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Biochemical Pharmacology 67 (2004) 1111–1121
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Evaluation of the binding of the tricyclic isoxazole photoaffinity label LY475776 to multidrug resistance associated protein 1 (MRP1) orthologs and several ATP-binding cassette (ABC) drug transporters

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Received 13 June 2003; accepted 4 November 2003

Abstract

Several of the ATP-binding cassette (ABC) transporters confer resistance to anticancer agents and/or antiviral agents when over-expressed in drug-sensitive cells. Recently a MRP1 (ABCC1) tricyclic isoxazole inhibitor, LY475776 was shown to be a glutathione-dependent photoaffinity label of human MRP1 and showed poor labeling of murine mrp1, an ortholog that does not confer anthracycline resistance. In the present study, the specificity of LY475776 was examined for its ability to modulate or photolabel orthologs of MRP1 and several other drug efflux transporters of the ABC transporter family. LY475776 modulated MRP1 and Pgp-mediated resistance (MDR, ABCB1) in, respectively, HeLa-T5 and CEM/VLB$_{100}$ cells to both vincristine and doxorubicin. LY475776 photolabeled 170 kDa Pgp and was inhibited by the potent Pgp inhibitor LY335979 (Zosuquidar.3HCl). The labeling of the 190 kDa MRP1 protein in membranes of HeLa-T5 cells was inhibited by substrates of MRP1 such as leukotriene C_4, vincrisine, and doxorubicin and by the inhibitor, MK571. LY475776 did not photolabel human MRP2 (ABCC2), MRP3 (ABCC3), MRP5 (ABCC5) or breast cancer resistance protein (ABCG2). Because LY475776 photolabels murine mrp1 less well than human MRP1 and binds to a region believed important for anthracycline binding, studies were conducted with monkey and canine MRP1 which also show a reduced ability to confer resistance to anthracyclines. Unlike murine mrp1, both orthologs were photolabeled well by LY475776. These studies indicate that the specificity of LY475776 is fairly limited to Pgp and MRP1 and further studies will help to define the binding regions.

Keywords: Photoaffinity label; ABC transporter; MRP1 ortholog; P-glycoprotein; Breast cancer resistance protein; LY475776

1. Introduction

The ABC transporter superfamily is comprised of seven families of transport proteins containing ~50 human transporters, many of which actively extrude substrates from cells. Alterations in several have been linked to human diseases [1]; for example, cystic fibrosis transmembrane conductance regulator CFTR is defective in cystic fibrosis patients, cholesterol efflux regulatory protein ABCA1 has an important role in arteriosclerosis, MRP6 (ABCC6) is mutated in a connective tissue disorder called Pseudoxanthoma elasticum, and MRP2 (ABCC2) is mutated in Dubin–Johnson syndrome resulting in hyperbilirubinemia [2–6]. This superfamily also contains several efflux

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Abbreviations: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; DOX, doxorubicin; EC_{50}, the concentration that gives 50% of the maximal response; FBS, fetal bovine serum; G418, geneticin; GSH, glutathione; LTC_4, leukotriene C_4; MEM, modified Eagle's medium; Mab, monoclonal antibody; MK571, 3-(3-(2-[7-chloro-2-quinolinyl]-ethenyl)phenyl)-[[(3-dimethylmino-3-oxopropyl)thio]-methyl]thio propanoic acid; MRP1, multidrug resistance associated protein 1 (ABCC1); PAGE, polyacrylamide gel electrophoresis; Pgp, P-glycoprotein; SDS, sodium dodecylsulfate; tet, tetracycline; VCR, vincristine; LY475776, *N*-(4-azido-3-[^{125}I]-phenyl)-2-[3-(9-chloro-3-methyl-4-oxo-4*H*-isoxazolo[4,3-*c*]quinolin-5-yl)-cyclohexyl]-acetamide; LY465803, *N*-[3-(9-chloro-3-methyl-4-oxo-4*H*-isoxazolo[4,3-*c*]quinolin-5-yl)-cyclohexylmethyl]-benzamide.

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doi:10.1016/j.bcp.2003.11.006

transporters that are capable of conferring drug resistance to anticancer and/or antiviral agents when overexpressed in drug-sensitive cell lines. These transporters include P-glycoprotein (ABCB1) in the "B" family, BCRP (ABCG2) a member of the "White" family and seven MRP1–5 (ABCC1-5) members of the "C" family as well as a recently identified MRP7 (ABCC10) and MRP8 (ABCC11) [7–9]. P-glycoprotein confers resistance to vinca alkaloids, anthracyclines, podophyllotoxins, and taxanes; the MRPs 1, 2, 3, and 6 confer resistance to anthracyclines, Vinca alkaloids, methotrexate, and both MRP4 and MRP5 give resistance to nucleoside antiviral and anticancer agents [10–12]. Overexpression of BCRP (ABCG2) confers resistance to topotecan and camptothecin [13–15]. Despite the overlapping substrate specificity of certain superfamily members, the homology of transporters with similar function is 15% or less [16]. Interestingly, differences in substrate specificity have been observed in orthologs of MRP1. Even though the identity of human, murine, canine, monkey is 88% or greater, only human MRP1 confers resistance to anthracyclines, rodent (both murine and rat) and canine MRP1 do not; and monkey has a reduced ability to do so [17–22]. There appear to be important differences in the binding site(s) within these MRP1 orthologs for this clinically important class of anticancer agents.

Selective transport inhibitors can be used to study the physiological role of each transporter in the laboratory as well as to determine their clinical importance. Because Pgp is overexpressed in numerous tumor types and effluxes drugs from four major classes of oncolytics, inhibitors of Pgp have long been sought [23–25]. Several inhibitors (LY335979, Zosuquidar·3HCl); XR9576 (tariquidar) and OC144-093 are more selective for Pgp than MRP1 and other family members [25]. MRP1 confers resistance to many of the same drugs and is overexpressed in lung tumors and neuroblastomas [26,27]. We have reported previously that the tricyclic isoxazole compounds inhibit MRP1-mediated transport of its physiological substrate, LTC_4 and sensitize MRP1-expressing transfectants to anticancer drugs that are effluxed by this transporter [28]. In addition, a [^{125}I]-radiolabeled tricyclic isoxazole photoactiveable analog LY475776 derived from LY465803 shown in Fig. 1 labels MRP1 in a GSH-dependent fashion and binds to the COOH-terminal half of MRP1 in the third membrane spanning domain (MSD3) [29]. Coincubation of MRP1 with LY475776 and vanadate inhibits photolabeling suggesting that an ATP-dependent conformation of MRP1 is labeled. Additional studies done with the murine mrp1 have indicated that this ortholog binds LY475776 less well than the human form [30]. This is particularly interesting since murine mrp1 does not confer resistance to anthracyclines unlike human MRP1 [17,18,31]. Mutational analysis and hybrids of human MRP1 and murine mrp1 identified a likely binding region for anthracycline and LY475776 to be within the same 572 amino acid region (residues 959–1531) [19,29,31].

