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# MRP1 mutated in the $L_0$ region transports SN-38 but not leukotriene $C_4$ or estradiol-17 (β-D-glucuronate)

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## Abstract

Multidrug resistance protein 1 (MRP1) is an ATP-binding cassette transporter that confers multidrug resistance on tumor cells. Much convincing evidence has accumulated that MRP1 transports most substances in a GSH-dependent manner. On the other hand, several reports have revealed that MRP1 can transport some substrates independently of GSH; however, the importance of GSH-independent transport activity is not well established and the mechanistic differences between GSH-dependent and -independent transport by MRP1 are unclear.

We previously demonstrated that the amino acids $W_{261}$ and $K_{267}$ in the $L_0$ region of MRP1 were important for leukotriene $C_4$ ($LTC_4$) transport activity of MRP1 and for GSH-dependent photolabeling of MRP1 with azidophenyl agosterol-A (azidoAG-A). In this paper, we further tested the effect of W222L, W223L and R230A mutations in MRP1, designated $dmL_0$MRP1, on MRP1 transport activity. SN-38 is an active metabolic form of CPT-11 that is one of the most promising anti-cancer drugs. Membrane vesicles prepared from cells expressing $dmL_0$MRP1 could transport SN-38, but not $LTC_4$ or estradiol-17 (β-D-glucuronate), and could not be photolabeled with azidoAG-A. These data suggested that SN-38 was transported by a different mechanism than that of GSH-dependent transport. Understanding the GSH-independent transport mechanism of MRP1, and identification of drugs that are transported by this mechanism, will be critical for combating MRP1-mediated drug resistance. We performed a pairwise comparison

*Abbreviations:* ABC transporter, ATP-binding cassette transporter; ACNU, 3-[(4-amino-2-methyl-5-pyrimidinyl) methyl]-1-(2-chloroethyl)-1-nitrosourea; ADM, adriamycin; AG-A, agosterol A; azidoAG-A, [$^{125}$I] 11-azidophenyl agosterol A; AZT, azidothymidine; BSO, buthionine sulfoximine; BSP, sulfobromophthalein; C, COOH; CDDP, *cis*-platinum(II) diammine dichloride; d4T, 2′,3′-didehydro-3′-deoxythymidine; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; DTT, dithiothreitol; $E_2$17β-G, estradiol-17 (β-D-glucuronate); γGCS, γ-glutamylcysteine synthetase; IAARh123, iodoaryl azidorhodamine 123; IAAQ, *N*-{4-[1-hydroxy-2-(dibutylamino) ethyl] quinolin-8-yl}-4-azidosalicylamide; IACI, *N*-(hydrocinchonidin-8′-yl)-4-azido-2-hydroxybenzamide; $LTC_4$, leukotriene $C_4$; $LTD_4$, leukotriene $D_4$; $LTE_4$, leukotriene $E_4$; mAMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; MDR, multidrug resistance; MRP1, multidrug resistance protein 1; MV, membrane vesicles; N, $NH_2$; NBD, nucleotide-binding domain; NNAL-*o*-glucuronide, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol-*o*-glucuronide; P-gp, P-glycoprotein; PMEA, 9-(2-phosphonylmethoxyethyl) adenine; PMEG, 9-(2-phosphonylmethoxyethyl) guanine; 3TC, 2′,3′-dideoxy-3′-thiacytidine; TM, transmembrane segment; TMD, transmembrane domain; VCR, vincristine; VP-16, etoposide; Rh123, rhodamine 123

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of compounds that are transported by MRP1 in a GSH-dependent or -independent manner. These data indicated that it may be possible to predict compounds that are transported by MRP1 in a GSH-independent manner.


*Keywords:* MRP1; SN-38; GSH; $L_0$; ABC transporter; Drug resistance; $LTC_4$; $E_2$17β-G

