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Review Article

Inhibitors of MRP1-mediated multidrug resistance

Bryan H. Norman
Eli Lilly and Compan y, Discovery Research, Lilly Corporate Cente r, Indianapolis, IN 46285, USA

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Introduction

The successful treatment of cancer by chemotherapy requires that the chemotherapeutic agent display potent and sustained activity against the tumor, such that the tumor cells are completely eradicated by the treatment. However, it is common for cancer patients to initially respond, then experience relapse, due to the fact that their tumors have become resistant and chemotherapy is no longer effective (1). In many cases, these tumors become multidrug resistant (MDR), meaning that in addition to being resistant to the oncolytic treatment, they show cross-resistance to many classes of agents that were never part of their treatment (2, 3). For many years, P-glycoprotein (Pgp) was the focus of most MDR studies because it was shown that Pgp, a member of the superfamily of ATP Binding Cassette (ABC) proteins, conferred resistance to MDR oncolytics in overexpressing cells (4). Research over the past 2 decades has shown that Pgp, which is located in the plasma membrane of Pgp-overexpressing tumor cells, is an ATP-dependent transporter of various natural product oncolytics, such as the vinca alkaloids, epipodophyllotoxins, anthracyclines and taxanes (5). This transport activity results in a lower intracellular concentration of the oncolytic and subsequent MDR phenotype. Agents which can inhibit the action of Pgp have been developed and several of these MDR reversal agents are currently undergoing clinical evaluation for the treatment of resistant tumors (6).

Another ABC transporter, the multidrug resistance-associated protein, or multidrug resistance protein (MRP1), shows functional similarity to Pgp, in spite of its lack of sequence similarity (15% homology) (7, 8). Like Pgp, MRP1 is a large (190 kD) plasma membrane-bound protein (8) which confers resistance to the anthracyclines, epipodophyllotoxins and some vinca alkaloids (9). However, taxanes are apparently not involved in MRP1-mediated resistance (9-12). Additionally, it has been hypothesized that some MRP1 oncolytics may require conjugative metabolism prior to transport (13), but this issue remains controversial (14). Due to the differential expression and tissue specificities of MRP1 and Pgp, MRP1 has recently become an important new target for the modulation of MDR (15). Several recent reviews on the biology of MRP1 have appeared (15-18) and, therefore, this account will focus on the various agents which can reverse MRP1-mediated MDR. Most of these agents are “off the shelf” compounds, which have not been optimized for MRP1 reversal activity. It is anticipated that focused research will result in MRP1 inhibitors of greater potency and selectivity. These agents will be extremely useful in elucidating the role of MRP1 in clinical resistance. Finally, it should be pointed out that compounds which are known to modulate MRP1 indirectly, such as the glutathione depleting agent buthionine sulfoximine (12, 19), will not be included in this review.

MRP1 substrates

It should come as no surprise that the normal physiological function of ABC transporters is not to confound chemotherapeutic advances. In fact, of the many known ABC transporters, only Pgp and MRP1 are presently known to confer drug resistance in cells. While the primary function of Pgp appears to be associated with the removal of toxic xenobiotics (1-4), MRP1 seems to have a more distinct role in normal physiology. Thus, several endogenous MRP1 substrates have been identified by measuring the ATP-dependent transport of these substrates into membrane vesicles, prepared from MRP1-overexpressing cells (13, 20-23). Both drug-selected (13, 22) and MRP1-transfected (21-23) cell lines have been used for these studies. Several important known MRP1 substrates are shown in Figure 1. The most thoroughly investigated MRP1 substrate is the glutathione-conjugated leukotriene, LTC_4 (K_m approx. 100 nM) (21, 22). Since MRP1 can also transport the related eicosanoids LTD_4 and LTE_4, albeit with less efficiency (21), it seems likely that a major physiological role for MRP1 is leukotriene transport. While the glutathione (GS)

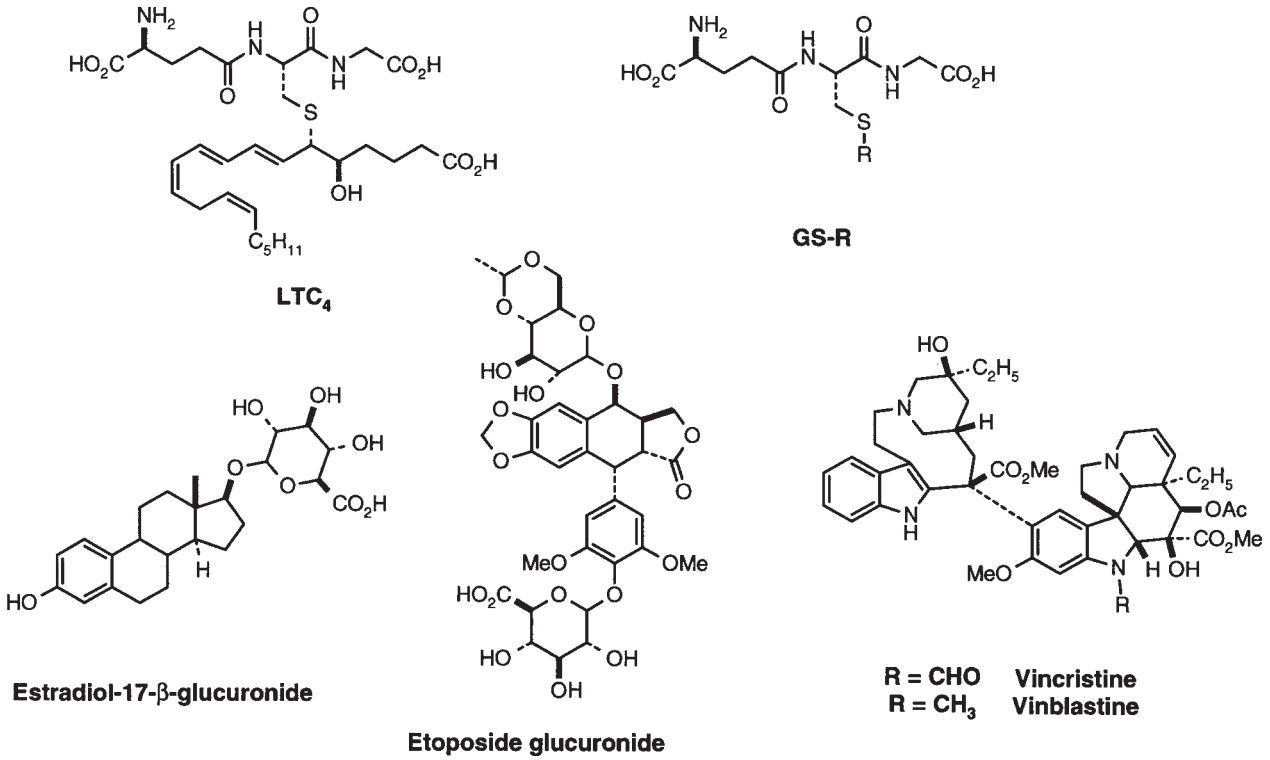


Fig. 1. MRP1 substrates.

moiety seems important for efficient transport, the functionality appended to the cysteine of GS is less critical to substrate specificity. In fact, several GS-R MRP1 substrates have been found, such as the 2,4-dinitrophenyl thioether of GS (GS-DNP) and the GS-melphalan conjugate (21). Even the oxidized glutathione GSSG is transported by MRP1 (21), but with greatly diminished efficiency. Finally, it was shown that GS-n-decyl is a potent and competitive inhibitor of LTC_4 transport (22), suggesting that it is also an MRP1 substrate.