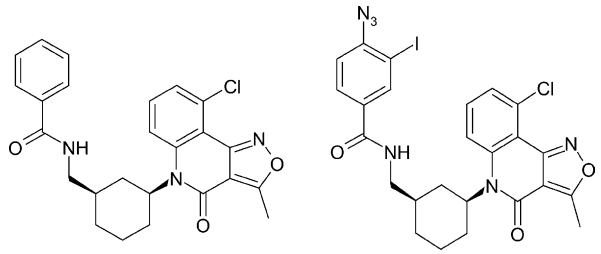

Fig. 1. Structure of tricyclic isoxazole modulators, the photoaffinity affinity MRP1 inhibitor, [^{125}I]LY475776 and a close structural analog LY465803 that lacks both the azido- and iodo-groups.

The present study was undertaken to explore the binding of this photolabel to two other orthologs of MRP1. Canine MRP1 does not confer anthracycline resistance while monkey confers reduced anthracycline resistance relative to human MRP1 [20,21]. The specificity of photolabeling by LY475776 for several other ABC drug efflux transporters is also examined.

2. Materials and methods

2.1. Materials

The ECL detection kit was purchased from Amersham Pharmacia Biotech. E.M. Science was the supplier for $MgCl_2$. The pRev-TRE retroviral vector and tetracycline-system approved FBS was purchased from Clontech while the other serums were purchased from Hyclone laboratories. Improved minimum essential medium was purchased from BioSource International. Gibco was the supplier of all other media and media components. Lipofectamine 2000 was purchased from Invitrogen. The Pierce BCA Protein Assay Reagent was purchased from Pierce. The anti-MRP4 polyclonal antibody that was obtained from Kamiya Biomedical Company and the other antibodies to the transporters were purchased from Alexis Biochemicals. The tricyclic isoxazole compounds LY475776 and LY465803 were prepared by Eli Lilly and Company. The radiolabeled azido-iodinated LY475776 photoaffinity probe was prepared via an iododestannylation technique using chloramine-T and Na[^{125}I]. Radiolabeled LTC_4 was purchased from Perkin-Elmer. Puromycin was obtained from Edge Biosystems. LY335979 (Zosuquidar trihydrochloride) was prepared by Eli Lilly and Co. and is licensed from Syntex as RS-33295-198 [32,33]. All other reagents were purchased from Sigma.

2.2. Cell lines and cell culture

These studies employed a variety of selected and transfected cell lines. The human MRP1- and vector-transfected HeLa cells, respectively, HeLa-T5 and HeLa-C1, and the murine MRP1- and vector-transfected HEK cells

(respectively, HEK-mrp1 and HEK-PC7) were provided by Drs. Susan Cole and Roger Deeley at Queen's University Cancer Research Institute and were grown as previously described [19,34–36]. MRP2- and vector-transfected HEK cells were obtained from Dr. Dietrich Keppler (Deutsches Krebsforschungszentrum) and were maintained as indicated previously [37]. Two alleles for the monkey MRP1 (alleles A and B) were cloned from kidney tissue of a Cynomolgus monkey and transfected into HEK-derived PEAK^{STABLE} cells (Edge Biosystems) using an episomal vector to give PEAK^{STABLE}-monMRP1-A and PEAK^{STABLE}-monMRP1-B [21]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 50 μg/mL G418 and 0.5 μg/mL puromycin. Two canine MRP1 alleles were also cloned from Beagle dog heart tissue and transfected into HeLa cells (HeLa canMRP1-A and HeLa canMRP1-B) and maintained as previously described [20]. CCRF-CEM and CEM/VLB$_{100}$ cells were provided by Dr. William T. Beck at the University of Illinois at Chicago and were maintained in culture as previously described [38]. HL60 cell lines were obtained from Dr. Melvin Center at University of Kansas and maintained as previously reported [39]. MCF-7/pcDNA3 and MCF-7/BCRP clone 8 were generously provided by Drs. Douglas Ross and Austin Doyle at the University of Maryland at Baltimore and were grown as reported previously [40]. HEK cells were transfected with a MRP5 cDNA cloned into a pMiNeo vector or the empty vector using Lipofectamine 2000 according to the manufacture's instructions. MRP5- and vector-transfected HEK cells were cultured in DMEM containing 2 mM L-glutamine and 1500 μg/mL G418 with 10% FBS. MRP3- and vector-transfected HeLa cells were prepared by cloning MRP3 cDNA into pRev-TRE retroviral vector using the manufacturer's instructions. Retroviral packaging and infection of HeLa-Tet-On cells to generate the Tet-inducible MRP3 and vector HeLa Tet-On cells that were grown and maintained in DMEM containing high glucose, pyridoxine·HCl, 110 mg/mL sodium pyruvate, 2 mM L-glutamine, 100 mg/mL G418, 250 mg/mL hygromycin and 10% Tet-system approved FBS. To induce the expression of MRP3, MRP3-transfected HeLa Tet-On cells were grown for 5 days in medium containing 1 mg/mL doxycycline.

2.3. Cytotoxicity assays

Cytotoxicity assays were conducted as previously report using AQ$_{ueous}$ One solution cell proliferation assay (Promega Corp.) [28,38].

2.4. Preparation of membrane vesicles

Inside-out membrane vesicles were prepared from CCRF-CEM, CEM/VLB$_{100}$, HeLa-C1 and HeLa-T5 cells as previously reported [41]. Plasma membranes for the other cell lines were prepared by a modification of that protocol. Instead of disrupting the cells by nitrogen cavitation, cells were lysed using a hypotonic solution. After centrifugation at 5000 *g*, cells were incubated for 10 min at room temperature in 1 mM sodium bicarbonate, followed by centrifugation in a tabletop Sorvall centrifuge ~5000 *g* for 5 min. The resulting supernatant was placed on a 38% sucrose gradient and membrane vesicles were collected at the interface and stored at −70° under argon [38,40].

2.5. MRP1-mediated uptake

The uptake of 50 nM [^{3}H]LTC$_4$ (158 Ci/mmol) was measured into inside-out membrane vesicles using a procedure that was previously published [42]. Uptake was measured in triplicate in the absence and presence of increasing concentrations of the tricyclic isoxazole inhibitor LY465803 (0.002–5 μM) and the effective concentration that gave 50% of the maximum inhibition (EC_{50}) was calculated by a Bravo curve-fitting program.