## 1. Introduction

Multidrug resistance (MDR) is a major obstacle to successful cancer chemotherapy and is mediated by MDR transporters [1]. Human multidrug resistance protein 1 (MRP1) is frequently over-expressed in MDR cells that do not express the P-glycoprotein (P-gp) transporter [2]. MRP1 is a member of the family of ATP-binding cassette (ABC) transporters [3,4]. Over-expression of MRP1 in cultured cells resulted in a reduction of drug accumulation and an increase in the ATP-dependent efflux rate of drugs. As an organic anion transporter, MRP1 actively transports a wide variety of diverse anionic compounds [3,4]. Leukotriene $C_4$ ($LTC_4$) is an endogenous substrate of MRP1 with the highest known affinity for MRP1 ($K_m$ about 100 nM) [5]. By using the in vitro inside-out membrane vesicle system, it was also found that physiological concentrations of GSH stimulated the ATP-dependent transport of certain drugs such as vincristine (VCR) [5–7], adriamycin (ADM) [7,8] and aflatoxin $B_1$ [9] as well as certain endogenous hydrophilic anionic conjugates such as 3-α-sulfatolithocholyl taurine, estrone-3-sulfate [10] and a tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol-*o*-glucuronide (NNAL-*o*-glucuronide) [11]. GSH is supposed to be transported as conjugates or co-transported with endo- and xenotoxins by MRP1, however, since GSSG ($K_m$ = 100 μM) is a preferential substrate of MRP1 rather than GSH. Under the oxidative stress condition, MRP1 pumps out cytoplasmic GSSG and may protect thiol group of other proteins from GSSG reactivity or affect to GSH metabolism [6,12].

Agosterol A (AG-A) is a novel inhibitor of MRP1 function and reverses MRP1-mediated MDR [13,14]. We synthesized a photoaffinity analog of AG-A, [$^{125}$I] azidoAG-A, and showed that photolabeling of MRP1 with [$^{125}$I] azidoAG-A was dependent on GSH. We and other group demonstrated that the $L_0$ region of MRP1 was important for the GSH-dependent photolabeling of MRP1 with azdioAG-A as well as for the $LTC_4$ transport activity of MRP1 [15–17].

Some evidence exists that MRP1 may be able to transport some molecules in a GSH-independent manner. Thus, MRP1 expressing cells were reported to be resistant to calcein, special toxic peptides 4A6 and SN-38 even in the presence of buthionine sulfoximine (BSO), a γ-glutamylcysteine synthetase (γGCS) inhibitor [18–20]. Furthermore, MRP1 was photolabeled even in the absence of GSH with the quinoline-based drugs, *N*-{4-[1-hydroxy-2-(dibutylamino) ethyl] quinolin-8-yl}-4-azidosalicylamide (IAAQ), *N*-(hydrocinchonidin-8′-yl)-4-azido-2-hydroxybenzamide (IACI) and iodoaryl azidorhodamine 123 (IAARh123) [21–23]. However, the importance and mechanism(s) of GSH-independent transport by MRP1 are still unclear.

SN-38 is a metabolized active molecule of CPT-11 which is now widely used as an effective drug for treatment of cancers. We could not detect any glucuronide conjugated SN-38 by HPLC analysis of either KB/MRP1 cells or of the culture media [20]. These data suggested that SN-38 could be transported without glucuronization. However, we could not rule out the possibility that SN-38 might be transported in the presence of another, as yet unidentified, molecule that might not require covalent binding of SN-38 for transport.

In this study, we find that resistance to SN-38 in MRP1-expressing cells cannot be effectively reversed with AG-A or BSO; these data suggest that SN-38 can be transported without GSH, and demonstrate the importance of GSH-independent transport by MRP1. Using a mutant MRP1, $dmL_0$MRP1, we show that GSH-independent transport of SN-38 is clearly distinct from GSH-dependent transport of other drugs by MRP1.

## 2. Materials and methods

### 2.1. Materials

[$^{125}$I] NaI (3.7 GBq/ml, [6,7-$^3$H (*N*)] estradiol-17β-glucuronide (1.11 TBq/mmol) and [14,15,19,20-$^3$H (*N*)]-$LTC_4$ (5.4 GBq/mmol) were purchased from Perkin-Elmer Life Sciences (Boston, MA). The synthesis and use of [$^{125}$I] 11-azidophenyl agosterol A ([$^{125}$I] azidoAG-A) were described in a previous paper [15]. Cellfectin, competent DH10Bac *E. coli* cells and Lipofectamine were purchased from Invitrogen Corp. (Carlsbad, CA). G418 was purchased from Nacalai Tesque Inc. (Kyoto, Japan). MRPm6, a monoclonal antibody against MRP1 and Tetra-His, anti-His monoclonal antibody were purchased from Progen Biotechnick (Heidelberg, Germany) and Qiagen GmbH (Hilden, Germany), respectively [24]. SN-38 and CPT-11 were kindly provided by Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan) and Yakult Pharmaceutical Ind. Co. Ltd. (Tokyo, Japan). Other drugs and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

### 2.2. Cell culture, membrane vesicle preparation and cytotoxicity assay

KB-3-1 parentel cells and KB/MRP cells, that are MRP1 transfected KB-3-1 cells, were cultured in Minimal Essen-

tial Medium (MEM) (Nissui Seiyaku Co., Tokyo, Japan) containing 10% newborn calf serum. Sf21 insect cells were cultured in serum-free Sf-900 II SFM Medium (Invitrogen Corp.). Membrane vesicles (MV) were prepared as previously described [14]. Protein concentrations were determined by the method of Bradford [25]. The cytotoxicity assay was conducted as previously described [20].

