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REVERSAL OF RESISTANCE IN MULTIDRUG RESISTANCE PROTEIN (MRP1)-OVEREXPRESSING CELLS BY LY329146.

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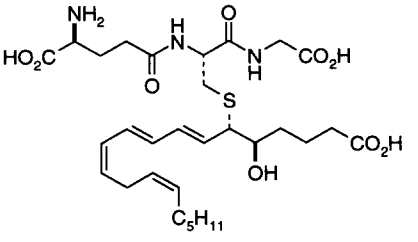
Abstract: The benzothiophene LY329146 reverses the drug resistance phenotype in multidrug resistance protein (MRP1)-overexpressing cells when dosed in combination with MRP1-associated oncolytics doxorubicin and vincristine. Additionally, LY329146 inhibited MRP1-mediated uptake of the MRP1 substrate LTC_4 into membrane vesicles prepared from MRP1-overexpressing cells.

Introduction

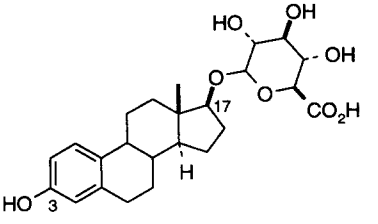
The multidrug resistance-associated protein (MRP1) is a member of the superfamily of ATP-binding cassette (ABC) transporters and is structurally and functionally related to the multidrug resistance (MDR) protein, P-glycoprotein (Pgp).[1] Like Pgp, MRP1 has been shown to confer cellular resistance to various natural product oncolytics, such as the anthracyclines, epipodophylotoxins and some *Vinca* alkaloids.[2] However, MRP1 differs from Pgp in that taxanes are apparently not involved in MRP1-mediated resistance.[2] Both Pgp and MRP1 act as efflux pumps for the corresponding oncolytics. This action results in a lower intracellular concentration of the cytotoxic agent, thus producing a drug resistant phenotype. Therefore, cells overexpressing the MRP1 protein require greater concentrations of oncolytic to achieve cytotoxic endpoints (such as IC_{50}'s), relative to cells that do not overexpress MRP1. This difference in IC_{50} is referred to as *relative resistance.*

In addition to its role in clinical drug resistance, MRP1 transports various endogenous substrates (Figure 1).[3-7] It has been shown that several hydrophobic glutathione conjugates, including the eicosanoid leukotriene C_4 (LTC_4),[3-7] are transported by MRP1. Of particular interest is the discovery that estradiol glucuronides are

Figure 1. Endogenous MRP1 Substrates.



LTC_4



Estradiol-17-β-glucuronide

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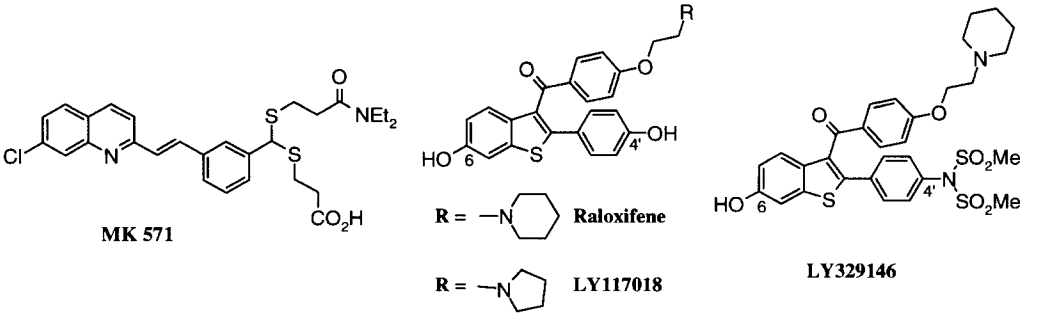
MRP1 substrates[4,6] and that the 17-β-glucuronide of estradiol, but not the 3-glucuronide, is transported by MRP1. As part of our studies in the drug resistance arena, we sought a potent inhibitor of MRP1 for functional studies of this protein.[8] Thus, we felt that one approach to finding potent inhibitors of MRP1 would be to use the molecular information of MRP1 substates for SAR studies. This tactic has been used successfully in the identification of the leukotriene antagonist, MK 571.[7] Unfortunately, while this compound inhibits MRP1 in direct transport assays, its cellular activity is limited.[9] This report describes the activity of LY329146, a potent MRP1 inhibitor, which inhibits MRP1 directly and, more importantly, reverses cellular resistance to MRP1-associated oncolytics.