2.6. Photolabeling of MRP1

The azido-iodinated MRP1 inhibitor (LY475776) was photoaffinity-linked to MRP1 in membranes prepared from the indicated MRP1-transfected cell line by exposure to UV light. Membranes (40–50 μg) in 250 mM sucrose, 0.05 M Tris–HCl (pH 7.4) were incubated in the dark for 5 min at 37° with 0.5–3.3 nM [^{125}I]LY475776 (2175 Ci/mmol) in the absence or presence of 1 mM GSH. Depending upon the experiment, compounds known to be MRP1 substrates or inhibitors were preincubated 5 min with membranes then added to the reaction mixture. The final incubation volume was 100 μL. After incubation, lids were removed, and the reaction tubes were placed on ice in a covered container. In a darkened room, tubes were individually exposed to 254 nm UV light for 5 min to assure the same exposure. Afterwards, the UV-treated membranes were washed and precipitated with 1 mL ice-cold 80% methanol, stored at 4° overnight, and then centrifuged for 15–20 min at ~12,000 *g* in a Beckman microfuge followed by drying of the pellet a vacuum centrifuge. The dried pellets were dissolved in 3.58 M urea, 10% SDS, 25 mg/mL dithiothreitol, and 50 mM Tris–HCl (pH 6.8). The samples were warmed to 50° for 5 min, loaded onto a 4–20% precast gel (OWL Separation Systems) and electrophoresed at 170 V for 2 hr. Gels were stained using the Pierce Gel Code Blue reagent, vacuum dried and exposed to Cronex IV film in a −70° freezer.

2.7. Western analysis

Proteins from membrane vesicle preparations (20 μg) used in the photoaffinity labeling studies were separated by SDS–PAGE as described above and subsequently transferred to nitrocellulose. The primary antibodies with the indicated dilution were as follows: anti-Pgp (C219, 1:250),

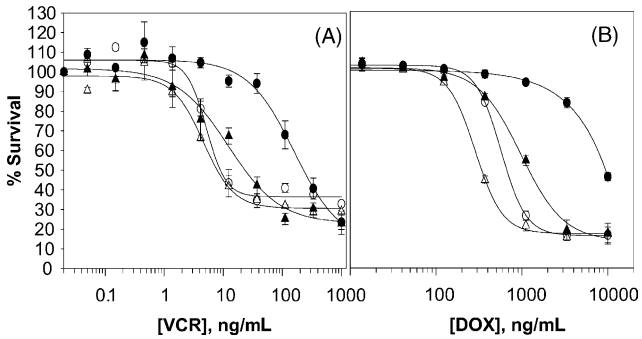

Fig. 2. Effect of LY475776 on MRP1-mediated drug resistance. Three day cytotoxicity assays were conducted with the indicated cell line in the presence of increasing concentrations of vincristine (VCR) or doxorubicin (DOX) in the absence (circles) or presence (triangles) of 1 μM LY475776. Drug sensitivity of MRP1-transfected (solid circles and solid triangles) and vector-transfected (open circles and triangles) HeLa cells respectively, HeLa-T5 and HeLa-C1, to VCR (A) and DOX (B). Curves are representative of two independent experiments conducted in duplicate.

anti-human MRP1 (QCRL-1, 1:200), anti-murine mrp1 polyclonal antibody (1:1000), anti-MRP2 (M_2 III-6, 1:200), anti-MRP3 (M_3 II-9, 1:200), anti-MRP4 polyclonal antibody (1:1000), anti-MRP5 (M_5 I-1, 1:200) and anti-BCRP (BXP-21, 1:200). The secondary antibodies, horseradish peroxidase-conjugated anti-mouse IgG antibody (1:1000) or horseradish peroxidase-conjugated anti-rat IgG antibody (1:5000) were used with anti-MRP5 and anti-murine mrp1 (MRPr1) primary antibodies, respectively. ECL detection was performed as indicated by the manufacturer's instructions.

3. Results

3.1. Effect on cytotoxicity of MRP1 and Pgp expressing cell lines

The ability of LY475776 (Fig. 1) to modulate the cytotoxicity of two anticancer agents that are pumped by MRP1 or Pgp was examined [43]. As shown in Fig. 2, the presence of 1 μM LY475776 enhanced the cytotoxicity of vincristine (Fig. 2A) and doxorubicin (Fig. 2B), to MRP1-transfected HeLa cells (HeLa-T5) by 13- and 7.8-fold, respectively, and close to the drug sensitivity of the vector-transfected HeLa cells, HeLa-C1. In addition, when the effect of this MRP1 modulator was examined on Pgp-mediated resistance in CEM/VLB$_{100}$ cells (Fig. 3), 1 μM LY475776 modulated the drug sensitivity to VCR by 25-fold and to DOX by 2.3-fold with little to no effect on the parental CCRF-CEM cells (Fig. 3A and B). By contrast, the presence of 0.5 μM LY335979, the potent and selective Pgp-specific inhibitor that has a K_i