### 2.3. Generation of constructs and viral infection

*pFastBac-MRP1-His* containing the His tagged MRP1 coding region was constructed as previously described [26]. The strategies employed for site-directed mutagenesis of the MRP1 cDNA were previously described [15].

A mutant $L_0$ MRP1 without $TMD_0$ had a decreased ability to transport $LTC_4$ and attenuated GSH-dependent photoaffinity labeling with azidoAG-A compared with wild type (wt) MRP1. However, the mutant MRP1 still retained some $LTC_4$ transport activity [15]. In order to completely disrupt $LTC_4$ transport activity, we created additional mutations in the $L_0$ region of MRP1 that already expressed the W261A and K267M mutations. The extra mutations created, W222L, W223L and R230A, were located within WxxxxxK sequences in the $L_0$ region that have been reported to an interacting site of glutathione-S-transferase with GSH. Furthermore, in order to avoid deletion of the $TMD_0$ region, the new mutant MRP1, named $dmL_0MRP1$, was designed to carry the original $TMD_0$ region as shown in Fig. 1. Practically, the W261A and K267M mutations were first introduced into wt MRP1 by PCR using primers described in a previous paper [15]. W222L, W223L and R230A mutations were then introduced into this construct by PCR using the forward primers, 5′-GGGTTGATTGTC**GC**GGGCTACCGCC-3′, and reverse primer 5′-TGTGATCA**A**CA**A**GAAGGTGATCC-TC-3′ (bold denotes mismatched bases encoding the mutation). Baculoviruses encoding the wt and mutant MRP1 described above were generated using the Bac to Bac expression system (Invitrogen Corp.) as described previously [26].

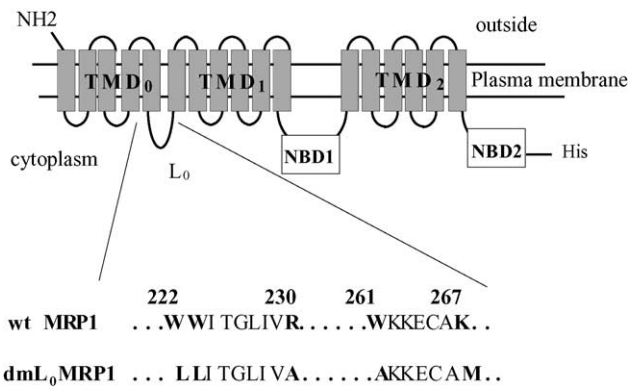



Fig. 1. Schematic presentation of wt and mutant structure of MRP1. Mutated amino acids in $L_0$ region of $dmL_0MRP1$ indicate in bold. Numbers above the amino acid sequences are position of MRP1.

### 2.4. Immunofluororescent cell staining

Sf21 cells were infected with wt or mutant MRP1 coding baculovirus. Three days after infection, the infected cells and Sf21 cells were fixed with phosphate buffered 4% paraformaldehyde at room temperature for 30 min. After washing with PBS, the cells were permeabilized with 100% methanol for 30 min and washed with PBS once. Following blocking with 3% BSA and 7% normal goat serum in PBS for 30 min, the cells were incubated with anti-His antibody (1:500) in PBS containing 3% BSA at 4 °C overnight. Following three times gentle wash with PBS containing 0.02% Tween-20, the cells were incubated 200-fold diluted Alexa Fluor® 546 labeled anti mouse IgG (Invitrogen Corp.) and 6 μM 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) in PBS containing 3% BSA, for 30 min. The cells were washed with 0.02% Tween-20 containing PBS three times and examined by confocal microscopy (FV500, Olympus Corporation, Tokyo, Japan).

### 2.5. Photoaffinity labeling of MRP1 with [$^{125}$I] azidoAG-A

[$^{125}$I] azidoAG-A (7.2 μCi/nmol) was used for photolabeling studies that were carried out as previously described [15]. Autoradiograms were exposed for a period of 10 h–3 days.