RESULTS

Reversal of Resistance in Drug Resistant Cells[10]

Raloxifene, a selective estrogen receptor modulator (SERM),[11] is indicated for the prevention of osteoporosis in post-menopausal women.[12,13] The biological activity of raloxifene results from this molecule's ability to competitively bind to the estrogen receptor (ER).[14] Raloxifene binds to the ER with about one third the binding affinity of estradiol (E2).[15] The benzothiophene LY117018,[16] which shows *in vitro* estrogen antagonism activity comparable to raloxifene,[17] was tested for its ability to modulate MRP1-mediated resistance in cells. These experiments are run by measuring the IC_{50} of doxorubicin (DOX) in the presence of various concentrations of the inhibitor (reversal agent). A reversal agent will enhance the cytotoxicity of the oncolytic by some factor, usually referrred to as the reversal factor (RF). LY117018 reversed doxorubicin resistance in the MRP1-expressing HL60/ADR cell line by about 3-fold at a modulator concentration of 5 μM (Figure 2, Table 1).[18] The LTC_4 receptor antagonist, MK 571 had no effect on doxorubicin resistance in HL60/ADR cells up to 5 μM, in spite of the fact that this compound is a sub-micromolar inhibitor of LTC_4 transport into MRP1 membrane vesicles[7] (*vide infra*). Using LY117018 as a lead, we examined other benzothiophenes for their ability to modulate cellular resistance to MRP1-associated oncolytics, such as doxorubicin and vincristine.

Figure 2. MRP1 Inhibitors.



The 4'-bis-methanesulfonanilide LY329146 (Figure 2, Table 1)[16,18] showed enhanced reversal activity in the HL60/ADR cell line (MRP1 highly expressed, Pgp not expressed), relative to LY117018. In fact, LY329146 reversed the resistance to doxorubicin in HL60/ADR cells by 13.3-fold at 5 μM, 2.7-fold at 1.0 μM, and 1.7-fold at 0.5 μM. LY329146 had no significant effect on the drug-sensitive parent HL60/S cells, which do not express either MRP1 or Pgp. LY329146 did, however, have marked reversal activity on the HL60/*Vinc* cell line (Pgp highly expressed, MRP1 not expressed), reversing the resistance to doxorubicin 69-fold (complete reversal) at 5 μM. The Pgp reversal activity was greatly diminished, however, at 1.0 and 0.5 μM. To confirm the modulator activity against Pgp, we examined the effect of LY329146 in the CEM/VLB_{100} cell line (Pgp highly expressed).[19] LY329146 reversed Pgp-mediated resistance to paclitaxel in these cells by 380-fold at 5 μM. (CEM/VLB_{100} is about 6000-fold resistant to paclitaxel.) Furthermore, LY329146 displaced [^{3}H]vinblastine binding to Pgp in an equilibrium binding assay employing CEM/VLB_{100} plasma membranes[19] with an IC_{50} of 20 μM.

Table 1. Modulation of Cytotoxicity by LY329146 and LY117018 in Drug Sensitive and Resistant HL60 Cell lines.[a]

	HL60/S[b]		HL60/ADR[d]		HL60/*Vinc*[e]	
	IC_{50} (μg/ml)	RF[c]	IC_{50} (μg/ml)	RF	IC_{50} (μg/ml)	RF
DOX	0.028	-	2.97	-	1.88	-
DOX + 5.0 μM LY329146	0.011	2.4	0.224	13.3	0.027	69
DOX + 1.0 μM LY329146	0.018	1.5	1.107	2.7	0.444	4.2
DOX + 0.5 μM LY329146	0.023	1.2	1.715	1.7	1.44	1.3
DOX + 5.0 μM LY117018			1.002	3.0		
DOX + 1.0 μM LY117018			3.20	1.0		

[a]LY329146 showed no cytotoxicity when dosed alone in HL60 cells.
[b]HL60/S (parental line; MRP1-, Pgp-).
[c]Reversal Factor (RF) = IC_{50} (oncolytic) / IC_{50} (oncoytic + modulator). This nomenclature is thoroughly described in Ref. 8.
[d]HL60/ADR (MRP1+, Pgp-) is 106-fold resistant to DOX.
[e]HL60/Vinc (MRP1-, Pgp+) is 67-fold resistant to DOX.

