**2g**, **2h**, **2i**, and **2j**) exhibited a relatively lower MRP1-modulating activity (Table 1). MRP1 modulation patterns are similar to what we observed for the apigenin homodimers **1**, suggesting that these two series of flavonoid dimers are probably binding to the same sites on MRP1. The fact that monomer **2d-M** showed no modulating activity compared to its dimer **2d** (Table 1; RF = 1.0), even when it was added at a double concentration, suggests that the modulating activity of flavonoid dimers is due to their bivalent interaction with MRP1, not just simply because of increasing the ligand concentration itself.

3. Lead Optimization of Flavonoid Dimer on Reversing DOX Resistance in 2008/MRP1 Cells. Because replacement of the hydrophilic OH groups in **1** by H in **2** led to enhanced activities, we were interested in introducing hydrophobic substitutions into the flavonoid dimers. On the basis of the analogy with the structure activity studies on P-gp,[52] such modifications included: (1) ethyl group at C-6 of A-ring (compounds **3d**−**3f**), (2) methyl group at C-6 of A-ring (compounds **4d**−**4f**), (3) methyl group at C-7 of A-ring (compounds **5d**−**5f**), (4) methyl group at C-3 of C-ring (compounds **6e**−**6f**), and (5) fluorine group at C-7 of A-ring (compounds **7e**−**7f**). The MRP1-modulating activities of the above compounds are summarized in Table 1. In general, flavonoid dimers with PEG linker length of 5 or 6 EG units showed better modulating activity than those with 4 EG units, irrespective of their substituent on A-ring of the flavonoid moiety.

Among those flavonoid dimers with optimal PEG linker lengths of 5 or 6, addition of methyl group at either C-6 (**4e** and **4f**) or C-7 position of A-ring (**5e** and **5f**) resulted in the highest MRP1-modulating activity (RF ranging from 10.7 to 13.7) (Table 1). Addition of 0.5 μM of these modulators completely reversed the IC_{50} of DOX of 2008/MRP1 to that

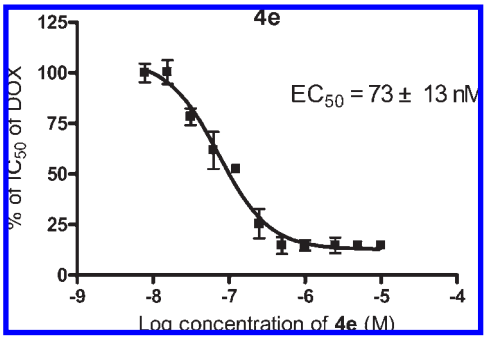


Figure 2. EC_{50} value of **4e** in lowering DOX resistance in 2008/MRP1 cells. The percent of IC_{50} of DOX was plotted against log concentration of **4e**. The IC_{50} value of DOX of 2008/MRP1 cells in the presence of modulator (**4e**) was determined as described in Experimental Section. The percent of IC_{50} of DOX = [(IC_{50} of DOX at each modulator concentration/IC_{50} of DOX without modulator) × 100%)]. The EC_{50} of modulator was the concentration of modulator that can reduce the % of IC_{50} of DOX by half. Each data point represents the average of three independent experiments, and the values are presented as mean ± standard error of mean. Nonlinear regression (sigmoidal dose−response assuming variable slope) performed using Prism 4.0 was used to determine EC_{50} value.

of parental 2008/P's level (Table 1). On the other hand, methyl group at C-3 position of C-ring showed a poorer modulating activity, suggesting that substitution other than a proton at this position is not favored. Interestingly, increasing substituent bulkiness from methyl (**4d**−**f**) to ethyl group at C-6 (**3d**−**f**) also resulted in a poorer modulating activity. Flavonoid dimers **7** with a fluorine group at C-7 (**7e** and **7f**) also showed promising modulating activity with a RF of 9.5−11.5.

Comparison was made with known MRP1 inhibitors including verapamil,[57−59] **9**,[60−62] and probenecid.[63,64] They all showed insignificant modulating activity when tested at 0.5 μM (Table 1). Higher concentrations were therefore used for these MRP1 inhibitors. Verapamil at 10 and 20 μM gave a RF of 4.4 and 7.5, respectively (Table 1). Compound **9** at 25 and 50 μM gave a RF of 3.6 and 3.9, respectively (Table 1). Probenecid gave a very poor MRP1-modulating activity with a RF of 1.7 and 1.9 at the concentration of 50 and 500 μM, respectively (Table 1). We cannot determine the concentration of **9** and probenecid at which they can give similar efficacy as **4e** because **9** at 100 μM and probenecid at 1000 μM were cytotoxic to 2008/MRP1 cells (data not shown).

To provide a quantitative measurement of the MRP1-modulating efficacy, we have determined the EC_{50} values for the four flavonoid dimers with the highest potency (**4e**, **4f**, **5e**, and **5f**) as well as several known MRP1 inhibitors. Among different flavonoid dimers, **4e** has the lowest EC_{50} (73 ± 13 nM) in reversing DOX resistance in 2008/MRP1 cells (Figure 2). This is comparable to other highly potent MRP1 modulators like **12** (EC_{50} = 93 nM; reversing DOX resistance in HeLa-T5 cells).[34,35] The other three flavonoid dimers **4f**, **5e**, and **5f** were also very potent, with EC_{50} of 105 ± 20, 133 ± 16, and 86 ± 20 nM, respectively (Table 2 and see Supporting Information for raw data). In contrast, verapamil, **9**, and probenecid have higher EC_{50} values of 2.6, 18, and >500 μM, respectively (Table 2 and see Supporting Information for raw data). Compound **4e** is about 36-, 247- and 6849-fold more potent than verapamil, **9**, and probenecid in reversing MRP1-mediated DOX resistance, respectively.

Table 2. EC_{50} Values of **4e**, **4f**, **5e**, **5f**, Verapamil, **9**, and Probenecid[a]

modulators	EC_{50}[b] for lowering DOX resistance of in 2008/MRP1
4e	73 ± 13 nM
4f	105 ± 20 nM
5e	133 ± 16 nM
5f	86 ± 23 nM
verapamil	2.6 ± 1.0 μM
9	18 μM[c]
probenecid	> 500 μM[c]

[a] Each EC_{50} value represents the mean of three independent experiments. [b] EC_{50} values are determined using the method described in Figure 2. [c] EC_{50} values of **9** and probenecid were a rough estimation because there was cytotoxicity effect observed when **9** and probenecid were used at a concentration of 100 and 500 μM respectively.

Table 3. VP16 Cytotoxicity and RF Using 0.5 μM Modulator in 2008/MRP1 Cells[a]

compd	IC_{50} (μM)	RF	compd	IC_{50} (μM)	RF
3d	23.9 ± 9.9	2.0	**5d**	12.6 ± 3.3	3.8
3e	13.5 ± 1.5	3.6	**5e**	4.3 ± 1.0	11.2
3f	15.9 ± 1.5	3.0	**5f**	4.7 ± 1.4	10.2
4d	13.3 ± 1.1	3.6	2008/P[b]	4.4 ± 0.3	10.9
4e	4.7 ± 0.6	10.2	2008/MRP1[c]	48.0 ± 6.3	1.0
4f	6.0 ± 0.7	8.0	0.05% DMSO [c]	44.0 ± 13.6	1.1

[a] IC_{50} value was determined after exposure to a series of VP16 concentration with different flavonoid dimers at 0.5 μM, as described in the Experimental Section. Relative fold (RF) = (IC_{50} without modulator)/(IC_{50} with 0.5 μM modulator). [b] 2008/P cells were used without addition of any modulator. [c] 2008/MRP1 cells were used without addition of modulator. $N = 3$ independent experiments and the values were presented as mean ± standard error mean.

4. Effect of Flavonoid Dimers on Reversing VP16 in 2008/MRP1 Cells. We are interested in determining if flavonoid dimers can also reverse the MRP1-mediated resistance to other drugs like etoposide (VP16; $C_{29}H_{32}O_{13}$), a known substrate of MRP1.[56] We found that 2008/MRP1 cells (IC_{50} = 48.0 ± 6.3 μM) were 11-fold more resistant to VP16 than 2008/P cells (IC_{50} = 4.4 ± 0.3 μM) (Table 3). Flavonoid dimers **3**, **4**, and **5** were tested for their modulating effects of MRP1-mediated VP16 resistance in 2008/MRP1 cells (Table 3). The results obtained are similar to those obtained for DOX. First, flavonoid dimers with 5 or 6 EG units have a higher modulating activity than those with 4 EG units, irrespective of their substitutions in ring A. Second, among the flavonoid dimers with 5 or 6 ethylene glycol linkers, addition of a methyl group at either position C-6 or C-7 increases the MRP1-modulating activity, whereas addition of ethyl group at C-6 position lowers it. Treatment with **4e**−**f** and **5e**−**f** at a concentration of 0.5 μM was sufficient to reverse VP16 resistance of 2008/MRP1 cells to 2008/P parental level. This result suggests that **4** and **5** can effectively modulate MRP1-mediated resistance to both DOX and VP16.