### 2.6. Vesicle uptake experiments of [$^3$H] $LTC_4$ and [$^3$H] $E_2$17β-G

[$^3$H] $LTC_4$ and [$^3$H] estradiol-17 (β-D-glucuronate) ($E_2$17β-G) uptake was measured using a rapid filtration technique as previously described [27]. Briefly, isolated MV (25 μg) were incubated with or without 4 mM ATP in 50 μl transport buffer [100 nM [$^3$H] $LTC_4$, or 2.5 μM [$^3$H] $E_2$17β-G, 0.25 M sucrose, 10 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM phosphocreatine and 100 μg/ml creatin phosphokinase] at 37 °C. The reaction was stopped at the indicated times with 3 ml of ice-cold stop solution [0.25 M sucrose, 0.1 M NaCl, 10 mM Tris–HCl (pH 7.5)]. The samples were passed through Millipore filters (GVWP, 0.22 μM pore size, Millipore Co., Bedford, MA) under a light vacuum. Following three rinses with 3 ml of cold stop solution, the filters were dissolved in liquid scintillation fluid and their radioactivities were measured.

### 2.7. Vesicle uptake experiments of SN-38

SN-38 uptake was measured by a rapid filtration technique as described previously with some modifications [27]. Briefly, isolated MV (50 μg protein) were incubated with 4 mM ATP and 10 μM SN-38 in 50 μl transport buffer [0.25 M sucrose, 10 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM phosphocreatine and 100 μg/ml creatine phosphokinase] at 37 °C. The reaction was

stopped with 3 ml ice-cold stop solution [0.25 M sucrose, 0.1 M NaCl, 10 mM Tris–HCl (pH 7.5)] at the indicated times. The samples were passed through Millipore filters (0.44 μm, HAWP02500) under a light vacuum, which were subsequently washed twice with an additional 5 ml of ice-cold stop solution [10 mM Tris–HCl (pH 7.4), 0.25 M sucrose and 0.1 M NaCl]. After the filters were dried, SN-38 was extracted with 600 μl 50 mM potassium phosphate buffer (pH 6.0) and 900 μl methanol. Following the removal of filter and debris by centrifugation, the extract was concentrated with a rotary concentrator under a light vacuum. Non-specific binding of drugs to the filters was determined in the absence of MV. Values for MV uptake were obtained by subtracting this non-specific binding from the apparent uptake.

### 2.8. Measurement of SN-38 by HPLC analysis

The analysis of SN-38 was accomplished as described previously with some modifications. Briefly, for analysis of filter samples, 500 μl ice-cold 50 mM phosphate buffer (pH 6.0) was added to the filter followed by 50 μl of 25 μM camptothecine as an internal standard and 950 μl methanol. The mixture was vortex-mixed for 1.5 min, to extract the drugs on the filters. HCl (0.3 M, 350 μl) was added to 500 μl of the extract, and then analyzed by HPLC (Shimadzu, Kyoto, Japan) using a TSG gel ODS 80Ts (4.6 mm i.d. × 150 mm) with a guard column (Tosoh, Tokyo, Japan). The excitation and emission settings for the analyses were 380 nm and 550 nm, respectively. The mobile phase solvent was THF/50 mM potassium phosphate buffer, 5 mM heptasulfonate (pH 4.4) = 32/68.

### 2.9. Calculating of CLogP and clustering of MRP substrates

CLogP values were calculated using pcmodel.cgi (Daylight Chemical Information System Inc., Mission Viejo, CA).

The clustering of MRP1 substrates was performed using the Ward hierarchical clustering and the Tanimoto pairwise similarity coefficient. The Ward clustering dendrogram was obtained using *R* (http://www.r-project.org). The Tanimoto coefficient was calculated using the SIMCOMP program [28].

## 3. Results

### 3.1. Inhibition of azidoAG-A photolabeling of MRP1 by CPT-11 and SN-38

To analyze the mechanism of MRP1 transport of CPT-11 and SN-38, we first determined the relative ability of CPT-11 and SN-38 to inhibit azidoAG-A photolabeling of MRP1. Inhibition of AG-A photolabeling was quantified by measuring the radioactivity of photolabeled MRP1 bands. At a concentration of 200 μM, CPT-11 and SN-38 inhibited the photolabeling of MRP1 by 51% and 57%, respectively, in contrast with ADM and VCR by 7% and 20%, respectively. The calculated $IC_{50}$s were 10 times higher than those (33.9 μM and 30.4 μM) of VCR and ADM (Fig. 2). This result suggested that either the binding site(s) of CPT-11 and SN-38 to MRP1 might be slightly different from the azidoAG-A binding site(s) or that these two drugs have a lower affinity for MRP1 than VCR or ADM. Etoposide (VP-16) also only weakly inhibited the photolabeling with azidoAG-A of MRP1; these data are consistent with the previous report that VP-16 glucuronide is, but VP-16 is not, an excellent substrate of MRP1 [29].