Although compounds such as LY329146 effectively modulate resistance in HL60/ADR cells in a dose-response manner, we have observed that none of our modulators to date are able to fully reverse resistance in this cell line. Alternative unknown resistance mechanisms may contribute to the resistance observed in this drug selected cell line, thus making it unlikely that 100% reversal would be observed with a selective MRP1 modulator. In an effort to determine if a potent MRP1 modulator, such as LY329146, could achieve full reversal of resistance in cells in which MRP1 is the only mechanism of drug resistance, we evaluated this compound in the *MRP1* transfected cell line HeLa T5.[20] These results are summarized in Table 2. HeLa T5 cells are 2.9-fold resistant to doxorubicin, relative to HeLa C1 (vector control) and 3.6-fold resistant to vincristine. When the HeLa T5 cells were treated with doxorubicin in the presence of 2.5 μM LY329146, full reversal (RF = 2.9) was observed. Similarly, full reversal was observed at 2.5 μM (RF = 3.9) when vincristine was used as the

oncolytic. The reversal activity was diminished at 1.0 μM, consistent with the HL60/ADR data. It should be pointed out that LY329146 has a modest modulatory effect on the activity of doxorubicin and vincristine in HeLa C1 cells. Although this was somewhat unexpected, it is consistent with the observations of others.[21] One explanation is that other ABC transporters (e.g. MRP2)[22] may be functioning in HeLa cells. If compounds such as LY329146 are active against any of these other putative transporters, modulation would be expected in the sensitive HeLa C1 cell line. Additional studies are needed to further address this hypothesis. However, this does not diminish the fact that LY329146 fully sensitizes the HeLa T5 cells to doxorubicin and vincristine at 2.5 μM, consistent with potent activity against MRP1.

Table 2. Modulation of Cytotoxicity in HeLa C1 and T5 Cells.[a]

	HeLa C1		HeLa T5	
	IC_{50} (μg/ml)	RF	IC_{50} (μg/ml)	RF[b]
DOX	0.339	-	0.988	-
DOX + 2.5 μM LY329146	0.213	1.6	0.241	2.9
DOX + 1.0 μM LY329146	0.315	1.1	1.063	1.0
VCR	0.014	-	0.051	-
VCR + 2.5 μM LY329146	0.005	2.8	0.013	3.9
VCR + 1.0 μM LY329146	0.007	2.0	0.031	1.6

[a]LY329146 showed no cytotoxicity when dosed alone in HeLa C1 and HeLa T5.
[b]HeLa T5 is 2.9-fold resistant to DOX. Therefore a Reversal Factor (RF) of 2.9 represents full (100%) reversal.

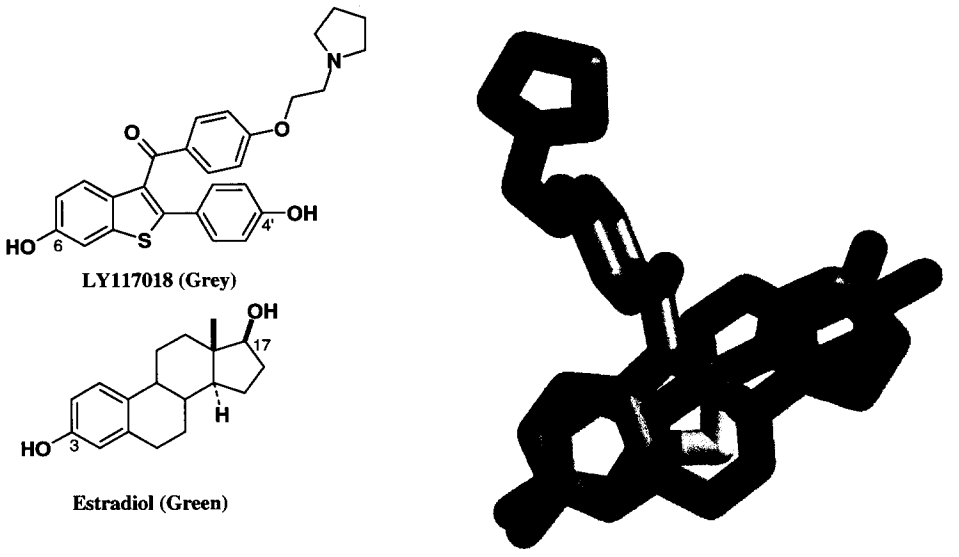
Inhibition of LTC_4 transport by MRP1 into Membrane Vesicles