5. Dosage-Effect of Flavonoid Dimers on Intracellular Accumulation of DOX in 2008/MRP1 Cells. The above results showed that the flavonoid dimers are effective MRP1 modulators. We are interested in determining if the modulation of MRP1-mediated drug resistance is associated with a concomitant increase in drug accumulation. DOX is a fluorescent drug that can be used to monitor drug accumulation in cells. Because **4e** is one of the most effective MRP1

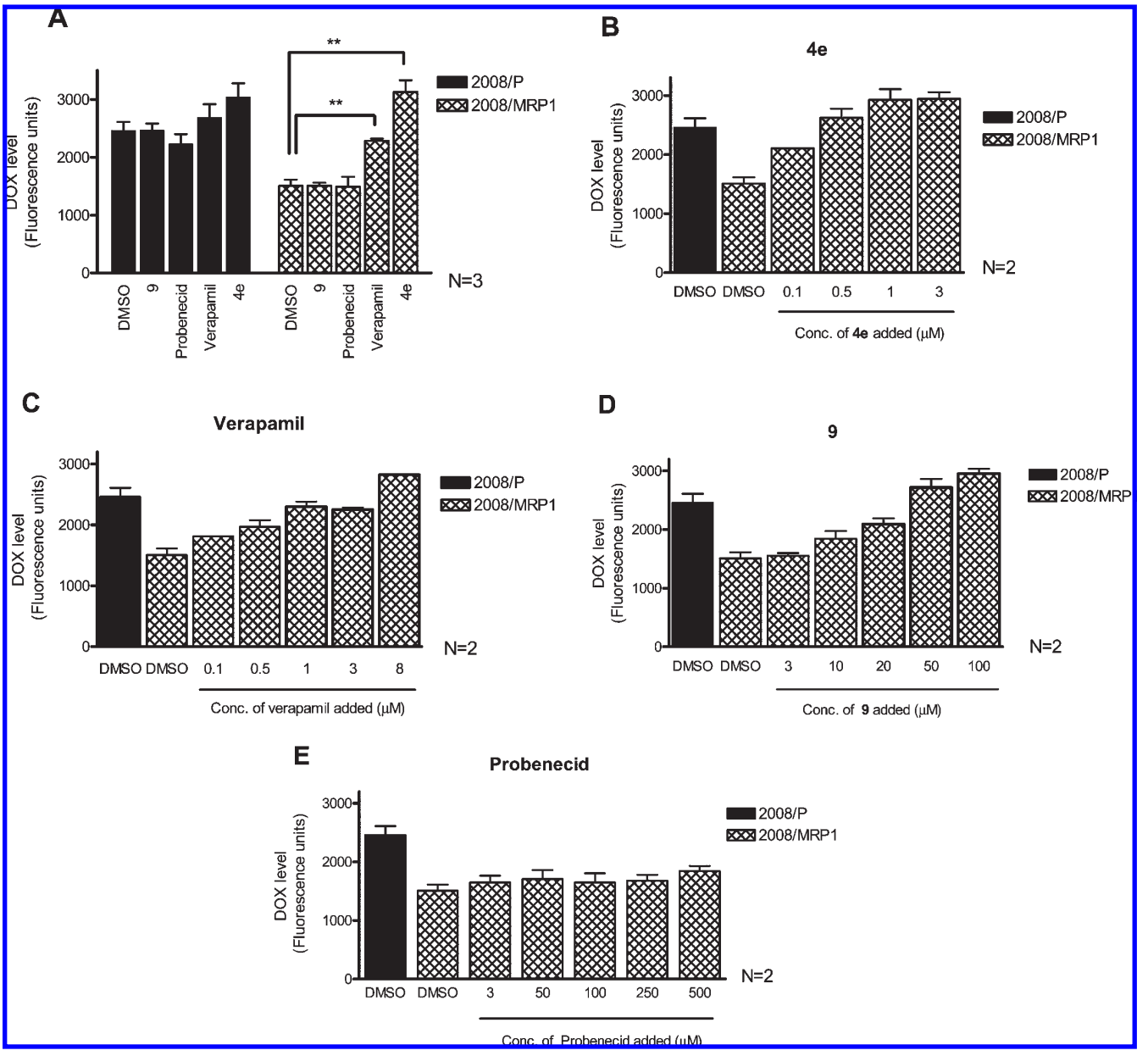


Figure 3. Effect of modulators added at different concentrations on intracellular DOX accumulation in 2008/P and 2008/MRP1 cells. Figure 3A: 2008/P or 2008/MRP1 cells were incubated with 20 μM DOX for 120 min at 37 °C with or without 3 μM of **4e**, **9**, probenecid or verapamil. DMSO 0.3% was used as negative control. After the incubation period, we lysed the cells and used the supernatant to measure the DOX level by spectrofluorometry. Experiments were performed in duplicate and repeated thrice. The results are presented as mean ± standard error of mean. $^{**}P < 0.005$ relative to negative control. The dosage-effect of modulators on intracellular DOX accumulation was also studied by incubating 2008/MRP1 cells with different concentration of modulators including **4e** (Figure 3B), verapamil (Figure 3C), **9** (Figure 3D), and probenecid (Figure 3E). DMSO 0.3% was used as negative control.

modulators in the series, we selected this compound for further characterization. As shown in Figure 3A, the level of DOX accumulation in 2008/P cells was about 1.6-fold higher than that of 2008/MRP1 in the absence of modulators. This is because MRP1 can pump DOX out of the cells, thereby lowering intracellular DOX level. Treatment of 2008/P cells with 3 μM verapamil, **9**, or probenecid did not increase DOX accumulation, whereas treatment with **4e** resulted in a slight increase in DOX accumulation, probably due to the inhibition of basal P-gp and/or MRP1 activity in 2008/P cells. In contrast, treatment of 2008/MRP1 cells with 3 μM of compound **4e** resulted in 2.1-fold ($P < 0.005$) increase in intracellular DOX accumulation (Figure 3A). Verapamil, being a less potent MRP1 modulator than **4e**, only increased the DOX accumulation by about 1.5-fold ($P < 0.005$) (Figure 3A). Compound **9** and probenecid did not increase DOX accumulation in 2008/MRP1 cells (Figure 3A). This result suggests that the mechanism by which flavonoid dimers inhibit MRP1 is by virtue of their inhibition on MRP1's transport activity. More importantly, these flavonoid dimers are more effective than other MRP1 inhibitors in restoring DOX accumulation.

We found that restoration of DOX accumulation in 2008/MRP1 cells by **4e**, verapamil, and **9** was dose dependent (Figure 3B,C,D). At 0.5 μM, **4e** was sufficient to restore DOX accumulation of 2008/MRP1 to parental 2008/P's level (Figure 3B). For verapamil and **9**, the minimum concentration needed to restore DOX accumu-

lation of 2008/MRP1 to parental 2008/P's level was about 8 and 50 μM, respectively (Figure 3C,D). Compound **4e** is therefore roughly 16- and 100-fold more potent than verapamil and **9**, respectively, in inhibiting MRP1's DOX transport activity. Surprisingly, probenecid did not have any effect on increasing DOX accumulation of 2008/MRP1 even at 500 μM (Figure 3E) although probenecid, when used at 500 μM, has a weak modulatory activity on 2008/MRP1's DOX cytotoxicity (RF = 1.9 in Table 1).

6. Lineweaver−Burk and Dixon Plots Suggest a Competitive Relationship between 4e and DOX in Binding to MRP1. We have employed Lineweaver−Burk and Dixon analysis to further characterize the inhibition mechanism of **4e**, verapamil, and **9** on MRP1's DOX transport activity. Generally, the increase in DOX retention in 2008/MRP1 cells was dependent on the concentration of **4e**, verapamil, and **9** used. Lineweaver−Burk analysis showed that there is likely a competitive relationship between DOX and **4e** (Figure 4A), verapamil (Figure 4B), and **9** (Figure 4C). In all three cases, V_{max} appeared unaffected whereas the K_m decreased with increasing concentration of modulator. K_i can be obtained by plotting the slope of double reciprocal plot versus modulator concentration (insets in Figure 4A,B,C). Compound **4e** exhibited the smallest K_i (0.2 μM) compared to verapamil (K_i = 13.2 μM) and **9** (K_i = 134.7 μM), suggesting that **4e** has a higher affinity than verapamil and **9** for the DOX recognition site on MRP1.