Another class of endogenous MRP1 substrates are the steroid glucuronides, specifically 17β-glucuronosyl estradiol (K_m = 1.5-2.5 μM, Fig. 1) (21, 23). In contrast to the GS conjugate MRP1 substrates, the substrate specificity of the steroid glucuronides seems most dependent on the nonconjugated portion of the molecules. In fact, it has been shown that only the 17β-glucuronide of estradiol (E_2) is an MRP1 substrate while the 3-substituted E_2-glucuronide is not transported by MRP1 (23).

Due to the fact that MRP1 is known to confer drug resistance in whole cells, it is expected that the MRP1-associated oncolytics would be readily transported into vesicles prepared from the membranes of MRP1-overexpressing cells. Unfortunately, efforts to establish this connection have been met with difficulty. It has been speculated that MRP1 transports oncolytics which are first conjugated via glutathionation or glucuronidation (13). This notion is supported by the report that the epipodophyllotoxin, etoposide (VP-16), when conjugated by glucuronidation (Fig. 1), is transported by MRP1 into vesicles in an ATP-dependent fashion (21). Additionally, vincristine (VCR) is directly transported by MRP1 into vesicles, but only when coincubated in the presence of physiological levels of glutathione (22). It has been suggested that equimolar amounts of VCR and GSH may be cotransported via a ternary complex (22), but this has yet to be proven. Doxorubicin (DOX) is apparently not transported in this type of assay (21); however, it was reported that a putative GS metabolite of DOX, which was prepared by synthesis, inhibits LTC_4 transport by MRP1 into vesicles (24, 25). This is consistent with direct transport of this species; however, the GS conjugate has never been established as a DOX metabolite. Clearly, there is much to be learned about the specific mechanisms of drug resistance conferred by MRP1.

Criteria for MRP1 inhibition

The reversal of MRP1-mediated MDR can be established using MRP1-overexpressing tumor cells, which have been either selected for resistance by exposure to a suitable oncolytic or transfected with the MRP1 gene, thus producing a MRP1 drug-resistant phenotype. These cells must demonstrate measurable resistance to an MRP1 oncolytic relative to the parent (MRP1 negative) cell line. An MRP1 reversal agent (inhibitor) should sensitize the resistant cell line to the oncolytic in a dose-dependent fashion. In drug-selected cells, other resistance mechanisms may contribute to the acquired

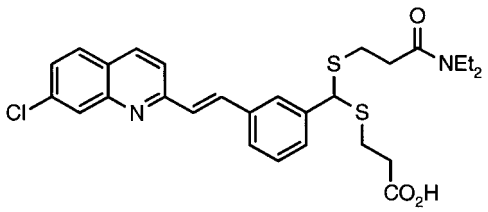
resistance; therefore, full reversal by a selective MRP1 inhibitor might be impossible. However, since MRP1 transfected cells differ from the parent or control cells by MRP1 status only, reversal should be still attainable. In addition to reversal studies on MRP1-overexpressing cells, much effort has focused on finding compounds which directly inhibit the ATP-dependent transport of an MRP1 substrate (usually LTC_4) by MRP1 into vesicles prepared from the plasma membranes of MRP1-overexpressing cells. This assay is particularly useful when interpreting cellular reversal activity of MRP1 inhibitors. Thus, a selective MRP1 reversal agent should inhibit the transport of MRP1 substrates in this type of assay.

Over the past several years, many publications describing the reversal activity of MRP1 inhibitors have appeared. These studies have utilized a variety of MRP1-overexpressing cell lines which show varying degrees of resistance to the appropriate oncolytics. Additionally, these data have been presented in many different ways. When possible, this review will summarize the data in the most commonly accepted format, the RF (reversal factor or fold reversal) value. RF is defined as the IC_{50} of the oncolytic (alone)/the IC_{50} of the oncolytic in the presence of a given concentration of modulator in the resistant cell line. Additionally, to put the RF data into proper perspective, the relative resistance of the cell line to the cytotoxic agent will also be reported. This number is the IC_{50} of the oncolytic in the resistant cell line/the IC_{50} in the parent (sensitive) cell line. Therefore, full reversal of resistance is observed when the RF value is equal to or greater than the relative resistance in the given cell line. RF values for many MRP1 modulators are summarized in Table I, along with the relative resistance values, in parentheses, for the given cell line. This table includes data for the most common MRP1-associated oncolytics DOX, daunorubicin (DNR), VCR and VP-16. Additional data on the ability of these agents to modulate other oncolytics is described in the text, along with additional *in vitro* results, such as substrate transport inhibition and drug accumulation/efflux studies.

Leukotriene antagonists

MK-571

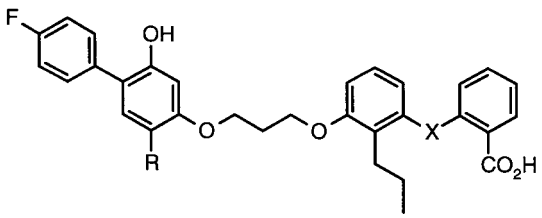
Due to the fact that leukotrienes are important MRP1 substrates, it seems logical that leukotriene antagonists might be a source of potential MRP1 inhibitors. This is indeed the case, as the LTD_4 receptor antagonist, MK-571, was identified as a potent inhibitor of LTC_4 transport into vesicles prepared from MRP1-overexpressing cells (20). MK-571 inhibits the ATP-dependent transport of LTC_4 into MRP1 transfected HeLa T5 membrane vesicles (K_i = 0.6 μM) (20). Additionally, MK-571 inhibited the photoaffinity labelling of MRP1 by [^{3}H]-LTC_4 (20). Finally, in cellular reversal studies, MK-571 modulated drug resistance in two MRP1-overexpressing cell lines (HL60/AR and GLC4/ADR) (26). Although the IC_{50}s were not calculated in this report (thus, RF values cannot be calculated), the published curves indicate complete reversal of VCR resistance at 30 and 50 μM in the HL60/AR and GLC4/ADR cell lines, respectively. Since these concentrations of MK-571 are fairly close to the reported IC_{50} of MK-571 alone in these cell lines (IC_{50} = 70-90 μM), it is important to note that the reversal activity was dose-dependent and moderate modulation of resistance was observed at concentrations as low as 10-30 μM. Reversal of DOX in the GLC4/ADR cell line was observed only at a very high concentration of MK-571 (80 μM) (26). Once again, MK-571 shows inherent cytotoxicity at this concentration, making this data difficult to interpret. MK-571 did not reverse the Pgp-mediated resistance in the Pgp-overexpressing cell line BL60/DAU120.