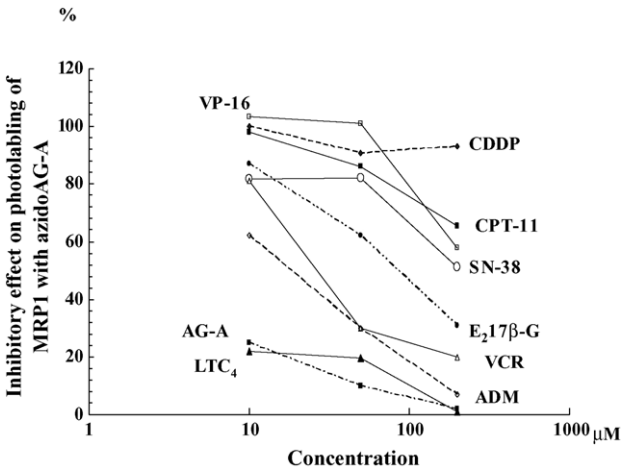



Fig. 2. Quantitation of the inhibitory effects of anti-cancer drugs on photolabeling of MRP1 with azidoAG-A. KB/MRP MV (100 μg) were incubated with [$^{125}$I] azidoAG-A in the absence or presence of the indicated concentration of the anti-cancer drugs at room temperature for 20 min with 2 mM GSH. Following 20 min UV irradiation, the samples were subjected to SDS-PAGE and [$^{125}$I] azidoAG-A-labeled MRP1 was detected by autoradiography. Gel slices corresponding to the 180-kDa-photolabeled MRP1 bands were excised and their radioactivities were determined with gamma-counting system ARC380 (Aloka, Japan). Background radioactivity from a random segment of the gel was deducted from the total count. The data are expressed as the percentage of radioactivity to that in the absence of any drugs.

### 3.2. Effect of AG-A and BSO on MRP-mediated resistance to SN-38

To know whether there are some differences between SN-38 and VCR transport by MRP1, we next tested if AG-A or BSO could reverse the resistance to SN-38. The addition of 10 μM AG-A or 100 μM BSO to KB/MRP cells could not reverse the resistance to SN-38, although it substantially reversed the resistance to VCR and VP-16 as previously described (Table 1) [14]. These data suggested that SN-38 could be transported by MRP1 in a GSH-independent manner through a mechanism distinct from that used for VCR transport.

Table 1
Drug resistance of KB/MRP1 cells to SN-38

| Cells | KB-3-1 | | | KB/MRP | | |
|---|---|---|---|---|---|---|
| | $IC_{50}$ | RR | DMF | $IC_{50}$ | RR | DMF |
| SN38 (nM) | 6.5 ± 0.67 | 1.0 | 1.0 | 83.9 ± 2.20 | 12.90 | 1.0 |
| SN38 + AGA 10 μM | 2.3 ± 0.05 | 0.35 | 2.83 | 49.4 ± 1.15 | 7.60 | 1.7 |
| SN38 + BSO 100 μM | 6.2 ± 0.62 | 0.95 | 1.05 | 60.8 ± 2.82 | 9.35 | 1.38 |

Survival of KB/MRP1 cells following SN-38 treatment in the presence or absence of AG-A or BSO was assayed by a cell survival assay as described in Section 2. Relative resistance (RR) was calculated as the $IC_{50}$ value for KB-3-1 or KB/MRP cells in the absence or presence of reversing agents, divided by the $IC_{50}$ value for KB-3-1 in the absence of reversing agents. DMF, the dose-modifying factor, was calculated as the $IC_{50}$ value in the presence of reversing agents divided by the $IC_{50}$ value in the absence of reversing agents.