K_i can also be obtained from Dixon plot (Figure 4D,E,F). K_i was determined to be 0.19 μM for **4e** (Figure 4D), 13.4 μM for verapamil (Figure 4E), and 139.6 μM for **9** (Figure 4F). K_i values obtained from Dixon plot are almost identical to those obtained from Lineweaver−Burk plot above. Linear regression line obtained from plotting the slopes of Dixon plot versus the reciprocal of substrate concentration (insets of Figure 4D,E,F) can distinguish between a competitive and noncompetitive or partially competitive relationship. A competitive inhibitor will give a plot with a linear regression line passing through the origin, whereas a noncompetitive inhibitor would not.[65,66] We found that the regression lines almost coincided with the origin for **4e** (inset of Figure 4D) and **9** (inset of Figure 4F), whereas it completely passed through the origin for the verapamil (inset of Figure 4E). These data suggests that **4e**, similar to other MRP1 inhibitors like verapamil and **9**, acts as a competitive inhibitor of MRP1-mediated DOX transport.

The K_i value for **4e** in inhibiting 2008/MRP1's DOX transport activity (K_i = 0.2 μM) is close to the EC_{50} value for modulating 2008/MRP1's DOX cytotoxicity (EC_{50} = 73 ± 13 nM). This suggests that the modulation mechanism by **4e** on 2008/MRP1's DOX cytotoxicity is very likely due to its inhibition on MRP1's DOX transport activity through a competitive inhibition mechanism.

Discussion

MRP1 was first demonstrated to be associated with MDR in 1992.[7] Flavonoids can modulate the transport activity of MRP1 and restore drug sensitivity.[38,39,41,43] For example, flavonolignan dehydrosilybin (DHS) at 10 μM has been demonstrated to completely reverse vincristine resistance of MRP1-transfected BHK-21 cells to parental level.[41] Quercetin at 10 μM reduced the IC_{50} of vincristine of MRP1-HeLa cells by 4-fold.[38] Compared to other MRP1 modulators, flavonoids have a relatively lower modulating activity. Nevertheless, flavonoids are still attractive targets for development of novel modulator of MRP1-mediated resistance because they are part of daily human diet and are presumably without any detrimental side effects.

Previously, we demonstrated that synthetic apigenin homodimers with optimal length of PEG linker can modulate not only P-gp-mediated drug resistance in cancer[51,52] but also in parasitic protozoan *Leishmania*.[53,54] Others have also demonstrated that bivalency approach is effective in making P-gp modulators (e.g., stipiamide dimer,[67] emetine dimer,[68] and quinine dimer[69]). Here we report the synthesis and characterization of flavonoid dimers as a new class of potent MRP1 modulator. Their efficacy in reversing MRP1-mediated DOX resistance in 2008/MRP1 cells (EC_{50} = 73−133 nM for compounds **4e**, **4f**, **5e**, and **5f**) are comparable to that of the most potent MRP1 inhibitors like tricyclic isoxazoles **12**.[34,35] At 0.5 μM, compounds **4e**, **4f**, **5e**, and **5f** can completely restore IC_{50} of DOX of 2008/MRP1 cells to sensitive cell's level (Table 1), indicating that the flavonoid dimers are at least 20-fold more effective than the aforesaid monomeric flavonoids such as dehydrosilybin in reversing MRP1-mediated drug resistance. We also demonstrated that monomeric flavonoid (**2d-M**) showed a much lower activity, even when added at a double concentration (Table 1). Therefore, it is believed that the high level of MRP1-modulating activity is achieved by the bivalent nature of the flavonoid dimer. This observation is reminiscent of our previous report where apigenin dimer, but not monomers, can reverse P-gp based MDR.[51,53]

We have compared the modulating activity of **4e** with other MRP1 inhibitors like verapamil, **9**, and probenecid. First, it has been reported that addition of verapamil at a concentration of 10 μM partially reversed DOX resistance in U-1285dox$_{900}$ human small lung carcinoma cell, which overexpresses MRP1 protein with a RF of 12.2, although this level is still lower than its parental cell (RF = 20.6).[57] In other MRP1-overexpressing model systems, verapamil has been observed to have a weak or no chemosensitization to DOX and daunomycin.[58,59] Second, it has been reported that treatment of **9** at a concentration of 10 μM gave a weak MRP1 reversing activity on DOX resistance in KB/MRP1 cells with a RF of 1.4.[60] In some MRP1-overexpressing cells, **9** at 10−30 μM has been demonstrated to moderately reverse vincristine, vinblastine, and VP16 resistance.[60−62] Third, addition of probenecid at 10 μM to 2008/MRP1 cells can partially reverse their resistance to methotrexate or hydrophobic peptide.[63,64] Here we report that the efficacy of **4e** in reversing DOX resistance in 2008/MRP1 cells (EC_{50} 73 ± 13 nM) is much higher than that of verapamil (2.6 μM), **9** (18 μM), and probenecid (>500 μM). The high level of MRP1-modulating activity of **4e** is because it can act as a competitive inhibitor to the DOX binding site of MRP1 with a high affinity (K_i = 0.2 μM).

Others have demonstrated that flavonoids can act as a competitive inhibitor of MRP1 transport. For example, robinetin can competitively inhibit calcein transport with apparent K_i of 5 μM for MRP1.[43] Moreover, naturally occurring flavonoids, including kaempferol, apigenin, quercetin, myricetin, and naringenin, were demonstrated to be competitive inhibitors of LTC_4 transport with apparent K_i ranging from 2.4 to 21 μM for MRP1.[39] Here we demonstrate that DOX transport is competitively inhibited by flavonoid dimer **4e** with apparent K_i of 0.2 μM for MRP1 (Figure 4A,D). The flavonoid dimer **4e** exhibits a greater inhibitory potency on