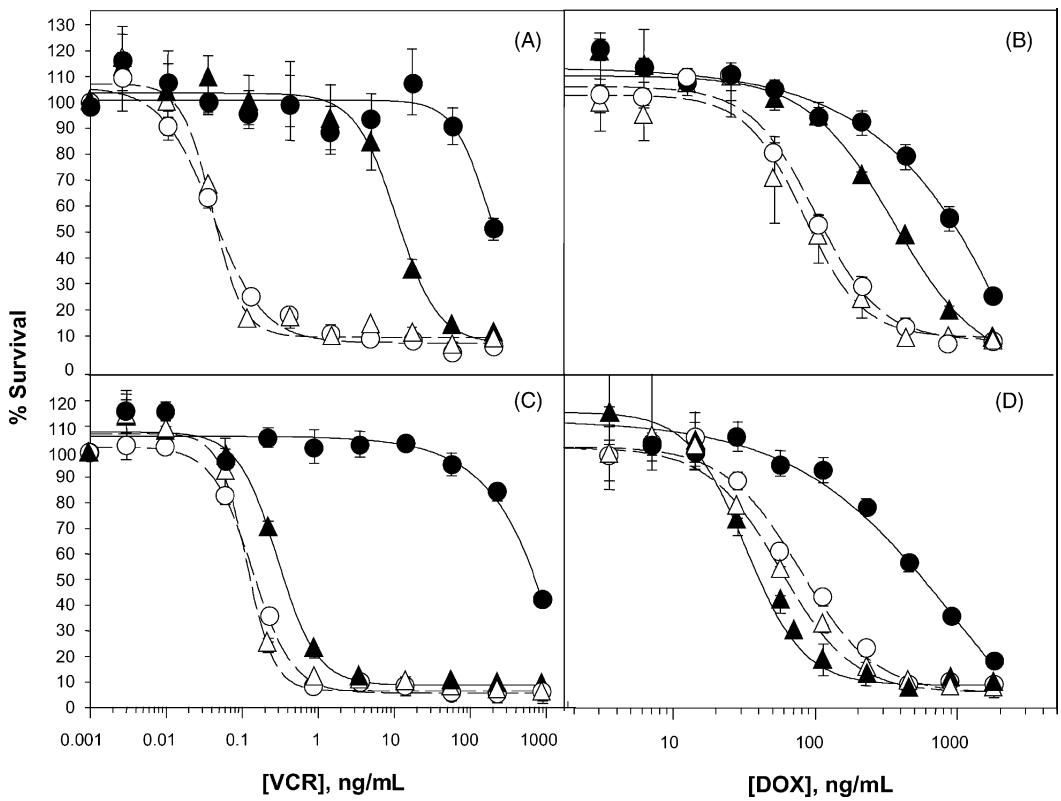

Fig. 3. Modulation of Pgp-mediated drug resistance. Drug sensitivity of Pgp-expressing CEM/VLB$_{100}$ and parental CCRF-CEM cells to VCR (A, C) or DOX (B, D) was measured in the absence or presence of the modulators 1 μM LY475776 (A, B) or 0.5 μM LY335979 (C, D). A 3-day cytotoxicity assay was employed. Symbols are: drug sensitive, CCRF-CEM cells (open circles) in the presence of modulator (open triangles) and drug resistant, CEM/VLB$_{100}$ cells in the absence (closed circles) or presence of modulator (closed triangles).

Table 1
Modulation of doxorubicin resistance of MRP1 and Pgp expressing HL60 cells[a]

Compound	HL60/Adr	HL60/Vinc
LY465803	0.01 ± 0.01	>1.91 ± 0.41
LY475776	0.08 ± 0.01	0.81 ± 0.08
LY335979	>2.0	0.06 ± 0.03

[a] The cytotoxicity assays were performed with the MRP1 expressing HL60/Adr and the Pgp expressing HL60/Vinc in the presence of fixed sublethal dose of doxorubicin and increasing concentrations of the indicated test compound and subsequently grown for 3 days. For the studies conducted with HL60/Adr cells, the doxorubicin concentration was 1 μM and for HL60/Vinc cells was 0.1 μM. The EC_{50} concentration necessary to achieve 50% of the maximal response is listed in μM. Values are the mean ± SEM of four independent experiments measured in quadruplicates.

of 0.059 μM, gave nearly full reversal to both anticancer agents (Fig. 3C and D). These data indicate that LY475776 modulates both MRP1 and Pgp although it is much less potent inhibitor of Pgp.

To assess the relative potency of LY475776 to LY335979 modulation studies were conducted with Pgp-expressing HL60/Vinc cell line in a 3-day cytotoxicity assay. The parent compound LY465803 to the photoactiveable analog was also included in these studies. A fixed, sublethal dose of doxorubicin was used with increasing concentrations of each modulator and EC_{50} values were determined. As shown in Table 1, LY475776 and LY465803 were much less potent Pgp modulators with EC_{50} values of 0.81–1.91 μM compared to approximately 0.06 μM for LY335979. The potency of LY475776 and LY465803 as MRP1 modulators on the HL60/Adr cells was 0.01–0.08 μM. Thus, these tricyclic isoxazole modulators are 14–32 times less potent Pgp modulators than LY335979 and are potent modulators of MRP1.

3.1.1. Effect of inhibitors and substrates of MRP1 on photoaffinity labeling

Figure 4 shows the photoactivateable labeling of MRP1 by LY475776 and the effect of the presence of known inhibitors and substrates of MRP1. Compounds were added to the preincubation mixture of HeLa-T5 membranes, GSH, and [^{125}I]LY475776 prior to cross-linking with UV-light. As shown in Fig. 4, photolabeling of the 190 kDa protein was completely blocked by excess of the unlabeled compound LY475776, as well as the MRP1 inhibitor, MK571 [44,45]. In addition, MRP1 substrates, LTC_4 and the anticancer agents, VCR and daunorubicin that are cotransported with GSH, reduced photolabeling of the 190 kDa protein [46–48].

3.2. Specificity of photoaffinity labeling of MRP1 from different species

Previously LY475776 was shown to label murine MRP1 less well than human MRP1 [30]. Consequently, we exam-

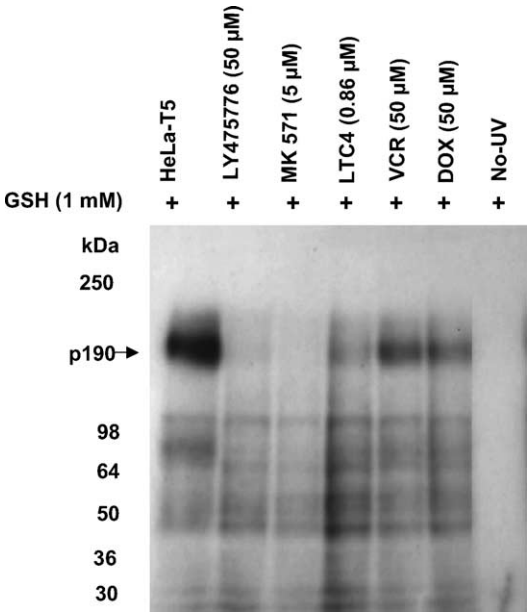

Fig. 4. Effect of MRP1 inhibitors and substrates on [^{125}I]LY475776 photoaffinity labeling of MRP1. HeLa-T5 plasma membranes (40 μg) were pre-incubated at 37° for 5 min with the compound at the final concentration indicated in the presence of 0.5 nM [^{125}I]LY475776 and 1 mM GSH and exposed to UV-light for 5 min on ice. The "No-UV" lane is a control that did not receive UV-light treatment. Molecular mass (kDa) markers are indicated; the arrow indicates the location of the ~190 kDa band corresponding to the MRP1 protein. In a separate experiment, the [^{125}I]LY475776-labeled protein was immunoprecipitated with the MAb QCRL-3 and protein A-sepharose beads that resulted in the detection of the ~190 kDa band after separation by SDS-PAGE and detection by autoradiography. No bands were detected with a sub-isotype matched negative control antibody (Data not shown).