MK 571
(Merck-Frosst)

LTB_4 receptor antagonists

Another class of MRP1 inhibitors are the LTB_4 receptor antagonists (27). Although these biphenyl substituted aromatic acids are structurally unrelated to MK-571, they have shown similar *in vitro* activity in the MRP1-overexpressing HL60/ADR cell line. Several compounds in this series have shown >50% inhibition of cell growth at 20 mcM in the presence of DOX.



LTB_4 Receptor Antagonists
(Lilly)

Steroids/estrogen receptor modulators

ZK112993

Because 17β-glucuronosyl estradiol was shown to be an MRP1 substrate, it seems likely that steroids and other estrogen mimics may offer insight into the identification of MRP1 modulators. This has proven to be the case as the

Table I: Modulation of MDR in MRP1-overexpressing cells.

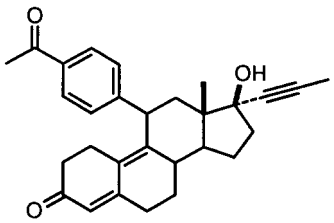
Modulator	Conc. (μM)	Cell line	RF[a] DOX	DNR	VCR	VP-16	Ref.
MK 571	50	HL60/AR			full (38)		26
	30	HL60/AR			full (38)		26
	50	GLC4/ADR			full (23)		26
	30-50	GLC4/ADR			full (23)		26
	50-80	GLC4/ADR	partial (683)				26
LTB_4 antag.	20	HL60/ADR	>50% inhib.				27
ZK112993	2	MCF7/VP	2.23 (12)				27
LY329146	5	HL60/ADR	13.3 (106)				29
	1	HL60/ADR	2.7 (106)				29
	0.5	HL60/ADR	1.7 (106)				29
	2.5	HeLa T5	2.9 (2.9)		3.9 (3.6)		29
	1.0	HeLa T5	1.0 (2.9)		1.6 (3.6)		29
Verapamil	10	HT1080/DR4	74 (88)				32
	3	HT1080/DR4	4.2 (88)				32
	1	HT1080/DR4	1.4 (88)				32
	10	H69AR	4.9 (66)				32
	3	H69AR	1.8 (66)				32
	1	H69AR	2.4 (66)				32
	7	POGB/DX	3.6 (14)	13.3 (53)			33
	5	HL60/ADR	7.3 (112)		17.6 (4.3)	6.3 (303)	34
	2.5	HL60/ADR	3.9 (112)		8.8 (4.3)	3.2 (303)	34
	1	HL60/ADR	2.0 (112)		2.6 (4.3)	2.1 (303)	34
	0.5	HL60/ADR	1.5 (112)		2.3 (4.3)	1.2 (303)	34
	3	KB-8-5	5.2 (42)		15.5 (162)		36
	3	C-A120	1.0 (61)		5.5 (87.5)		36
	5	UMCC-1/VP	2.1 (9)		14 (6.8)	2.2 (34)	49
	5	HL60/R	4.6 (125)		9.4 (53)	4.5 (491)	49
	3.3[b]	COR L23/R		4.4 (14)	9.1 (61)		35
Nicardipine	3	HT1080/DR4	1.0 (88)				32
	3	H69AR	4.6 (66)				32
	1	H69AR	4.2 (66)				32
NIK 250	10[b]	T98G	~full (9.1)		~full (6.1)	~full (12)	38
	10[b]	IN500	partial (10)		~full (5.4)	~full (12)	38
	20[b]	P/VP20	46 (23)			52 (62)	39
	10[b]	P/VP20	14 (23)			5.6 (62)	39
PAK-104P	5	HT1080/DR4	25 (255)				41
	1	HT1080/DR4	8.4 (255)				41
	5	HL60/ADR	31 (185)				41
	1	HL60/ADR	7.8 (185)				41
	10	KB-8-5	22 (42)		182 (162)		36
	3	KB-8-5	22 (42)		108 (162)		36
	10	C-A120	6.8 (61)		63 (88)		36
	3	C-A120	2.7 (61)		28 (88)		36
CSA	5	5637/DR5.5	3.4 (5.4)				43
	5	HL60/ADR	7.5 (112)		32 (4.3)	3.8 (303)	34
	2.5	HL60/ADR	3.1 (112)		4.9 (4.3)	2.0 (303)	34
	1	HL60/ADR	1.5 (112)		3.4 (4.3)	1.4 (303)	34
	0.5	HL60/ADR	1.5 (112)		2.1 (4.3)	1.2 (303)	34
	5[b]	COR L23/R		1.9 (14)	2.7 (61)		35
	3	UMCC-1/VP	1.1 (9)		1.8 (6.8)	1.3 (34)	49
	3	HL60/R	2.2 (125)		4.0 (53)	1.8 (491)	49
PSC 833	5[b]	COR L23/R		1.3 (14)	2.9 (61)		35
	0.4	GLC4-ADR	1.0 (91)		1.2 (22)		45
	2.5	HT1080/DR4	3.6 (255)				41
	0.5	HT1080/DR4	1.2 (255)				41
	2.5	HL60/ADR	1.6 (185)				41
	0.5	HL60/ADR	1.2 (185)				41
VX-710	5	HL60/ADR	15.7 (112)		69.4 (4.3)	12.4 (303)	34
	2.5	HL60/ADR	6.7 (112)		18.7 (4.3)	5.5 (303)	34
	1.0	HL60/ADR	2.2 (112)		7.5 (4.3)	2.5 (303)	34
	0.5	HL60/ADR	1.4 (112)		2.6 (4.3)	1.3 (303)	34
MS-209	5	UMCC-1/VP	3.9 (9)		25 (6.8)	3.7 (34)	49
	5	HL60R	8.2 (125)		13.3 (6.3)	5.3 (491)	49
	5	HL60/ADR	8.5 (112)		10.2 (4.3)	4.8 (303)	34
	2.5	HL60/ADR	4.2 (112)		7.0 (4.3)	2.7 (303)	34
	1.0	HL60/ADR	2.8 (112)		3.3 (4.3)	1.7 (303)	34
	0.5	HL60/ADR	1.2 (112)		2.0 (4.3)	1.2 (303)	34

(Continued)

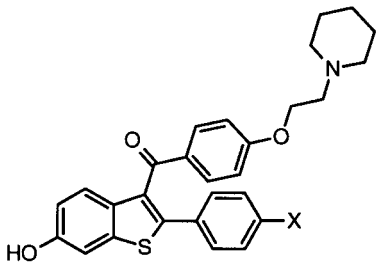
Table I: Modulation of MDR in MRP1-overexpressing cells (Continued).