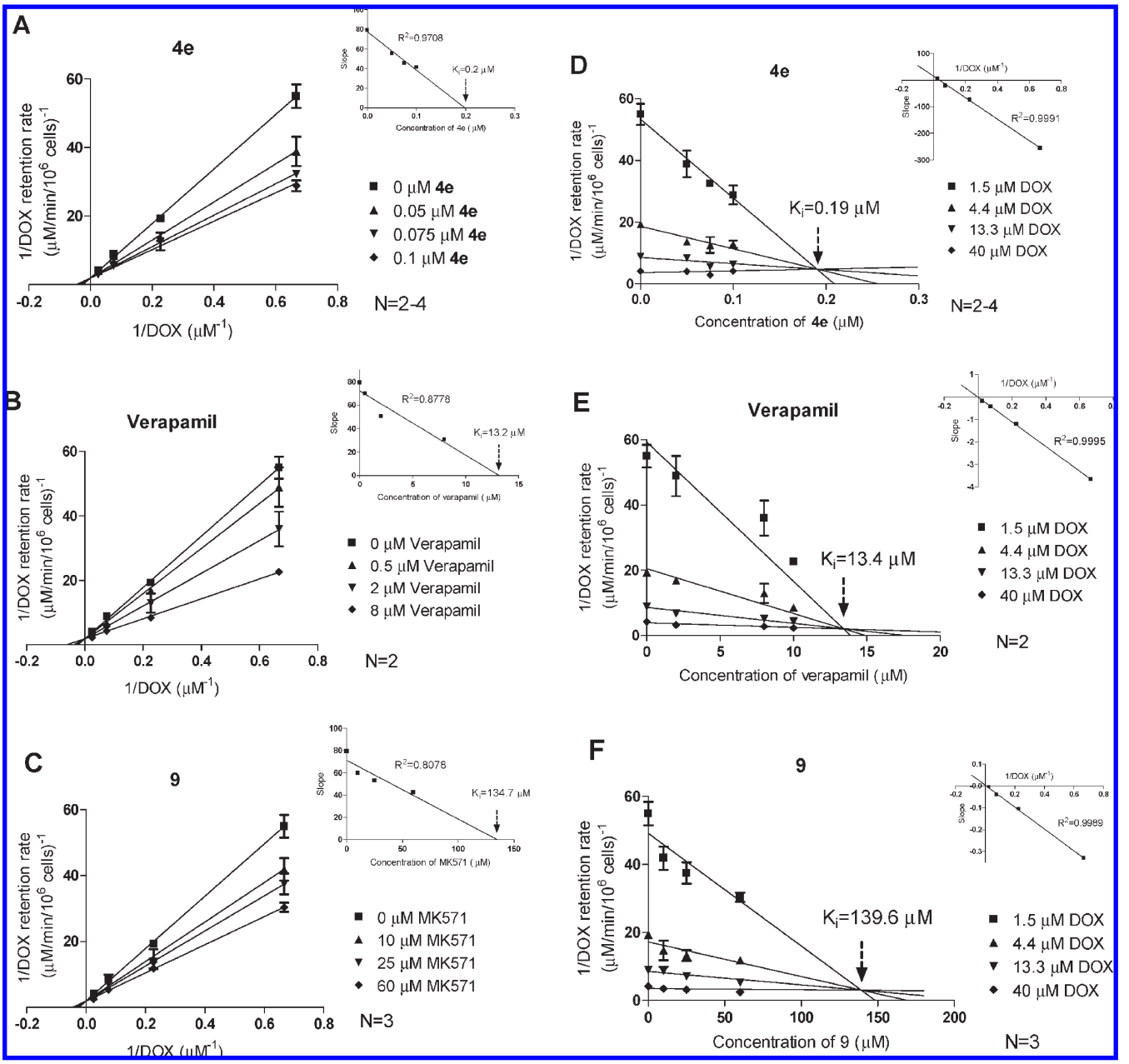


Figure 4. Biochemical study of relationship of **4e**, verapamil, and **9** with DOX. 2008/MRP1 cells were incubated with different concentrations of DOX (1.5, 4.4, 13.3, and 40 μM) in the presence of four concentrations of **4e** (0, 0.05, 0.075, and 0.1 μM), verapamil (0, 0.5, 2, 8 μM), or **9** (0, 10, 25, and 60 μM). After incubation, the amount of intracellular DOX accumulated was measured by spectrofluorometry after cell lysis. Lineweaver−Burk plots are shown in parts A, B, and C of Figure 4. The reciprocal of DOX retention rate is plotted against reciprocal of DOX concentration used. The apparent K_i value is determined by linear regression analysis from the slope of double reciprocal plots versus the concentration of modulator (insets of Figure 4A,B,C). The above experimental data is also analyzed by Dixon plot (Figure 4D,E,F). The reciprocal of DOX retention rate is plotted against concentration of modulator. The intersection point represents the K_i value. The slope of the Dixon plot is plotted against reciprocal of DOX concentration to determine their relationship (insets of Figure 4D,E,F). A linear line passing through origin suggests competitive relationship. Each data point represents two to four independent experiments and is presented as mean ± standard error of mean. The effect of **4e** at higher concentration (from 0.2 to 1.0 μM) on DOX retention rate was also tested, but it was found that DOX retention rate was saturated when **4e** was used at a concentration higher than 0.2 μM.

drug transport of MRP1 because it has about 12−105-fold lower K_i value than that of other monomeric flavonoids. This implies that bivalency approach markedly enhances the binding affinity of flavonoid for the substrate binding site of MRP1, thus a lower amount of flavonoid dimer is needed to inhibit the drug transport of MRP1 and eventually to restore the chemosensitization to anticancer drug. The K_i value of flavonoid dimer **4e** (K_i = 0.2 μM) (Figure 4A,D) correlates well with its EC_{50} value (EC_{50} = 73 ± 13 nM) (Figure 2). This suggests that restoration of DOX sensitivity by **4e** in 2008/MRP1 cells is mainly due to the inhibition of MRP1's DOX transport activity.

Lead optimization has generated the following limited structure−activity-relationship observations. First, removal of OH groups at the C-5 and C-7 positions of the A-ring (compounds **2b**−**2j**) from apigenin dimers (compounds **1b**−**1h**) resulted in an increase in MRP1-modulating activity. Second, flavonoid dimers with five or six EG units in the linker

are better than those with four. Third, methyl group at either C-6 or C-7 position or fluorine group at the C-7 position of the A-ring can increase MRP-modulating effect, whereas addition of ethyl group at C-6 position or methyl group at C-3 position of A-ring has the opposite effect (Table 1).

For future studies, we will investigate the in vivo efficacy of flavonoid dimers in suppressing tumor growth of MRP1-overexpressing cells when combined with anticancer drug. Such animal experiments will be critical for deciding if flavonoid dimers can be further developed to be used in clinical trials. Flavonoid dimers reported here have a similar in vitro potency as **12**, the most potent tricyclic isoxazole as MRP1 inhibitors.[34−36] It will be interesting to compare their in vivo potencies. Compound **12** at 8 mg/kg, when used together with 0.5 mg/kg vincristine, can suppress HeLa-T5 (MRP1-overexpressing) tumor regression by 63%.[34−36] No clinical trial data, however, is yet available for these compounds.

There are 13 members of ABCC families (ABCC1−ABCC13).[70] The closest member to ABCC1 is ABCC3 (58% amino acid identity), followed by ABCC2 (49%) and ABCC6 (45%)[71] and they are known to have the five transmembrane amino-terminal extension like ABCC1.[72] Recently, a series of natural flavonoids were studied in MRP1 and MRP2-mediated calcein transport in a cellular model.[43] It was found that while some flavonoids like diosmetin or chrysoeril can inhibit MRP1 with IC_{50} = 3−4 μM, they are less active against MRP2 (IC_{50} > 50 μM). On the other hand, some flavonoids like myricetin is equally active against both MRP1 (IC_{50} = 20 μM) and MRP2 (IC_{50} = 22 μM). At this stage, we do not know whether flavonoid dimers might also bind to other ABCC family members. This will be an interesting topic to investigate in the future in view of the much enhanced potency of the dimer over the monomer.

In summary, we have used bivalency approach to generate a new class of MRP1 modulator with an efficacy comparable to that of the most potent MRP1 inhibitors in the literature. These flavonoid dimers have a high affinity for the DOX recognition site of MRP1, leading to inhibiting the DOX transport and increasing the intracellular DOX accumulation and finally restoring DOX sensitivity in 2008/MRP1 cells. Overall, the present study demonstrates that the flavonoid dimers can be employed as an effective modulator of MRP1-mediated drug resistance in cancer cells.

Experimental Section

General. All NMR spectra were recorded on a Bruker MHz DPX400 spectrometer at 400.13 MHz for ^{1}H and 100.62 MHz for ^{13}C. All NMR measurements were carried out at room temperature, and the chemical shifts are reported as parts per million (ppm) in unit relative to the resonance of $CDCl_3$ (7.26 ppm in the ^{1}H, 77.0 ppm for the central line of the triplet in the ^{13}C modes, respectively). Low-resolution and high-resolution mass spectra were obtained on a Micromass Q-TOF-2 by electron spray ionization (ESI) mode or on Finnigan MAT95 ST by electron ionization (EI) mode. Melting points were measured using Electrothermal IA9100 digital melting point apparatus and were uncorrected. All reagents and solvents were reagent grade and were used without further purification unless otherwise stated. The plates used for thin-layer chromatography (TLC) were E. Merck Silica Gel 60F_{254} (0.25 mm thickness), and they were visualized under short (254 nm) UV light. Chromatographic purifications were carried out using MN silica gel 60 (230−400 mesh). The purity of synthesized compounds was determined by HPLC, which was performed using Agilent 1100 series installed with an analytic column of Agilent Prep-Sil Scalar column (4.6 mm × 250 mm, 5-μm) at UV detection of 315 nm (reference at 450 nm) with isocratic elution of hexane (42%)/ethyl acetate (50%)/methanol (8%) at a flow rate of 1.0 mL/min. Substituted 4′-hydroxyflavones **8a**−**f**, flavonoid dimers **1b**−**h**, **2d**, **3d**, **4d**, **5d**, and flavonoid monomer **2d-M** were prepared with purity >95% as previously reported.[51,52] All synthetic compounds were shown to have >95% purity except for compound **2j** (see below) according to HPLC.