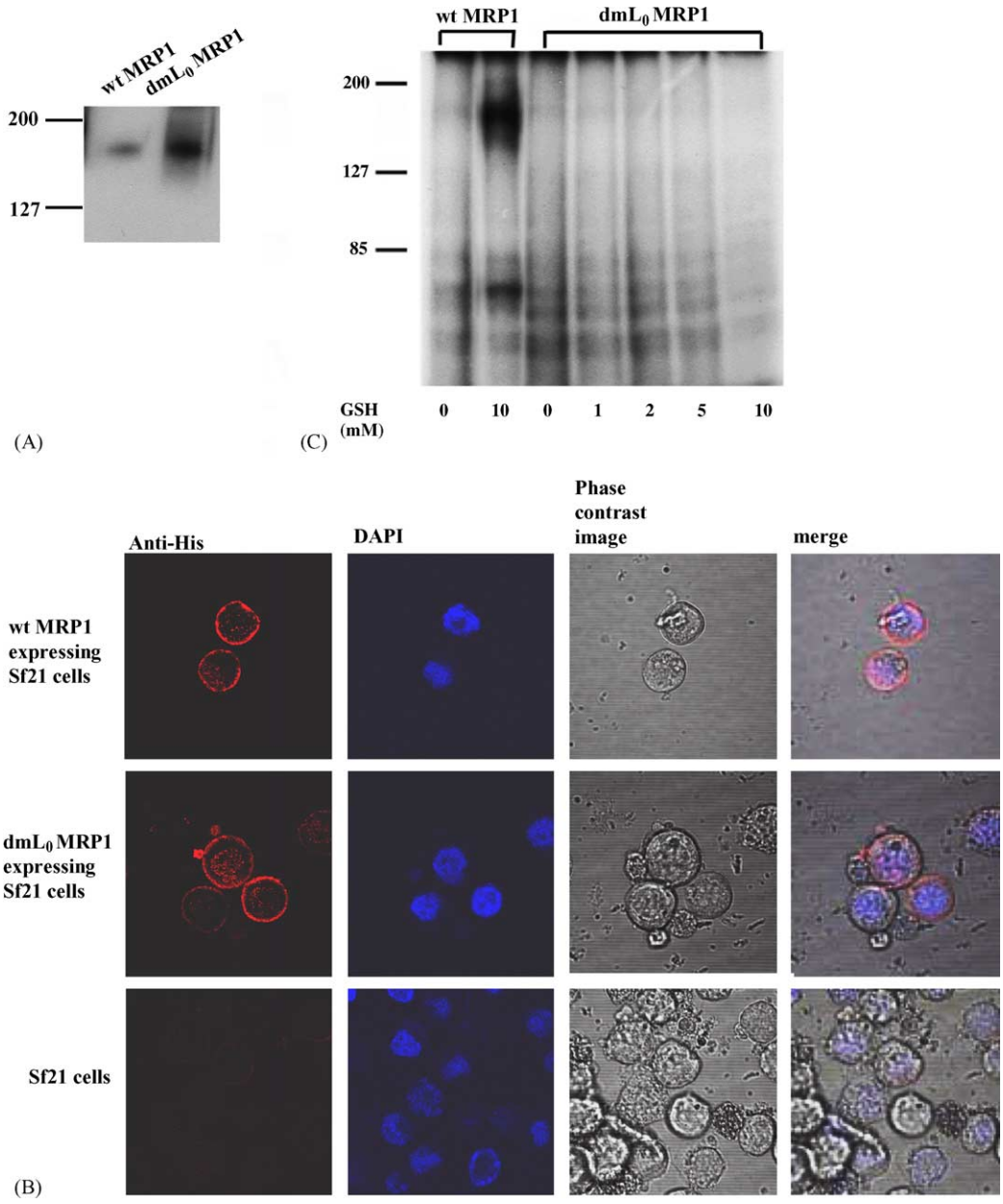



Fig. 3. Expression, subcellar localization and azidoAG-A-photolabeling of wt and mutant MRP1. (A) Expression levels of the wt and mutant MRP1 in membrane vesicles (100 μg) from Sf21 cells were determined by immunoblotting with the anti-MRP1 monoclonal antibody m6 as described in Section 2. MW markers are at left. (B) Immunofluorescent staining of wt (upper column), $dmL_0$ (middle) MRP1 expressing and virus-uninfected Sf21 cells (bottom). Cells were stained with immunofluorescence as described in Section 2. Nuclei were stained with DAPI (middle left panels) and wt and mutant MRP1 were stained with indirect immunofluorescence using anti-His antibody, MRP1 was detected in plasma membrane (left panels). Middle right panels: phase contrast images, right panels: merged images. (C) wt MRP1 or $dmL_0$MRP1 from MV of insect expressing cells were photoaffinity labeled as described in Section 2 in the absence or presence of the indicated amount of GSH. AzidoAG-A photoaffinity labeled wt MRP1 or $dmL_0$MRP1 were analyzed by electrophoresis and autoradiography. MW markers are at left.