Modulator	Conc. (μM)	Cell line	RF[a] DOX	DNR	VCR	VP-16	Ref.
ISIS-7597	0.3	HeLa T5	see text				53
Probenecid	500	HL60/AR		4.5 (6.7)	8.9 (6.9)		56
	250	HL60/AR		2.9 (6.7)	6.0 (6.9)		56
	100	HL60/AR		1.4 (6.7)	1.4 (6.9)		56
	500	H69/AR		6.0 (16.3)	13 (58)		56
	250	H69/AR		3.4 (16.3)	11.3 (58)		56
	100	H69/AR		2.9 (16.3)	8.8 (58)		56
Difloxacin	62.5	HL60/AR	15 (22)	18.1 (14.5)	6.0 (8.6)		59
	25	HL60/AR	7.1 (22)	11.1 (14.5)	4.5 (8.6)		59
	12.5	HL60/AR	4.5 (22)	5.4 (14.5)	1.8 (8.6)		59
	2.5	HL60/AR	1.4 (22)	2.2 (14.5)	1.4 (8.6)		59
Dipyridamole	10	COR L23/R	7.5 (12)			7.0 (19)	62
GF109203X	1-8	HL60/AR			~full (38)		65
	5-10	HL60/AR	partial (99)				65
	1-3	GLC4/ADR	partial (683)		partial (23)		65
Indomethacin	10	HL60/ADR	4.3 (100)		7.9 (21)		67
	10	PC-V40			5.4 (67)		67
Genistein	see text						68

[a]RF is defined as reversal factor or fold reversal (see text). Relative resistance of the cell line to the given oncolytic is shown in parentheses. When RF cannot be calculated from the reported data, partial or full reversal of resistance is noted. [b]Modulator concentration in μg/ml.



ZK 112993
(Zeneca)



X = OH Raloxifene
X = $N(SO_2Me)_2$ LY329146
(Lilly)

acetylenic steroid derivative ZK112993 has shown modest reversal of DOX resistance in the MRP1-overexpressing, non-Pgp cell line, MCF7/VP, at 2 μM (RF = 2.23) (28). This cell line, which was selected for resistance with VP-16, is approximately 12-fold resistant to DOX.

LY329146

A structural analog of the selective estrogen receptor modulator (SERM) raloxifene was shown to inhibit the function of MRP1 in MRP1-overexpressing cells. Thus, LY329146 showed reversal activity in the drug-selected HL60/ADR cell line (RF = 13.3 and 2.7) at 5 and 1 μM, respectively, in the presence of DOX (29). Additionally, this agent showed full reversal of resistance in the MRP1 transfected cell line HeLa T5 with an RF = 2.9 (DOX) and 3.9 (VCR) at a modulator concentration of 2.5 μM. LY329146 had no effect on the parent HL60/S and vector control HeLa C1 cell lines, which lack MRP1. Finally, LY329146 inhibited the ATP-dependent LTC_4 transport into membrane vesicles prepared from HL60/ADR cells (IC_{50} = 0.8 μM) (29).

P-glycoprotein modulators

Due to the functional similarity between Pgp and MRP1, many of the reported Pgp modulators have been evaluated for their ability to inhibit the function of MRP1. These compounds include calcium channel blockers and compounds that were optimized for their ability to inhibit Pgp. Several of these compounds show MRP1 activity, making them dual inhibitors of these transporters.

Calcium channel blockers (Fig. 2)

1) Verapamil

Verapamil was one of the first compounds to be identified as an inhibitor of Pgp (30). Similarly, an early search for useful MRP1 modulators found that this agent also reverses MRP1-mediated resistance (31). In the MRP1-overexpressing HT1080/DR4 cell line, verapamil showed modulation of drug resistance (RF = 7.4 and 4.2)

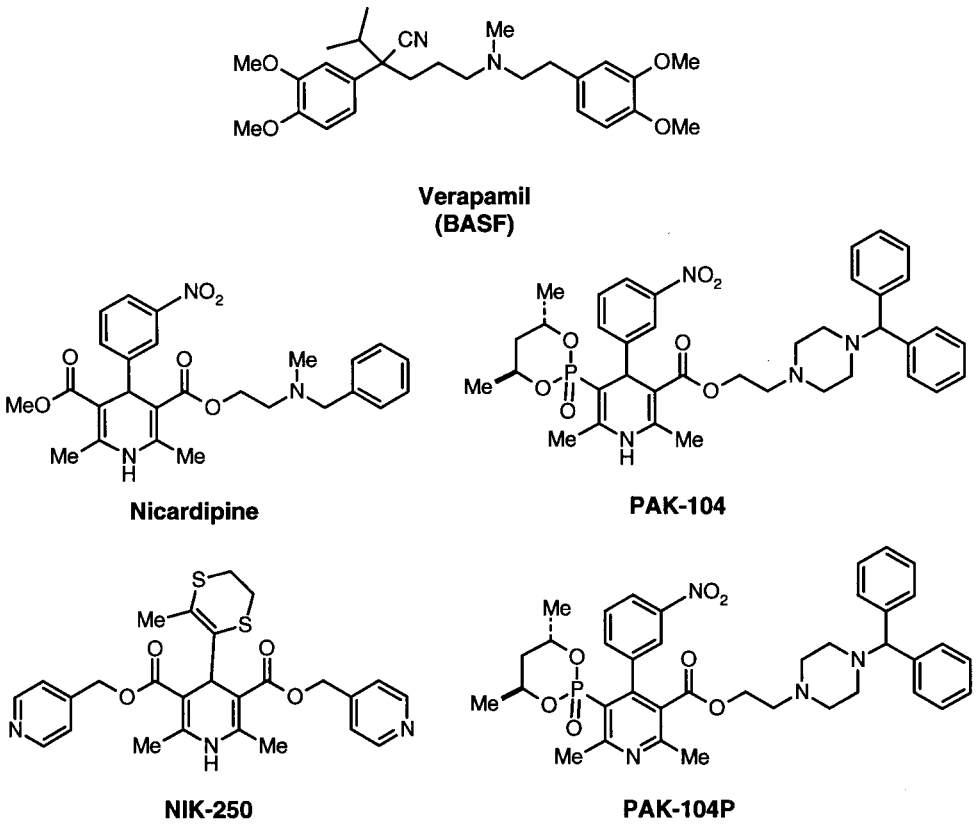


Fig. 2. Calcium antagonist modulators of MRP1.

at concentrations of 10 and 3 μM, respectively, when dosed with DOX (32). Reversal of MRP1-mediated resistance was also demonstrated in POGB/DX cells (33). At a verapamil concentration of 7 μM, an RF = 3.6 was observed in the presence of DOX, while an RF = 13.3 was observed in the presence of DNR (33). Verapamil has shown similar activity in several additional MRP1-overexpressing cell lines, such as H69AR (32), HL60/ADR (34) and COR L23/R (35) (see Table I). Supporting functional inhibition of MRP1 by verapamil is the demonstration that verapamil (100 μM) inhibited the ATP-dependent transport of [^{3}H]-LTC_4 into membrane vesicles prepared from C-A120 cell by 61% (36).