General Procedure for the Dimerization of 4′-Hydroxyflavones 8a−f to Flavonoid Dimers 2−7. To a round-bottom flask was charged with 4′-hydroxyflavone (2 equiv), $MsO(CH_2CH_2O)_n$-Ms (1 equiv), K_2CO_3 (2.5 equiv), and DMF. The reaction mixture was stirred at refluxing temperature for 3 h. During heating, the reaction mixture turned slowly from deep brown to milky in color. When TLC indicated complete consumption of starting material, the reaction mixture was poured into a separating funnel containing water. The mixture was continuously extracted with CH_2Cl_2. If the mixture could not be separated into two layers, a small amount of 1 M HCl was added. The combined organic layers were dried over $MgSO_4$, filtered, and evaporated to give a crude reaction mixture. Purification of the flavonoid dimer was performed by flash column chromatography on silica gel with 10−20% acetone in CH_2Cl_2 as eluent to furnish desired product.

1,7-Bis[4′-(4*H*-chromen-4-on-2-yl)phenyl]-1,4,7-trioxaheptane (2b). The titled compound **2b** (0.31 g, 55%) was obtained from 4′-hydroxyflavone **8a** (0.48 g, 2.0 mmol), diethylene glycol dimesylate (0.27 g, 1.0 mmol), and K_2CO_3 (0.40 g) as a pale-yellow solid according to the general procedure described above; mp 220−222 °C. ^{1}H NMR ($CDCl_3$) δ 3.98 (t, J = 4.8 Hz, 4H), 4.24 (t, J = 4.4 Hz, 4H), 6.71 (s, 2H), 7.03 (d, J = 8.8 Hz, 4H), 7.38 (dd, J = 7.6, 7.6 Hz, 2H), 7.52 (d, J = 8.0 Hz, 2H), 7.65 (ddd, J = 1.6, 7.6, 7.6 Hz, 2H), 7.85 (d, J = 8.8 Hz, 4H), 8.19 (dd, J = 1.2, 8.0 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 67.6, 69.8, 106.2, 115.0, 117.9, 123.8, 124.2, 125.0, 125.6, 127.9, 133.5, 156.1, 161.5, 163.2, 178.3. LRMS (ESI) m/z 547 (M^+ + H, 27), 569 (M^+ + Na, 100). HRMS (ESI) calcd for $C_{34}H_{27}O_7$ (M^+ + H) 547.1757, found 547.1760.

1,10-Bis[4′-(4*H*-chromen-4-on-2-yl)phenyl]-1,4,7,10-tetraoxadecane (2c). The titled compound **2c** (0.26 g, 43%) was obtained from 4′-hydroxyflavone **8a** (0.48 g, 2.0 mmol), triethylene glycol dimesylate (0.31 g, 1.0 mmol), and K_2CO_3 (0.40 g) as a pale-yellow solid according to the general procedure described above; mp 141−143 °C. ^{1}H NMR ($CDCl_3$) δ 3.78 (s, 4), 3.90 (t, J = 4.8 Hz, 4H), 4.19 (t, J = 4.4 Hz, 4H), 6.70 (s, 2H), 7.00 (d, J = 8.0 Hz, 4H), 7.35 (dd, J = 7.6, 7.6 Hz, 2H), 7.51 (d, J = 8.0 Hz, 2H), 7.65 (dd, J = 7.6, 7.6 Hz, 2H), 7.85 (d, J = 8.8 Hz, 4H), 8.19 (d, J = 8.0 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 67.6, 69.9, 70.9, 106.1, 115.0, 117.9, 123.8, 125.0, 125.6, 127.9, 133.5, 156.1, 161.5, 163.2, 178.3. LRMS (ESI) m/z 590 (M^+ + H, 40), (M^+ + Na, 58). HRMS (ESI) calcd for $C_{36}H_{31}O_8$ (M^+ + H) 591.2019, found 591.2002.

1,16-Bis[4′-(4*H*-chromen-4-on-2-yl)phenyl]-1,4,7,10,13,16-hexaoxahexadecane (2e). The titled compound **2e** (0.29 g, 46%) was obtained from 4′-hydroxyflavone **8a** (0.45 g, 1.89 mmol), pentaethylene glycol dimesylate (0.37 g, 0.94 mmol), and K_2CO_3 (0.35 g) as a pale-brown oil according to the general procedure described above. ^{1}H NMR ($CDCl_3$) δ 3.58−3.64 (m, 12H), 3.76 (t, J = 4.8 Hz, 4H), 4.05 (t, J = 4.4 Hz, 4H), 6.55 (s, 2H), 6.84 (d, J = 8.0 Hz, 4H), 7.24 (dd, J = 7.6, 7.6 Hz, 2H), 7.35 (d, J = 8.4 Hz, 2H), 7.51 (ddd, J = 1.6, 7.6, 7.6 Hz, 2H), 7.67 (d, J = 8.8 Hz, 4H), 8.19 (dd, J = 1.2, 8.0 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 67.5, 69.4, 70.5, 70.7, 105.8, 114.8, 117.8, 123.7, 123.7, 124.9, 125.3, 127.7, 133.4, 155.9, 161.5, 163.0, 178.0. LRMS (ESI) m/z 679 (M^+ + H, 25), 701 (M^+ + Na, 100). HRMS (ESI) calcd for $C_{40}H_{39}O_{10}$ (M^+ + H) 679.2543, found 679.2520.

1,19-Bis[4′-(4*H*-chromen-4-on-2-yl)phenyl]-1,4,7,10,13,16,19-heptaoxanonadecane (2f). The titled compound **2f** (0.68 g, 56%) was obtained from 4′-hydroxyflavone **8a** (0.80 g, 3.38 mmol), hexaethylene glycol dimesylate (0.74 g, 1.69 mmol), and K_2CO_3

(0.60 g) as a pale-brown oil according to the general procedure described above, ^{1}H NMR ($CDCl_3$) δ 3.64−3.72 (m, 16H), 3.86 (t, J = 4.8 Hz, 4H), 4.17 (t, J = 4.4 Hz, 4H), 6.69 (s, 2H), 6.99 (d, J = 8.8 Hz, 4H), 7.36 (dd, J = 7.6, 7.6 Hz, 2H), 7.50 (d, J = 7.6 Hz, 2H), 7.63 (ddd, J = 1.2, 7.6, 7.6 Hz, 2H), 7.82 (d, J = 8.8 Hz, 4H), 8.17 (dd, J = 1.6, 7.8 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 67.6, 69.5, 70.5, 70.6, 70.8, 106.1, 115.0, 117.9, 123.8, 124.0, 125.0, 125.5, 127.9, 133.5, 156.1, 161.6, 163.3, 178.3. LRMS (ESI) m/z 723 (M^+ + H, 89), 745 (M^+ + Na, 100). HRMS (ESI) calcd for $C_{42}H_{43}O_{11}$ (M^+ + H) 723.2805, found 723.2804.

1,22-Bis[4′-(4*H*-chromen-4-on-2-yl)phenyl]-1,4,7,10,13,16,19,22-octaoxadocosane (2g). The titled compound **2g** (0.33 g, 42%) was obtained from 4′-hydroxyflavone **8a** (0.48 g, 2.0 mmol), heptaethylene glycol dimesylate (0.49 g, 1.0 mmol), and K_2CO_3 (0.36 g) as a pale-brown oil according to the general procedure described above. ^{1}H NMR ($CDCl_3$) δ 3.60−3.70 (m, 20H), 3.84 (t, J = 4.8 Hz, 4H), 4.14 (t, J = 4.8 Hz, 4H), 6.67 (s, 2H), 6.97 (d, J = 8.8 Hz, 4H), 7.34 (dd, J = 7.6, 7.6 Hz, 2H), 7.47 (d, J = 8.0 Hz, 2H), 7.61 (ddd, J = 1.6, 7.6, 7.6 Hz, 2H), 7.79 (d, J = 8.8 Hz, 4H), 8.15 (dd, J = 1.6, 8.0 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 67.6, 69.5, 70.5, 70.5, 70.8, 106.0, 115.0, 117.9, 123.8, 124.0, 125.0, 125.5, 127.9, 133.5, 156.0, 161.6, 163.2, 178.2. LRMS (ESI) m/z 767 (M^+ + H, 49), 789 (M^+ + Na, 100). HRMS (ESI) calcd for $C_{44}H_{47}O_{12}$ (M^+ + H) 767.3068, found 767.3083.