ined photolabeling of orthologs of MRP1 from four species, human, monkey, canine and murine. Two orthologs exist within the coding region of MRP1 from both monkey and dog, therefore both forms of these orthologs were analyzed. Figure 5A shows the autoradiogram of proteins photolabeled with [^{125}I]LY475776 after incubation of membranes in the presence of 1 mM GSH. A protein band of ~190 kDa was identified in HeLa-T5 membranes, as indicated above and previously reported [29], and was also photolabeled in membranes of both orthologs A and B for monkey, PEAK^{STABLE}-monMRP1-A, PEAK^{STABLE}-monMRP1-B, and canine, HeLa-canMRP1-A and HeLa-canMRP1-B, and was not present in membranes from their respective vector control cell lines. By contrast in Fig. 5B, a faint ~190 kDa protein band was photoaffinity labeled in membranes from the murine mrp1- and vector-transfected HEK cells when compared to HeLa-T5; and labeling was dependent on the presence of 1 mM GSH. Confirmation of the presence of the murine mrp1 in the transfected HEK cells was made by Western blot analysis (Fig. 5B).

To examine the relative potency of the inhibitor, the uptake of 50 nM [^{3}H]LTC_4 was measured into inside-out membrane vesicles prepared from transfected cells expressing human MRP1 or the canine and monkey MRP1

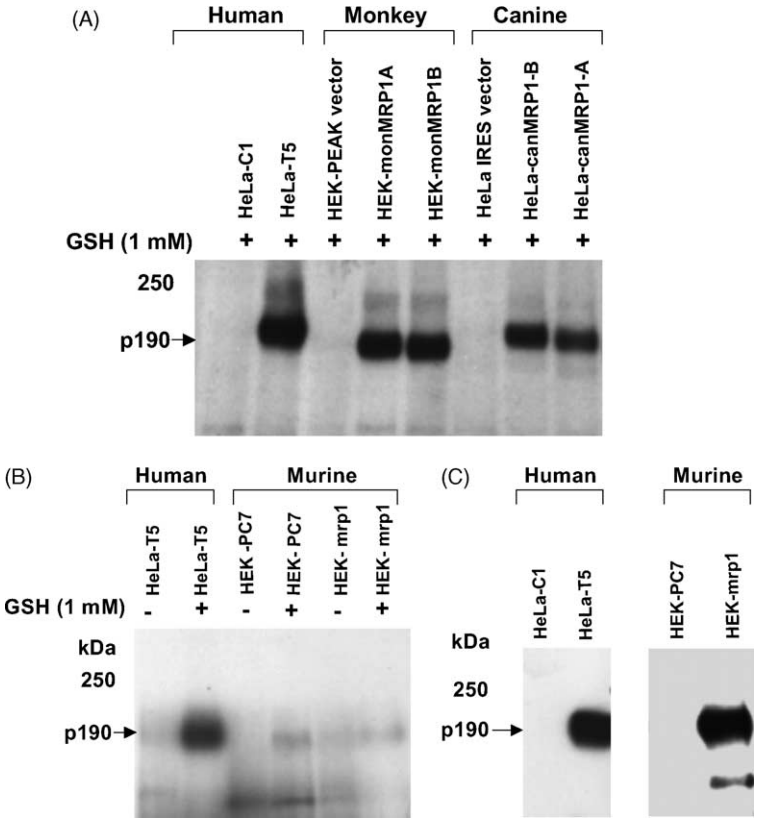

Fig. 5. [^{125}I]LY475776 photoaffinity labeling of orthologs of MRP1. Labeling of MRP1 protein was examined with this transporter cloned and expressed from four species, human, monkey, canine and murine. Monkey and canine each have two orthologs (A and B). (A) Membranes were incubated with [^{125}I]LY475776 with 1 mM GSH and the photolabeling of monkey and canine MRP1 was compared to human MRP1. Western blots of membrane vesicle preparations from these MRP1 ortholog transfectants have been previously shown to express the MRP1 protein [20,21]. (B) On the left, plasma membranes (40 μg) of murine mrp1 and human MRP1 were preincubated at 37° for 5 min indicated in the presence of 0.5 nM [^{125}I]LY475776 in the absence (−) or presence (+) of 1 mM GSH and exposed to UV-light for 5 min on ice. On the right, a Western blot was conducted with (20 μg) of the same plasma membrane preparations and probed with the anti-MRP1 antibody (QCRL-1) or with the anti-mrp1 polyclonal antibody as described in the Section 2.

orthologs. The close structural analog of LY475776, LY465803 (Fig. 1) was used that lacks the azido- and iodo-groups present in LY475776 necessary for photolabeling. This compound also blocks photolabeling of MRP1 when present in an excess of LY465803 similarly to LY475776 in Fig. 5A (data not shown) and the two tricyclic isoxazole inhibitors have similar MRP1 inhibitory activity for LTC_4 uptake (EC_{50} values of ∼50 nM). When the potency of LY465803 was examined for its effect on LTC_4 uptake by the human MRP1 and canine and monkey orthologs (Table 2), an EC_{50} value of 66 nM was obtained for human MRP1 and the values for the orthologs were within 1.7-fold. This indicates that the tricyclic isoxazole inhibitor binds similarly to human and the canine and monkey orthologs despite the fact that canine and monkey orthologs have a reduced ability to confer resistance to anthracyclines.

3.3. Photoaffinity labeling of Pgp

As previously shown in the cytotoxicity assays (Fig. 2), LY475776 modulated DOX and VCR resistance of Pgp expressing CEM/VLB_{100} cells. Therefore, LY475776 most likely inhibits Pgp-mediated efflux directly and would be expected to photolabel this transporter. To examine this possibility, plasma membranes from CCRF-CEM and CEM/VLB_{100} cells were prepared and incubated with 1 mM GSH and [^{125}I]LY475776 in the absence or presence of the highly potent and specific Pgp inhibitor, LY335979, or unlabeled LY475776 [38]. Figure 6 demonstrates that a protein of ∼170 kDa molecular mass was labeled in CEM/VLB_{100} membranes that was absent in the parental CCRF-CEM membranes and in HeLa-T5 membranes. The

Table 2
Inhibition of 50 nM [^{3}H]LTC_4 uptake by MRP1 orthologs by LY465803[a]

Cell line	EC_{50} (nM)
HeLa-T5	66 ± 13
HEK-monMRP1-A	108 ± 13
HEK-monMRP1-B	105 ± 22
HeLa-canMRP1-A	62 ± 11
HeLa-canMRP1-B	90 ± 13

[a] Uptake was performed using inside-out membrane vesicles prepared from each of the indicated MRP1 transfected cell lines. Values are the mean ± SE of triplicate data points obtained over a concentration range of 0.002–5 μM.