2) Dihydropyridines and pyridines

Another important class of calcium channel blockers are the dihydropyridines, which were identified as Pgp modulators in early studies of Pgp (37). Nicardipine was evaluated for its ability to modulate drug resistance in the MRP1-overexpressing cell lines HT1080/DR4 and H69AR. Although modulation was not observed in the HT1080/DR4 cells, a modest sensitization was observed in H69AR cells when dosed in the presence of DOX (32). However, this effect was not dose-dependent (RF = 4.6 and 4.2 at 3 and 1 μM, respectively) (32). The related dihydropyridine, NIK-250 (at 10 μg/ml), showed nearly full reversal of resistance to VCR and VP-16 in the MRP1-overexpressing glioma cell lines T98G and IN500 (38). Similar reversal activity was observed in the presence of DOX in T98G cells; however, only partial reversal of DOX resistance was observed in the IN500 cells at this concentration (38). Additionally, NIK-250 increased [^{3}H]-VP-16, but not [^{3}H]-VCR, accumulation in T98G cells (38). Finally, in the MRP1-overexpressing prostatic cell line PNP20, NIK-250 showed dose-dependent reversal of resistance at 10 and 20 μg/ml (39). Thus, at the higher concentration, partial to full reversal of resistance was observed in the presence of VP-16 (RF = 52), teniposide (RF = 27) and DOX (RF = 46) (39). NIK-250 also restored the decreased [^{3}H]-VP-16 uptake in the resistant cells to the same level as that observed in the sensitive PC-3 cells in the absence of NIK-250 (39).

In an effort to find more potent and selective Pgp reversal agents, structure-activity studies were performed on the dihydropyridine series of Pgp modulators. These efforts resulted in the identification of pyridine analogs which retained the Pgp-mediated MDR reversal activity but had greatly diminished calcium channel antagonistic activity (40). Specifically, PAK-104P (the pyridine analog of the dihydropyridine, PAK-104) showed promising activity as an MDR reversal agent with potentially reduced cardiovascular side effects (40). Subsequently, PAK-104P

was tested for its ability to modulate MRP1-mediated resistance in various cell lines. In the MRP1-overexpressing cell lines HT1080/DR4 and HL60/ADR, PAK-104P was able to partially reverse the resistance to DOX at 5 μM (HT1080/DR4: RF = 25; HL60/ADR: RF = 31), while having no modulatory effect on the parental lines, HT1080 and HL60, respectively (41). PAK-104P also restored cellular DOX concentrations in the resistant cell lines to parental levels. Of particular interest is the observed activity in these cell lines when dosed in the presence of paclitaxel. Although paclitaxel resistance is generally not believed to be mediated by MRP1 (9-12), each of these MRP1-overexpressing cell lines exhibited a moderate drug resistance profile to paclitaxel (6.1- and 9.0-fold relative resistance in HT1080/DR4 and HL60/ADR, respectively). It should be pointed out that these cell lines were negative for Pgp, using the MDR1-specific JSB-1 monoclonal antibody. PAK-104P fully restored sensitivity to paclitaxel in the HT1080/DR4 (RF = 4.3) and HL60/ADR (RF = 11.1) cell lines (41). In related studies, PAK-104P showed partial reversal to DOX and full reversal to VCR in MRP1-overexpressing C-A120 and KB-8-5 cells at 10 μM. An associated enhanced accumulation of [^{3}H]-VCR was observed in the presence of 10 μM PAK-104P (36). This agent fully reversed resistance to the heavy metals antimony, arenate and arsenite in the C-A120 cell line at 10 μM (42). Consistent with the findings of others, a corresponding increase of antimony accumulation and inhibition of ATP-dependent efflux was observed in C-A120 cells in the presence of 10 μM PAK-104P (42). Additionally, PAK-104P inhibited ATP-dependent transport of [^{3}H]-LTC_4 by MRP1 into membrane vesicles prepared from C-A120 cells at 100 μM (98% inhibition) and 50 μM (61% inhibition) (36). Lineweaver-Burke analysis indicated that this inhibition was competitive with LTC_4.

The demonstration of *in vivo* efficacy is a logical next step in the evaluation of MDR reversal agents which target MRP1. Vanhoefer and coworkers have shown that PAK-104P can modulate the resistance to DOX and paclitaxel in the MRP1-overexpressing human fibrosarcoma HT1080/DR4 xenograft model (41). Xenografts were also established using the parental HT1080 cell line as a control. Thus, it was shown that the HT1080 xenografts were sensitive to DOX and paclitaxel, using response rates (CR + PR), tumor doubling time (TD) and mean maximum inhibitory ratio (MIR) as endpoints. In the HT1080/DR4 xenograft model, a drug-resistant phenotype was observed relative to the HT1080 model. In the presence of DOX and paclitaxel, the overall response rates dropped, with a corresponding drop in TD and MIR. However, in the presence of PAK-104P (100 mg/kg i.v. x 4), overall response rates, TD and MIR increased, reversing the drug-resistant phenotype. PAK-104P showed no inherent toxicity (up to 150 mg/kg) in these models and showed no significant increase in toxicity when dosed with the oncolytics (41).

This study represents the only reported *in vivo* reversal study with an MRP1 modulator. Of particular interest in this study is the fact that drug resistance to paclitaxel was observed. Due to the fact that most studies have shown paclitaxel is not a substrate for MRP1, one might postulate that another resistance mechanism is operative in the HT1080/DR4 cell line. PAK-104P is known to be a nonselective inhibitor in that it modulates both Pgp and MRP1. Although Pgp was not detected in these cells, the reported data is consistent with very low Pgp levels in this cell line as Pgp is known to confer extremely high paclitaxel resistance levels in overexpressing cells. Studies of selective MRP1 and Pgp inhibitors in this model would be very useful in understanding these data.