To analyze the different mechanisms used by MRP1 to transport SN-38 and VCR, we focused on the $L_0$ region of MRP1, since we have previously shown that the $L_0$ region is important for GSH-dependent azidoAG-A photolabeling and for $LTC_4$ transport [15]. As described in Section 2, the new mutant MRP1, named $dmL_0$MRP1, was designed to carry the extra mutations, W222L, W223L and R230A, in addition to the previous mutants, W261A and K267M, in $L_0$ region, and the original $TMD_0$ region, in order to avoid the influence of deletion of the $TMD_0$ region (Fig. 1).

### 3.3. Expression, subcellar localization and azidoAG-A-photolabeling of wt and mutant MRP1

We then tested the mutant $dmL_0$MRP1 in functional assays of drug transport. The $dmL_0$MRP1, or the wt MRP1, were expressed in Sf21 insect cells with a baculovirus expression system and the proteins expressed in MV were detected by immunoblotting using a antibody against MRP1, m6 (Fig. 3A) and immunofluorescence staining of the cells. Both wt and mutant MRP1 were detected on the plasma membrane of Sf21 cells (Fig. 3B). Although the level of $dmL_0$MRP1 protein expressed in the virus-infected Sf21 MV was about 1.8 times higher than that of wt MRP1, the $dmL_0$MRP1 protein was not photoaffinity labeled with azidoAG-A even in the presence of a high concentration of GSH (Fig. 3C).

### 3.4. Uptake of [$^3$H] $LTC_4$ and [$^3$H] $E_2$17β-G by MV expressing wt and mutant MRP1

Next, we examined the transport of $LTC_4$ and $E_2$17β-G by $dmL_0$MRP1 and found that MV from $dmL_0$MRP1 expressing cells could transport neither $LTC_4$ nor $E_2$17β-G (Fig. 4A and B). These data suggested that the specific sequences that we mutated in the $L_0$ region of MRP1 are important not only for GSH-dependent transport but also for transport of substrates conjugated with glucuronide.

### 3.5. SN38 uptake by MV expressing wt and mutant MRP1

Prior to determination of the effect of $dmL_0$MRP1 on the transport of SN-38, we first examined the effect of GSH on the transport of SN-38 by MRP1. Previously, MV from MRP1 expressing cells were reported to transport SN-38 in the absence of GSH; however, the effect of GSH on the transport of SN-38 by MRP1 is unclear [27]. We found that transport of SN-38 in the MV from wt MRP1 expressing cells was similar in the presence or absence of GSH (Fig. 5A). We then compared the ability of the wt MRP1, and mutant $dmL_0$MRP1 expressing cells, to transport SN-38 in the absence of GSH. Both forms of MRP1 had similar SN-38 transport activity in the absence of GSH (Fig. 5B). These findings indicated that $dmL_0$MRP1 could

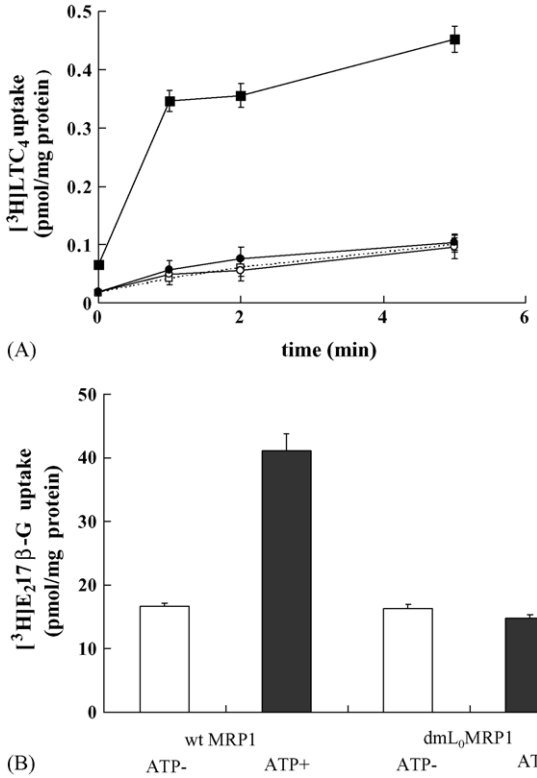



Fig. 4. Uptake of [$^3$H] $LTC_4$ and [$^3$H] $E_2$17β-G by MV expressing wt MRP1 or $dmL_0$MRP1. Uptake of $LTC_4$ and $E_2$17β-G in MV from Sf21 cells expressing wt or mutant $dmL_0$MRP1 were examined as described in Section 2. (A) Time course of ATP-dependent uptake of [$^3$H] $LTC_4$ by membrane vesicles (25 μg of protein) from wt MRP1 (squares) or $dmL_0$MRP1 (circles) expressing Sf21 cells in the presence (closed) or absence (open) of ATP. (B) ATP-dependent uptake of [$^3$H] $E_2$17β-G by MV from wt MRP1 or $dmL_0$MRP1-expressing Sf21 cells in the presence (closed) or absence (open) of ATP was examined at 5 min. Each data point and bar is the average and ±S.D. of triplicate measurements following subtraction of background from the Sf21 cells.

transport SN-38 and suggested that the intact $L_0$ region of MRP1 was not essential for transport of SN-38.