1,25-Bis[4′-(4*H*-chromen-4-on-2-yl)phenyl]-1,4,7,10,13,16,19,22,25-nonaoxapentacosane (2h). The titled compound **2h** (0.42 g, 51%) was obtained from 4′-hydroxyflavone **8a** (0.48 g, 2.0 mmol), octaethylene glycol dimesylate (0.53 g, 1.0 mmol), and K_2CO_3 (0.37 g) as pale-brown oil according to the general procedure described above. ^{1}H NMR ($CDCl_3$) δ 3.56−3.66 (m, 24H), 3.79 (t, J = 4.8 Hz, 4H), 4.09 (t, J = 4.8 Hz, 4H), 6.61 (s, 2H), 6.91 (d, J = 8.8 Hz, 4H), 7.28 (dd, J = 7.6, 7.6 Hz, 2H), 7.41 (d, J = 8.4 Hz, 2H), 7.56 (ddd, J = 1.6, 7.6, 7.6 Hz, 2H), 7.73 (d, J = 9.2 Hz, 4H), 8.09 (dd, J = 1.6, 7.8 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 67.5, 69.4, 70.4, 70.5, 70.7, 105.9, 114.9, 117.9, 123.7, 123.8, 125.0, 125.4, 127.8, 133.5, 156.0, 161.5, 163.2, 178.2. LRMS (ESI) m/z 811 (M^+ + H, 48), 833 (M^+ + Na, 100). HRMS (ESI) calcd for $C_{46}H_{51}O_{13}$ (M^+ + H) 811.3330, found 811.3309.

1,28-Bis[4′-(4*H*-chromen-4-on-2-yl)phenyl]-1,4,7,10,13,16,19,22,25,28-decaoxaoctacosane (2i). The titled compound **2i** (0.24 g, 55%) was obtained from 4′-hydroxyflavone **8a** (0.24 g, 1.0 mmol), nonaethylene glycol dimesylate (0.29 g, 0.5 mmol), and K_2CO_3 (0.19 g) as a pale-brown oil according to the general procedure described above. ^{1}H NMR ($CDCl_3$) δ 3.55−3.65 (m, 28H), 3.79 (t, J = 4.8 Hz, 4H), 4.09 (t, J = 4.8 Hz, 4H), 6.61 (s, 2H), 6.91 (d, J = 9.2 Hz, 4H), 7.28 (dd, J = 7.2, 7.2 Hz, 2H), 7.42 (d, J = 8.0 Hz, 2H), 7.56 (ddd, J = 1.6, 7.6, 7.6 Hz, 2H), 7.73 (d, J = 8.8 Hz, 4H), 8.09 (dd, J = 1.6, 7.8 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 67.5, 69.4, 70.4, 70.5, 70.7, 105.9, 114.9, 117.9, 123.7, 123.8, 125.0, 125.4, 127.8, 133.5, 156.0, 161.5, 163.2, 178.1. LRMS (ESI) m/z 855 (M^+ + H, 22), 877 (M^+ + Na, 100). HRMS (ESI) calcd for $C_{48}H_{55}O_{14}$ (M^+ + H) 855.3592, found 855.3571.

1,40-Bis[4′-(4*H*-chromen-4-on-2-yl)phenyl]-1,4,7,10,13,16,19,22,25,28,31,34,37,40-tetradecaoxatetracontane (2j). Tridecaethylene glycol dimesylate was prepared from polyethylene glycol 600 with an average number of ethylene glycol units of 13. The titled compound **2j** (0.29 g, 57%) was obtained from 4′-hydroxyflavone **8a** (0.24 g, 1.0 mmol), tridecaethylene glycol dimesylate (0.37 g, 0.5 mmol), and K_2CO_3 (0.18 g) as a pale-brown oil according to the general procedure described above. The HPLC chromatogram showed a number of peaks normally disbursed consistent with the use of PEG 600 having the most abundance species bearing 10−14 ethylene glycol units. ^{1}H NMR ($CDCl_3$) δ 3.55−3.67 (m, 44H), 3.80 (t, J = 4.8 Hz, 4H), 4.11 (t, J = 4.8 Hz, 4H), 6.62 (s, 2H), 6.93 (d, J = 8.8 Hz, 4H), 7.30 (dd, J = 7.2, 7.2 Hz, 2H), 7.44 (d, J = 8.4 Hz, 2H), 7.56 (dd, J = 7.6, 7.6 Hz, 2H), 7.76 (d, J = 8.4 Hz, 4H), 8.11 (d, J = 8.0 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 67.6, 69.4, 70.5, 70.5, 70.8, 106.0, 114.9, 117.9, 123.8, 123.9, 125.0, 125.4, 127.8, 133.5, 156.0, 161.5, 163.2, 178.1. LRMS (ESI) m/z 1031 (M^+ + H, 20). HRMS (ESI) calcd for $C_{56}H_{71}O_{18}$ (M^+ + H) 1031.4640, found 1031.4648.

1,16-Bis[4′-((6-ethyl)-4*H*-chromen-4-on-2-yl)phenyl]-1,4,7,10,13,16-hexaoxahexadecane (3e). The titled compound **3e** (0.21 g, 56%) was obtained from 6-ethyl-4′-hydroxyflavone **8b** (0.27 g, 1.0 mmol), pentaethylene glycol dimesylate (0.20 g, 0.5 mmol), and K_2CO_3 (0.18 g) as a colorless oil according to the general procedure described above. ^{1}H NMR ($CDCl_3$) δ 1.24 (t, J = 7.2 Hz, 6H), 2.69 (q, J = 7.6 Hz, 4H), 3.64−3.72 (m, 12H), 3.84 (t, J = 4.8 Hz, 4H), 4.14 (t, J = 4.8 Hz, 4H), 6.65 (s, 2H), 6.96 (d, J = 9.2 Hz, 4H), 7.38 (d, J = 8.8 Hz, 2H), 7.44 (dd, J = 2.4, 8.6 Hz, 2H), 7.78 (d, J = 8.8 Hz, 4H), 7.95 (d, J = 2.0 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 15.4, 28.3, 67.6, 69.5, 70.6, 70.8, 105.9, 114.9, 117.7, 123.5, 123.6, 124.1, 127.8, 133.7, 141.2, 154.4, 161.5, 163.0, 178.4. LRMS (ESI) m/z 735 (M^+ + H, 100), 757 (M^+ + Na, 13). HRMS (ESI) calcd for $C_{44}H_{47}O_{10}$ (M^+ + H) 735.3169, found 735.3143.

1,19-Bis[4′-((6-ethyl)-4*H*-chromen-4-on-2-yl)phenyl]-1,4,7,10,13,16,19-heptaoxanonadecane (3f). The titled compound **3f** (0.19 g, 49%) was obtained from 6-ethyl-4′-hydroxyflavone **8b** (0.27 g, 1.0 mmol), hexaethylene glycol dimesylate (0.22 g, 0.5 mmol), and K_2CO_3 (0.18 g) as a colorless oil according to the general procedure described above. ^{1}H NMR ($CDCl_3$) δ 1.19 (t, J = 7.2 Hz, 6H), 2.64 (q, J = 7.6 Hz, 4H), 3.58−3.66 (m, 16H), 3.79 (t, J = 4.8 Hz, 4H), 4.08 (t, J = 4.8 Hz, 4H), 6.58 (s, 2H), 6.90 (d, J = 8.8 Hz, 4H), 7.32 (d, J = 8.4 Hz, 2H), 7.38 (dd, J = 1.6, 8.6 Hz, 2H), 7.72 (d, J = 8.8 Hz, 4H), 7.89 (s, 2H). ^{13}C NMR ($CDCl_3$) δ 15.3, 28.2, 67.5, 69.4, 70.5, 70.5, 70.8, 105.8, 114.9, 117.7, 123.5, 123.5, 124.0, 127.7, 133.6, 141.1, 154.4, 161.4, 163.0, 178.3. LRMS (ESI) m/z 779 (M^+ + H, 100), 801 (M^+ + Na, 63). HRMS (ESI) calcd for $C_{46}H_{51}O_{11}$ (M^+ + H) 779.3431, found 779.3423.

1,16-Bis[4′-((6-methyl)-4*H*-chromen-4-on-2-yl)phenyl]-1,4,7,10,13,16-hexaoxahexadecane (4e). The titled compound **4e** (0.15 g, 42%) was obtained from 6-methyl-4′-hydroxyflavone **8c** (0.26 g, 1.0 mmol), pentaethylene glycol dimesylate (0.20 g, 0.5 mmol), and K_2CO_3 (0.18 g) as a colorless oil according to the general procedure described above. ^{1}H NMR ($CDCl_3$) δ 2.39 (s, 6H), 3.64−3.72 (m, 12H), 3.85 (t, J = 4.8 Hz, 4H), 4.14 (t, J = 4.8 Hz, 4H), 6.64 (s, 2H), 6.96 (d, J = 9.2 Hz, 4H), 7.35 (d, J = 8.4 Hz, 2H), 7.41 (dd, J = 2.0, 8.6 Hz, 2H), 7.77 (d, J = 8.8 Hz, 4H), 7.92 (d, J = 0.8 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 20.9, 67.6, 69.5, 70.6, 70.8, 105.9, 114.9, 117.6, 123.4, 124.1, 124.9, 127.8, 134.7, 134.9, 154.3, 161.5, 163.0, 178.3. LRMS (ESI) m/z 707 (M^+, 45), 729 (M^+ + Na, 100). HRMS (ESI) calcd for $C_{42}H_{43}O_{10}$ (M^+ + H) 707.2856, found 707.2835.