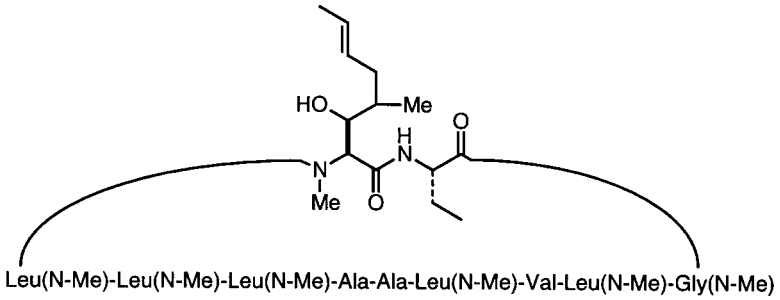
Ciclosporin analogs

1) Ciclosporin A

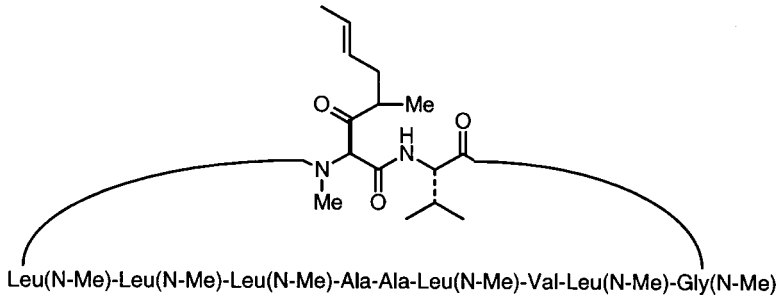
Ciclosporin A (CSA) was tested in the MRP1-overexpressing, non-Pgp human bladder cell line 5637/DR5.5, which is 5.4-fold resistant to DNR, relative to the parent 5637 cell line. In the presence of 5 μM CSA, DNR resistance was reversed (RF = 3.4) in 5637/DR5.5, while no effect was observed in the 5637 cells (43). Additionally, it was found that 10 μM CSA increased the cellular accumulation of [^{3}H]-DNR and also decreased [^{3}H]-DNR efflux in the resistant cells, while having no such effect on the parent cells (43). In a separate report, 5 μM CSA showed reversal activity in the MRP1-overexpressing HL60/ADR cell line in the presence of DOX (RF = 7.5), VP-16 (RF = 3.8) and VCR (RF = 32) (34). Consistent with other reports, reversal of resistance to VCR was observed at lower concentrations. Thus, CSA showed reversal activity (RF = 3.4) even at a concentration of 1 μM. Finally, CSA (7 μg/ml) showed modest reversal activity when dosed with VCR in the MRP1-transfected HeLa T5 cell line, which is 8.4-fold resistant to VCR (9). Interestingly, a similar effect was observed in the control, non-MRP1, HeLa C1 cells. This activity correlated with only a slight increase in the accumulation of VCR in HeLa T5 cells at a CSA concentration of 7 μg/ml. CSA activity was diminished even further when dosed with DOX in HeLa T5 cells (9). This data suggests that CSA may be acting as a chemosensitizing agent via pathways distinct from MRP1 in HeLa cells.

2) PSC-833

The nonimmunosuppressive ciclosporin analog PSC-833, originally developed as a Pgp modulator (44), only slightly increased the accumulation of [^{3}H]-DNR and [^{3}H]-VCR in the MRP1-overexpressing COR L23/R cell line at a concentration of 5 μg/ml (35). This activity was similar to that observed for CSA in this assay. A modest sensitization effect was observed in cellular reversal studies in the COR L23/R cell line at 5 μg/ml PSC-833 in the presence of DNR (RF = 1.9) and VCR (RF = 4.3) (35). In a related study, PSC-833 showed no reversal of resis-



Cyclosporine A
(Sandoz)



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tance to DOX and only slight reversal of resistance to VCR (RF = 1.2) in the MRP1-overexpressing cell line GLC4/ADR at a concentration of 0.4 μM (45). Finally, in the CEM human leukemia MRP1-overexpressing subline CEM/131000, 2 μM PSC-833 significantly increased the accumulation of DNR and idarubicin but had no effect on the accumulation of rhodamine-123. However, this cell line shows no decrease in idarubicin accumulation relative to parental CEM cells (46). These data indicate that PSC-833 is much less effective at modulating MRP1-mediated MDR relative to Pgp-mediated MDR.

Other P-glycoprotein modulators (Fig. 3)

1) VX-710

Another Pgp modulator which shows activity as a MRP inhibitor is VX-710 (47). This agent has shown reversal activity in the HL60/ADR cell line using DOX, VP-16 and VCR (34). Interestingly, in the presence of VCR, VX-710 sensitizes HL60/ADR cells far beyond their relative resistance of 4.3-fold. This observed hypersensitivity of this cell line to VCR in the presence of VX-710 is not well understood, but was not observed in the parent BL60 cell line (RF-5). It was suggested that VX-710 might interact with other cellular targets distinct from Pgp or MRP1 (34). Alternatively, the authors did not rule out the possibility that the activity of VCR (which targets tubulin) might increase the cytotoxicity of VX-710 itself. More studies will be required to fully understand this phenomena.

The ability of VX-710 to increase the intracellular accumulation of DNR in HL60/ADR cells was measured by flow cytometry. These studies indicate that 5 mcM VX-710 fully restored the daunorubicin accumulation in BL60/ADR cells to parent HL60/S levels (34). Additionally, 2.5 μM VX-710 completely restored calcein uptake in this cell line to the parent HL60/S levels while having no observable effect on the parent cell line (34). Finally, MRP1 was labelled by a VX-710 photoaffinity analog, [^{3}H]-VF-13,159 in HL60/ADR cells (34). This labelling could be inhibited by the addition of VX-710 in a dose-dependent manner, consistent with direct interaction with MRP1.

2) MS-209

Another Pgp inhibitor that has shown reversal activity in MRP1-overexpressing cells in MS-209 (48). In the UMCC-1/VP cell line, 5 mcM MS-209 restored sensitivity to VP-16 (RF = 3.7), DOX (RF = 3.5) and VCR (RF = 25) (49). Once again, hypersensitivity was observed for VCR at this concentration in this cell line. MS-209 also reversed oncolytic resistance in the MRP1-overexpressing cell line, HL60R. In the presence of VP-16, DOX and VCR, 5 mcM MS-209 had an RF = 5.3, 8.2 and 13.3, respectively. MS-209 had little effect on several wild type (low to no expression of MRP1) cell lines and the reversing effects of MS-209 correlated with MRP1 expression levels (49). Finally, the intracellular accumulation of [^{3}H]-VCR increased about 2.5-fold in HL60R cells in the