In addition to our studies on the reversal of resistance in MRP1-overexpressing cells, we have also found that LY329146 inhibits 50 nM LTC_4 uptake into HL60/ADR membrane vesicles (IC_{50} = 0.80 μM). This activity compares favorably to the IC_{50} of 0.50 μM for MK 571.[23] These data indicate that LY329146 acts directly on the MRP1 transporter and are consistent with our observed reversal activity in the MRP1-overexpressing cell lines.

Discussion

Although it is not apparent from the two dimensional representation that LY117108 and estradiol are conformationally similar, a three dimensional overlay (Figure 3)[24] clearly shows that the 6-hydroxy in LY117108 occupies a similar region of space as the 3-hydroxy substituent in estradiol. Furthermore, the 17-β-hydroxyl group in estradiol also overlaps nicely with the 4'-hydroxyl group in LY117018. This is consistent with the estrogenic activity observed for this benzothiophene in many tissues.[11,13] Thus, because it is the added functionality at the 17-position of estradiol (glucuronidation) which is required for estradiol to become an MRP1 substrate, it is not surprising that the added functionality of the bis(methylsulfonyl) group at the 4' position has a profound effect on the MRP1 activity relative to LY117018.

Figure 3. Structural Overlay of LY117018 and Estradiol.[24]



The concept of utilizing substrate information has been useful in the early studies of MRP1 and has led to the identification of several interesting MRP1 modulators. Most notable is the leukotriene antagonist MK 571,[7] which shows potent inhibition of LTC_4 uptake by MRP1 into membrane vesicles. However, leukotriene antagonists, such as MK 571, are usually negatively charged species which display diminished activity in whole cell assays. Focusing on other MRP1 substrates, such as the 17-β-glucuronide of estradiol represents another approach to the identification of new leads which may have improved MRP1 modulatory activity in whole cell assays.

The data presented here shows that LY329146 is a unique MRP1 modulator. In contrast to MK 571, LY329146 shows significant reversal activity in MRP1-overexpressing cells (HL60/ADR and HeLa T5) in addition to its ability to inhibit MRP1-mediated uptake of LTC_4. This represents a major advance in this field due to the historical difficulty that is typically encountered when trying to convert potent leads with low cellular permeability into useful therapeutic agents. Compounds such as LY329146 already contain this required feature, making them particularly exciting leads for further study. Additionally, the foundation has been laid for future SAR studies by showing that structural analysis of MRP1 substrates can provide useful information for the design of new MRP1 modulators.