1,19-Bis[4′-((6-methyl)-4*H*-chromen-4-on-2-yl)phenyl]-1,4,7,10,13,16,19-heptaoxanonadecane (4f). The titled compound **4f** (0.18 g, 48%) was obtained from 6-methyl-4′-hydroxyflavone **8c** (0.26 g, 1.0 mmol), hexaethylene glycol dimesylate (0.22 g, 0.5 mmol), and K_2CO_3 (0.18 g) as a colorless oil according to the general procedure described above. ^{1}H NMR ($CDCl_3$) δ 2.36 (s, 6H), 3.61−3.68 (m, 16H), 3.82 (t, J = 4.8 Hz, 4H), 4.11 (t, J = 4.8 Hz, 4H), 6.61 (s, 2H), 6.93 (d, J = 8.8 Hz, 4H), 7.33 (d, J = 8.8 Hz, 2H), 7.41 (dd, J = 2.0, 8.6 Hz, 2H), 7.75 (d, J = 8.8 Hz, 4H), 7.89 (s, 2H). ^{13}C NMR ($CDCl_3$) δ 20.8, 67.6, 69.5, 70.5, 70.6, 70.8, 105.8, 114.9, 117.6, 123.4, 124.0, 124.8, 127.8, 134.6, 134.9, 154.3, 161.5, 163.0, 178.3. LRMS (ESI) m/z 751 (M^+ + H, 45), 773 (M^+ + Na, 20). HRMS (ESI) calcd for $C_{44}H_{47}O_{11}$ (M^+ + H) 751.3118, found 751.3115.

1,16-Bis[4′-((7-methyl)-4*H*-chromen-4-on-2-yl)phenyl]-1,4,7,10,13,16-hexaoxahexadecane (5e). The titled compound **5e** (0.19 mg, 53%) was obtained from 7-methyl-4′-hydroxyflavone **8d** (0.26 g, 1.0 mmol), pentaethylene glycol dimesylate (0.20 g, 0.5 mmol), and K_2CO_3 (0.18 g) as a colorless oil according to the general procedure described above. ^{1}H NMR ($CDCl_3$) δ 2.47 (s, 6H), 3.67−3.74 (m, 12H), 3.87 (t, J = 4.8 Hz, 4H), 4.18 (t, J = 4.8 Hz, 4H), 6.67 (s, 2H), 6.99 (d, J = 8.8 Hz, 4H), 7.18 (d, J = 8.4 Hz, 2H), 7.30 (s, 2H), 7.81 (d, J = 8.8 Hz, 4H), 8.05 (d, J = 8.0 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 21.7, 67.6, 69.5, 70.6, 70.8, 105.9, 114.9, 117.6, 121.5, 124.1, 125.2, 126.4, 127.7, 144.8, 156.1, 161.4, 162.9, 178.1. LRMS (ESI) m/z 707 (M^+ + H, 100), 729 (M^+ + Na, 50). HRMS (ESI) calcd for $C_{42}H_{43}O_{10}$ (M^+ + H) 707.2856, found 707.2867.

1,19-Bis[4′-((7-methyl)-4*H*-chromen-4-on-2-yl)phenyl]-1,4,7,10,13,16,19-heptaoxanonadecane (5f). The titled compound **5f** (0.21 g, 56%) was obtained from 7-methyl-4′-hydroxyflavone **8d** (0.26 g, 1.0 mmol), hexaethylene glycol dimesylate (0.22 g, 0.5 mmol), and K_2CO_3 (0.18 g) as a colorless oil according to the general procedure described above. ^{1}H NMR ($CDCl_3$) δ 2.43 (s, 6H), 3.63−3.70 (m, 16H), 3.84 (t, J = 4.8 Hz, 4H), 4.14 (t, J = 4.8 Hz, 4H), 6.63 (s, 2H), 6.96 (d, J = 8.8 Hz, 4H), 7.14 (d, J = 8.4 Hz, 2H), 7.26 (s, 2H), 7.77 (d, J = 8.8 Hz, 4H), 8.02 (d, J = 8.0 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 21.7, 67.6, 69.5, 70.5, 70.6, 70.8, 105.9, 114.9, 117.7, 121.5, 124.1, 125.2, 126.5, 127.8, 144.8, 156.2, 161.5, 162.9, 178.2. LRMS (ESI) m/z 751 (M^+ + H, 55), 773 (M^+ + Na, 16). HRMS (ESI) calcd for $C_{44}H_{47}O_{11}$ (M^+ + H) 751.3118, found 751.3110.

1,16-Bis[4′-((3-methyl)-4*H*-chromen-4-on-2-yl)phenyl]-1,4,7,10,13,16-hexaoxahexadecane (6e). The titled compound **6e** (0.14 g, 39%) was obtained from 3-methyl-4′-hydroxyflavone **8e** (0.26 g, 1.0 mmol), pentaethylene glycol dimesylate (0.20 g, 0.5 mmol), and K_2CO_3 (0.18 g) as a color oil according to the general procedure described above. ^{1}H NMR ($CDCl_3$) δ 2.12 (s, 6H), 3.64−3.71 (m, 12H), 3.85 (t, J = 4.8 Hz, 4H), 4.15 (t, J = 4.8 Hz, 4H), 6.98 (d, J = 8.8 Hz, 4H), 7.31 (dd, J = 7.6, 7.6 Hz, 2H), 7.36 (d, J = 8.4 Hz, 2H), 7.54 (dd, J = 7.6, 7.6 Hz, 2H), 7.55 (d, J = 8.4 Hz, 4H), 8.17 (dd, J = 1.6, 8.0 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 11.9, 67.5, 69.5, 70.6, 70.8, 114.4, 116.7, 117.7, 122.4, 124.5, 125.7, 125.8, 130.5, 133.1, 155.9, 160.2, 160.8, 178.7. LRMS (ESI) m/z 707 (M^+ + H, 100), 729 (M^+ + Na, 12). HRMS (ESI) calcd for $C_{42}H_{43}O_{10}$ (M^+ + H) 707.2856, found 707.2837.

1,19-Bis[4′-((3-methyl)-4*H*-chromen-4-on-2-yl)phenyl]-1,4,7,10,13,16,19-heptaoxanonadecane (6f). The titled compound **6f** (0.17 g, 45%) was obtained from 3-methyl-4′-hydroxyflavone **8e** (0.26 g, 1.0 mmol), hexaethylene glycol dimesylate (0.22 g, 0.5 mmol), and K_2CO_3 (0.18 g) as a colorless oil according to the general procedure described above. ^{1}H NMR ($CDCl_3$) δ 2.14 (s, 6H), 3.64−3.72 (m, 16H), 3.86 (t, J = 4.8 Hz, 4H), 4.17 (t, J = 4.8 Hz, 4H), 7.00 (d, J = 8.8 Hz, 4H), 7.32 (dd, J = 7.6, 7.6 Hz, 2H), 7.39 (d, J = 8.0 Hz, 2H), 7.57 (dd, J = 7.6, 7.6 Hz, 2H), 7.58 (d, J = 8.4 Hz, 4H), 8.19 (dd, J = 1.6, 7.8 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 11.8, 67.5, 69.5, 70.5, 70.8, 114.3, 116.6, 117.7, 122.3, 124.5, 125.6, 125.7, 130.4, 133.1, 155.9, 160.1, 160.7, 178.6. LRMS (ESI) m/z 751 (M^+ + H, 100), 773 (M^+ + Na, 15). HRMS (ESI) calcd for $C_{44}H_{47}O_{11}$ (M^+ + H) 751.3118, found 751.3096.