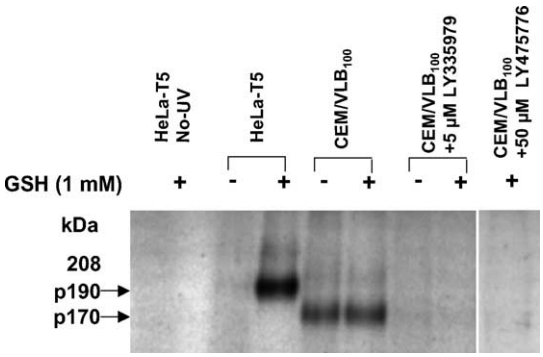

Fig. 6. Effect of the MDR-specific inhibitor, LY335979, on the photoaffinity labeling of Pgp. Plasma membranes (50 μg) prepared from MRP1-transfected HeLa-T5, drug-sensitive CCRF-CEM, Pgp-expressing drug-resistant CEM/VLB_{100} cells were preincubated with 0.5 nM [^{125}I]LY475776 in the absence (−) and presence (+) of 1 mM GSH and then cross-linked with UV-light for 5 min on ice. Plasma membranes from CEM/VLB_{100} cells were also preincubated in the absence (−) or presence (+) of 1 mM GSH with 5 μM LY335979 or 50 μM unlabeled LY475776. Molecular mass (kDa) markers are indicated; the arrows indicate that location of the ∼170 or ∼190 kDa protein bands corresponding to Pgp or MRP1, respectively.

labeling of the ∼170 kDa Pgp protein was GSH-independent and was inhibited by the presence of 5 μM LY335979 or 50 μM LY475776. Taken together, these data indicate that this photoaffinity probe does indeed bind to Pgp and cross-links to this transporter.

3.4. Specificity of photoaffinity labeling of several other ABC transport proteins

To examine whether LY475776 may also bind to other ABC transport proteins, photoaffinity labeling experiments were conducted with plasma membranes prepared from several transfected cell lines (MRPs 1, 2, 3, 5 and BCRP) along with their corresponding vector control cell lines. Figure 7A compares the photoaffinity labeling of membrane vesicles incubated with [^{125}I]LY475776 in the absence and presence of 1 mM GSH. As previously demonstrated, GSH-dependent photoaffinity labeling was observed of the ∼190 kDa protein band corresponding to MRP1 in HeLa-T5 membranes while there was faint labeling, if any, of the other ABC transporter proteins.

To confirm that the membrane vesicles used for these photolabeling experiments did indeed express the ABC transporter of interest, Western blot analysis of the SDS–PAGE separated proteins from the same membrane vesicle preparations was conducted using primary antibodies specific for MRPs 1–5 and BCRP (Fig. 7B). As expected, the ∼190 kDa protein was present in membranes of the MRP transfected cells and not in the corresponding vector control. Western analysis indicated that the membranes used in the photoaffinity labeling experiment did indeed express the transfected ABC transporter. Moreover, the finding that membranes of MRP5- and vector-transfected HEK cells

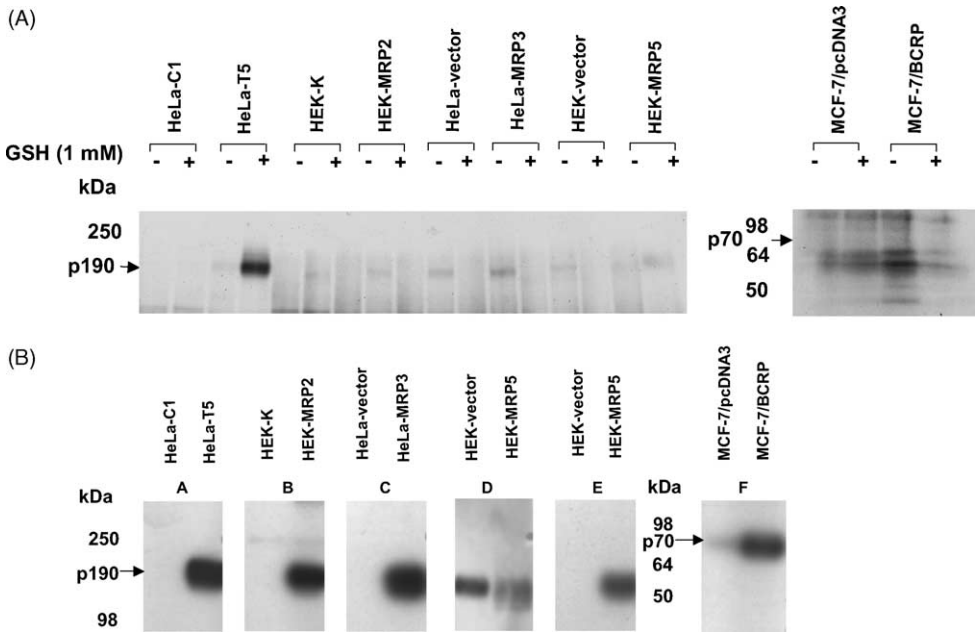

Fig. 7. Specificity of [^{125}I]LY475776 photoaffinity labeling for several drug transporting members of the ABC transporter superfamily. Plasma membranes were prepared from HeLa-T5 (MRP1), HEK-MRP2, HeLa Tet-On MRP3, HEK-MRP5, MCF-7/BCRP transfectants, and the corresponding vector control cell lines. Panel A: Plasma membranes (50 μg) were incubated at 37° for 5 min with 0.5 nM [^{125}I]LY475776 in the absence (−) or presence (+) of 1 mM GSH and cross-linked with UV-light for 5 min on ice. Molecular mass (kDa) markers are indicated; the arrow indicates the location of the ∼190 kDa MRP1 or the the ∼70 kDa BCRP protein bands. Panel B: Western blots were conducted using the same membrane preparations (20 μg) and probed with primary antibodies that were specific for each transporter as described in Section 2. The antibodies were as follows anti-MRP1 MAb QCRL-1, anti-MRP2 MAb M_2 III-6, anti-MRP3 MAb M_3 II-9, anti-MRP4 polyclonal antibody, anti-MRP5 MAb M_5 I-1 and anti-BCRP MAb BXP-21. Plasma membranes for vector and MRP5 transfected HEK cells were probed for MRP4 (D) and MRP5 (E). Molecular mass (kDa) markers are indicated; the arrows indicate that location of the ∼190 kDa protein bands corresponding to the various MRP transport proteins and ∼70 kDa protein band corresponding to BCRP.

also expressed MRP4 suggests that the photoaffinity label does not bind to this ABC transporter protein. Western blot analysis of plasma membranes from BCRP- and vector-transfected MCF-7 cells detected a ~70 kDa protein band consistent with the lower molecular mass of this "half transporter", BCRP [49]. Taken together, these data suggest that the photoaffinity label [^{125}I]LY475776 does not interact with MRPs 2, 3, 4, 5, or BCRP sufficiently to permit photolabeling.