References and Notes

1. Cole, S. P. C.; Bhardwaj, G.; Gerlach, J. H.; Mackie, J. E.; Grant, C. E.; Almquist, K.C.; Stewart, A. J.; Kurz, E. U.; Duncan, A. M. V.; Deeley, R. G. *Science* **1992**, *258*, 1650.
2. Cole, S. P. C.; Sparks, K. E.; Fraser, K.; Loe, D. W.; Grant, C. E.; Wilson, G. M.; Deeley, R. G. *Cancer Res.* **1994**, *54*, 5902.
3. Jedlitschky, G.; Leier, I.; Buchholz, U.; Center, M.; Keppler, D. *Cancer Res.* **1994**, *54*, 4833.
4. Jedlitschky, G., Leier, I.; Buchholz, U.; Barnouin, K.; Kurz, G.; Keppler, D. *Cancer Res.* **1996**, *56*, 988.
5. Loe, D. W.; Almquist, K. C.; Deeley, R. G.; Cole, S. P. C. *J. Biol. Chem.* **1996**, *271*, 9675.
6. Loe, D. W.; Almquist, K. C.; Deeley, R. G.; Cole, S. P. C. *J. Biol. Chem.* **1996**, *271*, 9683.
7. Leier, I.; Jedlitschky, G.; Buchholz, U.; Cole, S. P. C.; Deeley, R. G.; Keppler., D. *Biol. Chem.* **1994**, *269*, 27807.
8. For a recent review of MRP1 inhibitors, see Norman, B. H. *Drugs Fut.* **1998**, *23*, 1001.
9. Gekeler, V.; Ise, W.; Sanders, K. H.; Ulrich, W.; Beck, J. *Biochem. Biophys. Res. Commun.* **1995**, *208*, 345.
10. Experimental procedures for all assays have been described previously. See Ref. 2 for cellular chemosensitivity and reversal studies. See Ref. 3 for inhibition of membrane transport studies. See Ref. 19 for equilibrium binding displacement studies. Additionally, it should be pointed out that experiments were typically performed in duplicate, therefore appropriate statistical analysis is not possible. The use of standards in these experiments, however, indicates that the numbers are accurate to within 20%.
11. Kauffman, R. F.; Bryant, H. U. *Drug News Perspect.* **1995**, *8*, 531.
12. Jones, C. D.; Jevniker, M. G.; Pike, A. J.; Peters, M. K.; Black, L. J.; Thompson, A. R.; Falcone, J. F.; Clemens, J. A. *J. Med. Chem.* **1984**, *27*, 1057.
13. Delmas, P. D.; Bjarnason, N. H.; Mitlak, B. H.; Ravoux, A.; Shah, A. S.; Huster, W. J.; Draper, M.; Christiansen, C. *N. Engl. J. Med.* **1997**, *337*, 1641.
14. Glasebrook, A. L.; Phillips, D. L.; Sluka, J. P. *J. Bone Miner. Res.* **1993**, *8 (Suppl. 1)*, S268.
15. Grese, T. A.; Cho, S.; Finley, D. R.; Godfrey, A. G.; Jones, C. D.; Luger, C. W.; Martin, M. J.; Matsumoto, K.; Pennington, L. D.; Winter, M. A.; Adrian, M. D.; Cole, H. W.; Magee, D. E.; Phillips, D. L.; Rowley, E. R.; Short, L. L.; Glasebrook, A. L.; Bryant, H. U. *J. Med. Chem.* **1997**, *40*, 146.
16. LY117018 and LY329146 were prepared using chemistry outlined in Ref. 14.
17. Grese, T. A., J. P. Sluka, J. P.; H. U. Bryant, H. U.; G. J. Cullinan, G. J.; A. L. Glasebrook, A. L.;C. D. Jones, C. D.; Matsumoto, K.; Palkowitz, A. D.; Sato, M.; Termine, J. D.; Winter, M. A.; Yang, N. N.; Dodge, J. A. *Proc. Natl. Acad. Sci USA* **1997**, *94*, 14105.
18. Norman, B. H.; Dantzig, A.; Hauser, K.; Kroin, J.; Law, K; Palkowitz, A.; Shepard, R.; Sluka, J.; Starling, J.; Tabas, L.;Winter, M. *Proc. Am. Assoc. Cancer Res.* **1997**, *38*, 2949.
19. Dantzig, A. H.; Shepard, R. L.; Cao, J.; Law, K. L.; Ehlhardt, W. J.; Baughman, T, M.; Bumol, T. F.; Starling, J. J. *Cancer Res.* **1996**, *56*, 4171.
20. Grant, C. E.; Valdimarsson, G.; Hipfner, D. R.; Almquist, K. C.; Cole, S. P. C.; Deeley, R. G. *Cancer Res.* **1994**, *54*, 357.
21. Germann, U. A.; Ford, P. J.; Shlyakhter, D.; Mason, V. S.; Harding, M. W. *Anti-Cancer Drugs* **1997**, *8*, 141.
22. Keppler, D.; Konig, J. *FASEB J.* **1997**, *11*, 509.
23. Our IC_{50} for MK 571 is consistent with the published $K_i = 0.6$ μM (see ref. 7).
24. The overlay was calculated using a least squares technique using Insight-II (version 95.0.4, Molecular Simulations Inc., San Diego) from the x-ray crystal structures of raloxifene and estradiol.