1,16-Bis[4′-((7-fluoro)-4*H*-chromen-4-on-2-yl)phenyl]-1,4,7,10,13,16-hexaoxahexadecane (7e). The titled compound **7f** (0.19 mg, 53%) was obtained from 7-fluoro-4′-hydroxyflavone **8f** (0.26 g, 1.0 mmol), pentaethylene glycol dimesylate (0.20 g, 0.5 mmol), and K_2CO_3 (0.18 g) as a colorless oil according to the general procedure described above. ^{1}H NMR ($CDCl_3$) δ 3.65−3.76 (m, 12H), 3.86 (t, J = 4.8 Hz, 4H), 4.17 (t, J = 4.8 Hz, 4H), 6.64 (s, 2H), 6.98 (d, J = 9.2 Hz, 4H), 7.08 (ddd, J = 2.0, 8.4, 8.4 Hz, 2H), 7.17 (dd, J = 2.0, 8.8 Hz, 2H), 7.77 (d, J = 9.2 Hz, 4H), 8.17 (dd, J = 8.8, 8.8 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 67.6, 69.5, 70.6, 70.8, 104.7 (d, J = 27.3 Hz, C-5), 106.0, 113.8 (d, J = 23.9 Hz, C-8), 115.0, 120.7, 123.6, 127.8 (d, J = 15.5 Hz, C-5), 157.0 (d, J = 13.1 Hz, C-9), 161.6, 163.5, 165.5 (d, J = 253.1 Hz, C-7), 177.3. LRMS (ESI) m/z 715 (M^+ + H, 8), 737 (M^+ + Na, 100). HRMS (ESI) calcd for $C_{40}H_{36}O_{10}F_2Na$ (M^+ + Na) 737.2174, found 737.2177.

1,19-Bis[4′-((7-fluoro)-4*H*-chromen-4-on-2-yl)phenyl]-1,4,7,10,13,16,19-heptaoxanonadecane (7f). The titled compound **7f** (0.21 g, 56%) was obtained from 7-fluoro-4′-hydroxyflavone **8f** (0.26 g, 1.0 mmol), hexaethylene glycol dimesylate (0.22 g, 0.5 mmol), and K_2CO_3 (0.18 g) as a colorless oil according to the general procedure described above. ^{1}H NMR ($CDCl_3$) δ 3.60−3.73 (m, 16H), 3.86 (t, J = 4.8 Hz, 4H), 4.17 (t, J = 4.8 Hz, 4H), 6.66 (s, 2H), 6.99 (d, J = 9.2 Hz, 4H), 7.09 (ddd, J = 2.0, 8.4, 8.4 Hz, 2H), 7.18 (dd, J = 2.0, 8.8 Hz, 2H), 7.80 (d, J = 9.2 Hz, 4H), 8.18 (dd, J = 8.8, 8.8 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 67.6, 69.5, 70.5, 70.6, 70.8, 104.6 (d, J = 25.1 Hz, C-6), 106.1, 113.7 (d, J = 23.9 Hz, C-8), 115.0, 120.7, 123.6, 127.9 (d, J = 15.5 Hz, C-5), 157.0 (d, J = 13.3 Hz, C-9), 161.7, 163.6, 165.5 (d, J = 252.8 Hz, C-7), 177.3. LRMS (ESI) m/z 759 (M^+ + H, 5), 781 (M^+ + Na, 100). HRMS (ESI) calcd for $C_{42}H_{40}O_{11}F_2Na$ (M^+ + Na) 781.2436, found 781.2429.

Materials for Biological Studies. Dimethyl sulfoxide (DMSO), DOX ($C_{27}H_{29}NO_{11}$), VP16 ($C_{29}H_{32}O_{13}$), verapamil ($C_{27}H_{38}N_2O_4$), probenecid, and phenazine methosulfate (PMS) were purchased from Sigma-Aldrich. Dulbecco's Modified Eagle's Medium (DMEM), trypsin-ethylenediaminetetra-acetic acid (EDTA) and penicillin/streptomycin were purchased from Gibco BRL. Roswell Park Memorial Institute (RPMI) 1640 medium and fetal bovine serum (FBS) was purchased from HyClone Laboratories. 3-(4,5-Dimethylthiazol-2-yl)-5-[3-(carboxymethoxy)phenyl]-2-(4-sulfo-phenyl)-2*H*-tetrazolium (MTS) was purchased from Promega. The human ovarian carcinoma cell lines 2008/P and 2008/MRP1 were generous gifts from Prof. P. Borst (The Netherlands Cancer Institute, Amsterdam, Netherlands).

Cell Culture. 2008/P is a human ovarian carcinoma cell line while 2008/MRP1 is its stable MRP1 transfectant generated by retroviral transduction using pCMV-Neo-MRP1.[55] pCMV-Neo-MRP1 was constructed by inserting a *Sal*I-*Not*I DNA fragment containing the complete human MRP1 cDNA as a blunt-end fragment in pCMVneo.[55] 2008/P and 2008/MRP1 cells were cultured in RPMI 1640 medium with 10% FBS and 100 U/mL of penicillin and 100 μg/mL of streptomycin and maintained at 37 °C in a humidified atmosphere with 5% CO_2. The cells were split constantly after a confluent monolayer has been formed. To split cells, the plate was washed briefly with phosphate-buffered saline (PBS), treated with 0.05% trypsin-EDTA, and harvested by centrifugation.

Cell Proliferation Assay. Four thousand cells of 2008/P or 2008/MRP1 and anticancer drugs doxorubicin (DOX) or etoposide (VP16) were mixed with or without modulators to a final volume of 200 μL in each well of 96-well plates. The plates were then incubated for 5 days at 37 °C.

The CellTiter 96 AQ_{ueous} Assay (Promega) was used to measure the cell proliferation according to the manufacturer's instructions. MTS (2 mg/mL) and PMS (0.92 mg/mL) were mixed in a ratio of 20:1. An aliquot (10 μL) of the freshly prepared MTS/PMS mixture was added into each well, and the plate was incubated for 2 h at 37 °C. Optical absorbance at 490 nm was then recorded with microplate absorbance reader (Bio-Rad). IC_{50} values were calculated from the dose−response curves of MTS assays (Prism 4.0). All experiments were performed in triplicate and repeated at least thrice, and the results were represented as mean ± standard error mean.

DOX Accumulation and Kinetic Characterization for MRP1 Inhibition. DOX accumulation assay was done in 1 mL volume. A 5×10^5 cells of 2008/P and 2008/MRP1 cells were added in an Eppendorf and incubated with 20 μM DOX and 3 μM of modulators (**9**, probenecid, verapamil, and **4e**) at 37 °C for 120 min. A 0.3% DMSO was as a negative control. After incubation, the cells were spinned down and washed with cold PBS, pH 7.4, for 2 times and lysed with 100 μL of lysis buffer (0.75 M HCl, 0.2% Triton-X100 in isopropanol) with vigorous vortex. The lysate was further incubated at 37 °C for 20 min. After incubation, the lysate was spinned down and 100 μL of supernatant was saved and seeded into a black 96-well microtiter plate. The fluorescence level of DOX was determined by fluorescence spectrophotometer (BMG Technologies) using an excitation and an emission wavelength pair of 460 and 610 nm.

Kinetic parameters for MRP1 inhibition by modulators were determined by incubating the 5×10^5 2008/MRP1 cells with various concentration of DOX (1.5, 4.4, 13.3, and 40 μM) in the presence of **4e** (0, 0.05, 0.075, and 0.1 μM) or verapamil (0, 0.5, 2, and 8 μM) or **9** (0, 10, 25, and 50 μM) for 120 min at 37 °C. The cell lysis and fluorescence intensity was carried out as described previously. The relationship between DOX and modulators for MRP1 inhibition was analyzed by Lineweaver−Burk plot and Dixon plot. In Lineweaver−Burk analysis, the kinetics para-

meter (V_{max}, K_m) for each modulator were estimated from the linear regression of double reciprocal plot of the DOX retention rate versus DOX concentration. The inhibition constant (K_i) can be determined by the plotting the slope from the Lineweaver−Burk plot versus modulator concentration. In the Dixon plot, the K_i was estimated from the linear regression of reciprocal plot of DOX retention rate versus modulator concentration. The slope of the Dixon plot was plotted against the reciprocal of DOX concentration to define relationship between the modulator and DOX.

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Supporting Information Available: Copies of proton and carbon NMR spectra of new compounds, cytotoxicity data of selected compounds, EC_{50} values of **4f**, **5e**, **5f**, verapamil, **9**, and probenecid and the experimental evidence that flavonoid dimer **4e** is in molecular form, but not in aggregate form when it reversed MRP1-mediated drug resistance. This material is available free of charge via the Internet at http://pubs.acs.org.

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