4. Conclusions

Photoaffinity probes have been useful tools in the identification of transport proteins and in the characterization of substrates of a number of ABC transporter proteins [50]. Although Pgp and MRP1 confer resistance to many of the same anticancer agents, clear differences in the substrate binding properties of these two transporters became apparent when photoactiveable derivatives of doxorubicin, vinca alkaloids, or of the modulators verapamil or azidopine, that label Pgp quite effectively did not label MRP1 [50]. While Pgp pumps unmodified anticancer agents, MRP1 prefers organic anions of these drugs that are glutathione-conjugated, glucuronidated, or sulfated, or MRP1 cotransports certain drugs, such as VCR and daunorubicin with glutathione [46,47]. An important physiological substrate, LTC_4, was identified initially by photolabeling of the ~190 kDa protein after incubation of membranes of mastocytoma cells with radiolabeled LTC_4 [51–54]. A number of photoaffinity probes such as LTC_4 and agosterol A, have been used to label regions within MRP1 to define and map their respective binding sites [55–57]. Recently, tricyclic isoxazole compounds were identified as potent inhibitors of MRP1 transport function that sensitized MRP1-transfected cells to anticancer drugs [28]. A binding region for the tricyclic isoxazole inhibitor, iodinated azido analog LY475766, was identified in the terminal carboxy half of MRP1 encompassing the transmembrane helices 16 and 17 [29,30]. This is also the same MRP1 region that was identified to be important for conferring resistance to anthracyclines but not to Vinca alkaloids and is altered in murine mrp1. In the presence of glutathione, LY475776 inhibits murine mrp1-mediated transport of LTC_4 6-fold less well than human MRP1-mediated transport with an EC_{50} value of 300 nM vs. 50 nM, respectively [30]. These data support that this region is critical for both anthracycline resistance and LY475776 binding. Consequently, it was of interest to examine the photolabeling of the canine and monkey orthologs of MRP1 that confer, respectively, no resistance and reduced resistance to anthracyclines but do confer similar level of resistance to vinca alkaloids [20,21]. As shown in Fig. 5, human, canine, and monkey orthologs were photolabeled as well as human MRP1 while murine mrp1 was poorly labeled as previously reported [30]. Using the non-photoaffinity affinity analog LY465803 that lacks both the azido- and iodo-groups for competition studies for LTC_4 uptake, it was determined that the human MRP1 and canine and monkey orthologs of MRP1 all have EC_{50} values within 1.7-fold of one another (ranging from 62 to 108 nM). These data support that the tricyclic isoxazole modulator binds equally well to these orthologs although it apparently binds 6-fold less well to the murine mrp1 [30]. This is consistent with the similar photolabeling of MRP1 and the orthologs observed in Fig. 5A.

To try to understand the discrepancy between the binding of LY475776 and the canine and monkey orthologs sensitivity to anthracycline anticancer agents, a partial sequence alignment of these orthologs was made of their carboxy-terminal regions (Fig. 8). The photolabeling sites for LY475776 was previously mapped to this region for the mouse and human hybrid proteins [30]. Photolabeling of tryptic fragments of human MRP1 identified the region between amino acids 1150 and 1250 containing TM16 and TM17 as the major LY475776 binding site [29]. This region is completely conserved in the monkey MRP1 orthologs shown in Fig. 8 with the exception of murine mrp1, which contains two mismatches, and the canine orthologs that contain one mismatch. Substitution of Thr^{1242} in human MRP1 with Ala as it is in murine mrp1 decreased but did not eliminate photolabeling by LY476776, while the converse substitution of Ala^{1239} of murine mrp1 with Thr only slightly increased the photolabling of this protein [30]. However, Val^{1240} of human MRP1 and the other orthologs is an Ile in murine mrp1 (position 1237, Fig. 8), and its effect on photolabeling by LY475776 has not been evaluated. The combination of Ala^{1239} and Val^{1240} in murine mrp1 in this region may account for the major differences in photolabeling between mouse and the other MRP1 orthologs.

The survey of photolabeling by LY475776 of several other ABC transporters that efflux anticancer agents indicate that the inhibitor is not a general photoaffinity probe for ABC transporters. For example, LY475776 did not photolabel MRPs 2, 3, 5 or BCRP. In addition, since HEK cells express a fairly high level of MRP4 constitutively, one can infer from these studies with the vector control cells that LY475776 does not interact with MRP4. As shown in cytotoxicity assays, the tricyclic isoxazole inhibitor LY475776 modulates MRP1-mediated resistance to doxorubicin and vincristine and also partially modulates Pgp-mediated drug resistance. Photolabeling of Pgp expressing membranes of CEM/VLB_{100} cells indicated that indeed a 170 kDa protein corresponding to Pgp was labeled. Unlike MRP1, photolabeling of Pgp was not dependent on the presence of GSH, consistent with GSH independence of other known Pgp substrates and inhibitors. Labeling of the 170 kDa protein was reduced in the presence of the highly potent and selective MDR inhibitor, LY335979 (Zosuquidar trihydrochloric acid) confirming that Pgp was labeled [39,41,58]. Modulation of doxorubicin resistance of