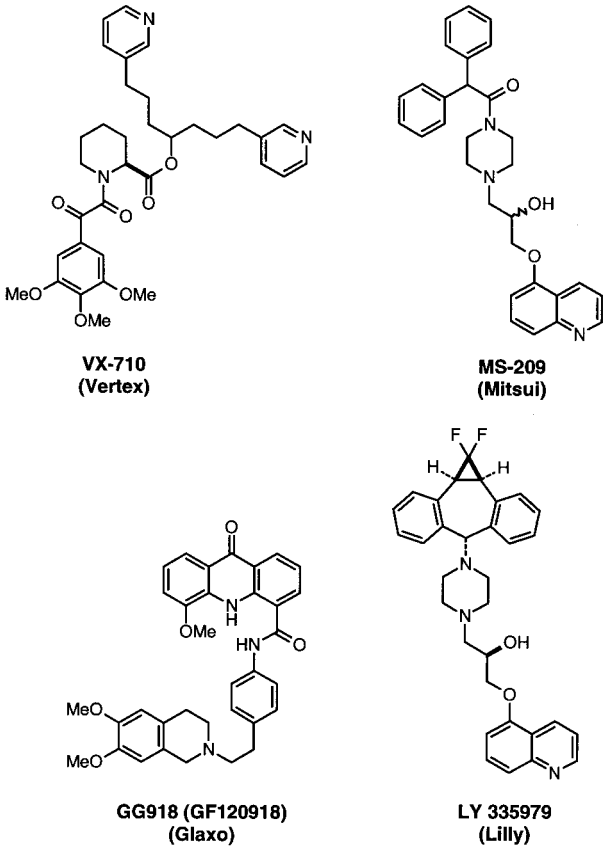

Fig. 3. P-glycoprotein modulators.

presence of 10 μM MS-209. Once again this effect was more pronounced in the higher expressing MRP1 cell lines (49). It should be pointed out that similar results have been reported for MS-209 in the MRP1-overexpressing cell lines KATO III (50), HGC27 (50) and HL60/ADR (34).

In contrast to VX-710 and MS-209, both LY335979 (51, 52) and GF120918 (GG918) (34) do not reverse resistance in MRP1-overexpressing cells, making them selective inhibitors of Pgp. It remains to be seen if selective MDR reversal agents will prove more useful than nonselective compounds. It is hoped that ongoing clinical evaluations of these compounds will help clarify these issues.

Antisense oligonucleotides

ISIS-7597

MRP1 reversal agents demonstrate their activity by inhibiting the protein function of MRP1. An alternative approach to overcoming resistance is to inhibit MRP1 protein synthesis with an antisense oligonucleotide. This approach has resulted in the identification of the antisense phosphorothioate oligonucleotide ISIS-7597 (53, 54), which targets the coding region of the MRP1 mRNA. ISIS-7597 (0.3 μM) was shown to decrease the MRP1 mRNA levels by >90%, with a comparable decrease in protein levels in the MRP1-transfected cell line HeLa T5 (53). This effect resulted in an increased sensitization of this cell line to 10 mcM DOX (82% cytotoxicity compared to 50% in untreated HeLa T5 cells). Similar results were reported for the drug-selected H69AR cell line, which also overexpresses MRP1 (53). These data indicate that antisense oligonucleotides may be useful in the reversal of MRP1-mediated resistance.

Miscellaneous compounds (Fig. 4)

Probenecid

Due to its activity as an organic anion transport inhibitor, probenecid (55) was evaluated for its ability to reverse MRP1-mediated MDR. In the HL60/AR cell line, probenecid reversed resistance to DNR (RF = 4.5) at 500 μM (56). Reversal activity diminished at 250 μM. However, in the presence of VCR, full resistance was observed at 500 and 250 μM (RF = 8.9 and 6.0, respectively) (56). Additionally, in the H69/AR cell line, probenecid reversed resistance to DNR (RF = 6.0 at 500 μM) and VCR (RF = 13.0 at 500 μM). Once again, reversal of resistance to VCR was observed at lower concentrations (RF = 11.3 at 250 μM and 8.8 at 100 μM) (56). Probenecid had no modulatory effects on either the Pgp-overexpressing HL60/Tax and P388/ADR cells or the parental non-Pgp expressing cells (56). Although the effective concentrations are fairly high, probenecid showed little to no toxicity when dosed alone, making it an interesting candidate for *in vivo* studies.

In support of direct inhibition of MRP1, probenecid increased the accumulation of DNR, [^{3}H]-VCR and calcein in the MRP1-overexpressing cell lines H69/AR, COR-L23/R and MOR/R in a dose-dependent fashion that paralleled the cellular reversal activity (56, 57). Additionally, probenecid corrected the altered subcellular distributions of DNR in H69/AR cells (56). Interestingly, it has recently been reported that probenecid inhibited LTC_4 secretion in normal murine mast cells (MC-9), which have no detectable levels of MRP1 (58). This data indicates that probenecid may also inhibit LTC_4 transport by a mechanism independent of MRP1.

Difloxacin

The quinolone antimicrobial agent, difloxacin, has shown MRP1 reversal activity in the HL60/AR cell line in a dose-dependent manner (59). When the HL60/AR cells were dosed with either DOX, DNR or VCR in the presence of 2.5-12.5 μM of difloxacin, a small, but measurable sensitization occurred. When the dose of difloxacin was increased to 62.5 μM, full to nearly full reversal was achieved for all of the oncolytics studied: RF = 15, 18 and

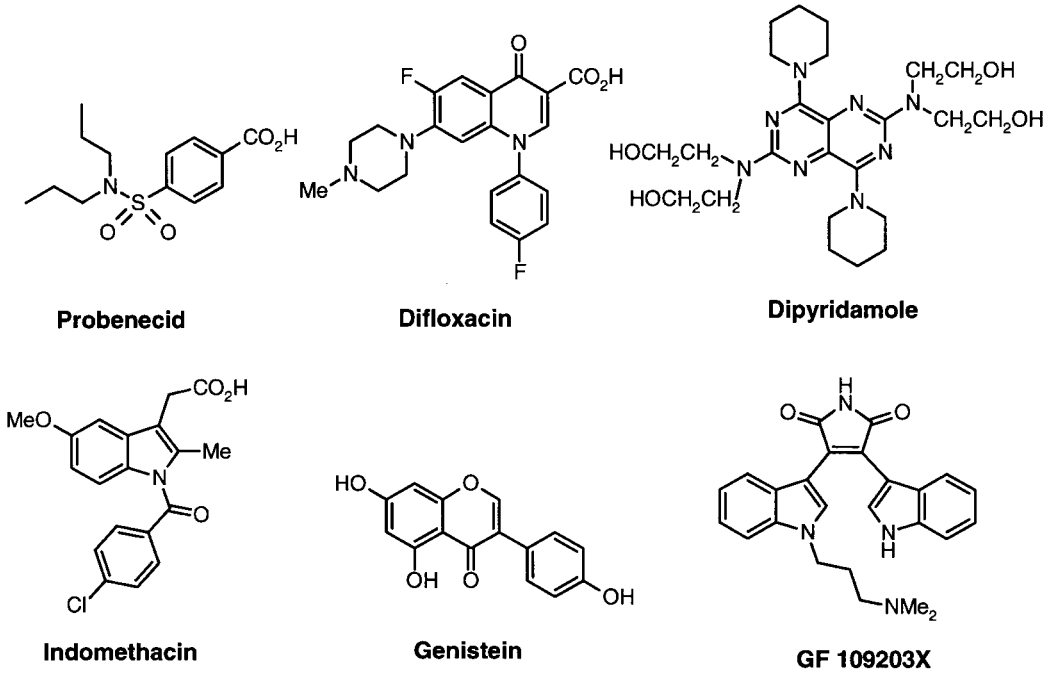

Fig. 4. Miscellaneous MRP1 modulators.