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hMRP1      959 :KLSVYWDYMKAIGLFISFLSIFLFMCNHVSALASNYWLSLWTDDP-IVNGTQEHTKVRLSVYGALGISQG : 1027
monMRP1-A  959 :.......................I....A.............-....................... : 1027
monMRP1-B  959 :.......................I....A.............-....................... : 1027
canMRP1    959 :.......................L....AS.V..........-.........I............ : 1027
muMRP1     955 :Q.....N.........T.......L.....................PV.....ANRNF..........L.. : 1024
                                                                          *
hMRP1     1028 :IAVFGYSMAVSIGGILASRCLHVDLLHSILRSPMSFFERTPSGNLVNRFSKELDTVDSMIPEVIKMFMGS : 1097
monMRP1-A 1028 :...................Y.................................................. : 1097
monMRP1-B 1028 :.................................................................... : 1097
canMRP1   1028 :.T.............F...R......QNV..............................Q........ : 1097
muMRP1    1025 :A.I............F...R..L...YNV..............................Q........ : 1094

hMRP1     1098 :LFNVIGACIVILLATPIAAIIIPPLGLIYFFVQRFYVASSRQLKRLESVSRSPVYSHFNETLLGVSVIRA : 1167
monMRP1-A 1098 :.................................................................... : 1167
monMRP1-B 1098 :.................................................................... : 1167
canMRP1   1098 :.........I........S.................................................. : 1167
muMRP1    1095 :..S....V.I.........V.......V.................................... : 1164

hMRP1     1168 :FEEQERFIHQSDLKVDENQKAYYPSIVANRWLAVRLECVGNCIVLFAALFAVISRHSLSAGLVGLSVSYS : 1237
monMRP1-A 1168 :.................................................................... : 1237
monMRP1-B 1168 :.................................................................... : 1237
canMRP1   1168 :........R.....................................S..................... : 1237
muMRP1    1165 :.................................................................... : 1234
                    *   *
hMRP1     1238 :LQVTTYLNWLVRMSSEMETNIVAVERLKEYSETEKEAPWQIQETAPPSSWPQVGRVEFRNYCLRYREDLD : 1307
monMRP1-A 1238 :.............................................N...................... : 1307
monMRP1-B 1238 :.............................................N...................... : 1307
canMRP1   1238 :.........................................M....T..........D.G.....N.. : 1307
muMRP1    1235 :..I.A.....................................T..HS......D.......... : 1304

hMRP1     1308 :FVLRHINVTINGGEKVGIVGRTGAGKSSLTLGLFRINESAEGEIIIDGINIAKIGLHDLRFKITIIPQDP : 1377
monMRP1-A 1308 :.....................................................R.............. : 1377
monMRP1-B 1308 :.....................................................R.............. : 1377
canMRP1   1308 :L..K...I.........................................D............V......... : 1377
muMRP1    1305 :L..K......E.....................................V........N............ : 1374

hMRP1     1378 :VLFSGSLRMNLDPFSQYSDEEVWTSLELAHLKDFVSALPDKLDHECAEGGENLSVGQRQLVCLARALLRK : 1447
monMRP1-A 1378 :.............................G...................................... : 1447
monMRP1-B 1378 :.............................G...................................... : 1447
canMRP1   1378 :.................................G.....NQ.......................... : 1447
muMRP1    1375 :.......................MA.......G.........N.......................... : 1444

hMRP1     1448 :TKILVLDEATAAVDLETDDLIQSTIRTQFEDCTVLTIAHRLNTIMDYTRVIVLDKGEIQEYGAPSDLLQQ : 1517
monMRP1-A 1448 :.................................................................... : 1517
monMRP1-B 1448 :.................................................................... : 1517
canMRP1   1448 :............................D.........................R.C.Q....... : 1517
muMRP1    1445 :..................N.....................................VR.C....E.... : 1514

hMRP1     1518 :RGLFYSMAKDAGLV : 1531
monMRP1-A 1518 :.....N..R..... : 1531
monMRP1-B 1518 :.....N..R..... : 1531
canMRP1   1518 :.............. : 1531
muMRP1    1515 :..I........... : 1528
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Fig. 8. Protein sequence alignment of the carboxy-terminal region of MRP1 orthologs. Sequence identities with human MRP1 (hMRP1, Genbank 2828206) are denoted with periods (".") while the residues are displayed at mismatched positions. Gaps introduced to optimize the alignment are displayed as dashes ("-"). The other sequences are as follows: MRP1 from Cynomolgus monkey (monMRP1) alleles A and B ([21]; Genbank AY146672 and AY146673, respectively); Canine MRP1 (canMRP1) [20]; Murine mrp1 (muMRP1) ([36], Genbank AF022908). Residues E^{1089}, T^{1242}, and $W^{1246}$of human MRP1 that were previously subjected to site-directed mutagenesis to determine effects of LY475776 photolabeling [29,30] as discussed in the text are marked with asterisks ("*"). The region implicated as the major LY475776 photolabeling site from trypsin digests of human MRP1 (residues 1150–1250) is underlined.

Pgp-expressing HL60/Vinc cells indicates that LY475776 is 14-fold less potent Pgp modulator than LY335979. While human MRPs 2, 3, 4, and 5 that are 32–57% identical to human MRP1 were not labeled, it is interesting that Pgp was photolabeled even though Pgp is only 15% identical to human MRP1 [56,59]. Alignments of Pgp with the carboxy-terminal region of MRP1 did not reveal an increased homology in the regions of residues previously found to be important for LY475776 photolabeling (data not shown). Further mapping of the LY475776 binding site(s) within Pgp may illuminate structural features common to both MRP1 and Pgp and important for LY475776 binding that are not evident from analysis of primary sequences.

Interestingly, agosterol A is another MRP1 inhibitor that binds the C-terminal region of MRP1. The photoaffinity analog of agosterol A is also dependent on glutathione for labeling of MRP1 and binds to the same region within MRP1 (amino acids C932–1531) and Arg^{1549} appears to

be critical for its binding [56,57]. Whether this MRP1 amino acid sequence falls within a hydrophobic pocket that is more accessible for drug binding remains to be determined.

Previous studies with a photoaffinity probe of Pgp inhibitor VX-710 indicate that labeling of more than one transporter was observed. VX-710 was developed as a Pgp inhibitor and was subsequently found to modulate MRP1-mediated resistance. The photoaffinity analog of VX-710 labels Pgp better than MRP1 [60]. Similarly, LY475776 inhibits and also photolabels both MRP and Pgp. Unlike VX-710, LY475776 shows a greater preference for MRP1 vs. Pgp in its ability to modulate resistance and to photolabel these transport proteins. Thus, both inhibitors have labeling patterns that are consistent with them being developed originally as an MRP1 inhibitor (LY475776) or as a Pgp inhibitor (VX-710).

In summary, the tricyclic isoxazole photoaffinity label LY475776 is a potent inhibitor of MRP1. The exact binding site of the label to MRP1 remains to be elucidated although it is localized to the terminal carboxy half. Studies with the monkey and canine orthologs indicate that residues within the MSD3 may influence its binding characteristics independently of whether the transporter confers resistance to anthracyclines. This probe is not MRP1 specific but can modulate and label Pgp although less effectively. The compound however does show selectivity with regards to other MRPs (MRPs 2, 3, 4, and 5) and BCRP that were not photolabeled. Additional studies are needed to further elucidate the residues important for LY475776 binding to MRP1 and Pgp.

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