6 for DOX, DNR and VCR, respectively (59). Difloxacin had little to no sensitization effects on the parent HL60 cell line at these concentrations. Additionally, difloxacin, in a concentration-dependent manner, increased the intracellular accumulation of DOX, DNR and [^{3}H]-VCR in HL60/AR cells, as measured by flow cytometry and scintillation cytometry (59). These effects were not observed in the parent HL60 cell line. Finally, it was reported that difloxacin can correct the subcellular localization defects of DOX in HL60AR cells, allowing DOX accumulation in the nucleus, as was observed in the sensitive parent cells. Interestingly, it should be pointed out that the related quinolone antimicrobial agents rufloxacin, ciprofloxacin and norfloxacin did not reverse MRP1-mediated resistance in these assays (59).

Dipyridamole

The antiplatelet agent and Pgp modulator, dipyridamole (60), has shown drug resistance reversal activity in the presence of antimetabolites such as 5-fluorouracil and methotrexate (61). This activity has been ascribed to the compound's ability to inhibit nucleoside transport. Recently, dipyridamole was evaluated for its ability to reverse MDR in the MRP1-overexpressing COR L23/R cell line in the presence of VP-16 and DOX. In the COR L23/R cell line, 10 μM dipyridamole showed an RF = 7.0 when dosed with VP-16, while a similar effect was observed (RF = 7.5) in the presence of DOX (62). The reversal effects of dipyridamole were reduced (approx. 2-fold) in the sensitive COR L23/P cell line. It appears that dipyridamole may not be acting directly on the MRP1 transporter, since only a modest effect on [^{3}H]-VP-16 accumulation and efflux was observed in the resistant cell line, and this effect was similar to that observed in the sensitive line (62). It was determined that dipyridamole caused enhanced depletion of GSH from resistant COR L23/R cells relative to the sensitive COR L23/P cells (62). Therefore, it has been suggested that dipyridamole may be acting via a glutathione depletion mechanism.

GF109203X

Some protein kinase C (PKC) inhibitors are known to be modulators of Pgp (63, 64). This activity has been ascribed to their potential ability to inhibit phosphorylation of Pgp or through modulation of MDR1 gene expression (63, 64). One PKC inhibitor, GF109203X, has been evaluated for its ability to modulate MRP1-mediated resistance in the HL60/AR and GLC4/ADR cell lines. In the HL60/AR cell line, GF109203X showed dose-dependent reversal of resistance to VCR in the 1-8 μM range, with nearly full reversal at 8 μM (65). Although this agent also showed an effect on HL60/AR cells when dosed with DOX, this effect was not as pronounced and showed only modest reversal at the highest concentration of 10 μM. A similar effect was observed in the GLC4/ADR cell line when GF109203X (1-3 μM) was dosed in the presence of VCR (65). Although the published curves clearly show this effect, the IC_{50} values were not reported, precluding the calculation of RF values. It should be pointed out that

the IC_{50} of GF109203X, when dosed alone, is approx. 14 μM (HL60 and HL60/AR) and approx. 7 μM (GLC4 and GLC4/ADR) (65). Therefore, one cannot rule out a synergistic cytotoxic effect at the higher concentrations, which clearly approach the modulator IC_{50} values. However, GF109203 had no effect on the parent HL60 cells when dosed in the presence of VCR up to 10 μM (65). Furthermore, GF109203X inhibited the rhodamine-123 efflux in HL60/AR at a concentration of 10 μM, consistent with functional inhibition of MRP1 (65).

Indomethacin

Indomethacin, which is a potent antiinflammatory agent (66), was shown to modulate MRP1-mediated resistance in the MRP-overexpressing cell lines, PC-V40 (murine) and HL60/ADR (human). Using VCR, 10 μM indomethacin reversed resistance in the PC-V40 cell line (RF = 5.4) and the HL60/ADR cell line (RF = 7.9) (67). Additionally, 10 μM indomethacin reversed the resistance to DOX (RF = 4.3) in HL60/ADR cells (67). Indomethacin showed no inherent toxicity at 10 μM in these cells and had IC_{50}s of approx. 20 μM (HL60 cells) and approx. 50 μM (PC cells) when dosed alone. Indomethacin (at 10 μM) showed no effects on the Pgp-overexpressing variants (PC4-80, PC-V160 and HL60/VCR) and also showed no significant effects on the sensitivity of the parent cells (PV4-WT and HL60) (67).

Consistent with its ability to reverse cellular resistance to MRP1-associated oncolytics, 10 μM indomethacin increased the accumulation and inhibited efflux of the fluorescent dye 2',7'-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) in both PC-V40 and HL60/ADR cells, while having little to no effects on the parental and Pgp-overexpressing variants (67). Additionally, 10 μM indomethacin increased [^{3}H]-VCR accumulation in the PC-V40 cells but not the parent PC4WT cells. It has been suggested that the MRP1 reversal activity of indomethacin may be due, in part, to its activity as an inhibitor of glutathione S-transferase (67). However, additional studies will be required to fully establish this mechanism.

Genistein

The isoflavonoid genistein and several related compounds were shown to inhibit DNR accumulation and efflux in the MRP1-overexpressing cell line, GLC4/ADR, when dosed at 200 μM. Genistein also increased VP-16 accumulation in this cell line, while having no effect on the parent GLC4 cells or several Pgp-overexpressing cells (68). It was further demonstrated that genistein increased the ATPase activity of GLC4/ADR membranes. These studies are consistent with MRP1-mediated transport of isoflavonoids such as genistein (69); however, this has yet to be proven. Finally, although genistein is a useful agent for transport studies, its relative toxicity (IC_{50} = 16 μM in GLC4/ADR cells) precludes its use in cellular reversal studies.

Conclusions/Future Directions

Clearly, the search for MRP1 modulators is still in its infancy, relative to the more than 20 years of effort that has gone into finding MDR reversal agents targeting Pgp. However, as is evident from this review, a wealth of interesting chemical structures have already emerged from these early efforts and the prospect of finding more potent and selective agents is encouraging. It has already been shown that structural information on MRP1 substrate specificity can provide important clues for the design of more effective agents. These agents will become useful tools in future studies aimed at determining the importance of MRP1-mediated MDR in clinical resistance. Additionally, it is hoped that a greater understanding of MRP1-mediated resistance to anthracyclines will be forthcoming, due to the obvious clinical importance of these oncolytics. This information will add greatly to our understanding of the MRP1 substrate pharmacophore, which in turn will enhance our ability to design potent and selective MRP1 inhibitors. This knowledge will enable the clinical evaluation of MRP1 as a new drug target in oncology.

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