### 3.6. Clustering of substrates of MRP1

We postulated that the ability of drugs to be transported in a GSH-dependent or -independent manner may depend on the chemical character of the drugs. To determine if it was possible to define distinguishing chemical characteristics of the different drugs transported by MRP1, we first calculated the CLogP, an index of hydrophobicity. The CLogP values of the GSH-independent substrates, 4A6, SN-38, Rh123, IAARh123, IAAQ, IACI and calcein, were 1.341, 2.503, 2.329, 6.125, 6.502, 7.761 and 2.129, respectively. In contrast, CLogP values of the GSH-dependent substrates, VCR, ADM, aflatoxin B1, estrone-sulfate and NNAL-*o*-glucuronide, were 3.948, −2.111, 1.157, 1.262 and −2.377, respectively. A trend towards higher CLogP values ($p < 0.05$) of the drugs transported in a GSH-independent manner suggested that GSH-independent transport

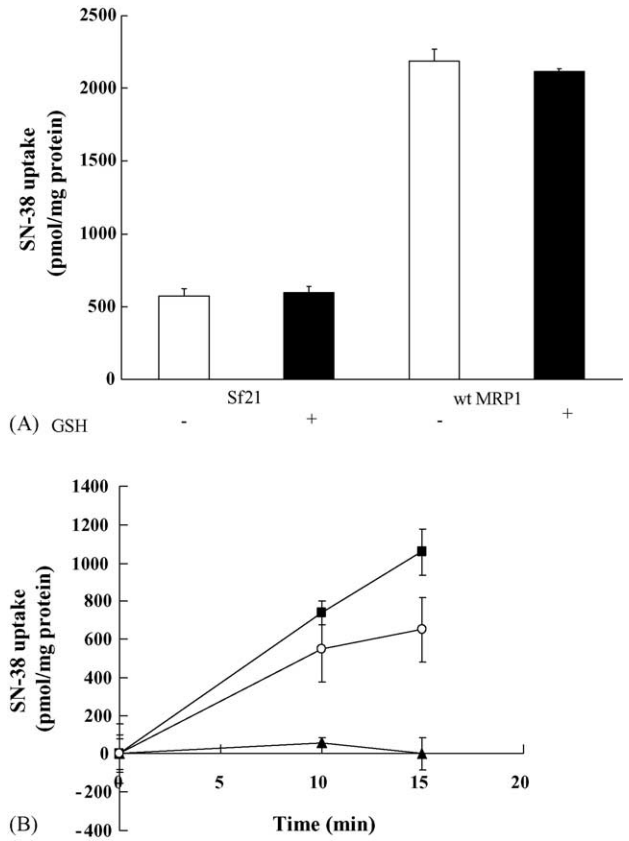



Fig. 5. Uptake of SN-38 by MV expressing wt MRP1 or dmL$_0$MRP1. (A) Uptake of SN-38 by MV from control, or wt MRP1 expressing Sf21cells, was examined in the presence (close bars) or absence (open bars) of 2 mM GSH over 10 min. Each data point and bar is an average and ±S.D. of a triplicate study. Differences between data in the presence and absence of GSH were not significant for Student's *t*-test. (B) Uptake of SN-38 by MV from Sf21 cells (triangles), wt MRP1 (close squares) or dmL$_0$MRP1 (open circles) expressing insect cells. Each data point and bar is the average and ±S.D. of triplicate measurements following subtraction of background from filter only. The data between MV from Sf21 and dmL$_0$MRP1expressing cells were statistically significant ($p < 0.01$) for Student's *t*-test in 10 min and 15 min.

might be somehow related to hydrophobicity of the drug. However, since the CLogP value of VCR was higher than that of SN-38, CLogP values are not the only predictor of GSH-independent transport. We therefore attempted to cluster the substrates using a new, pairwise comparison program SIMCOMP [28]. Using this program, we successfully clustered substrates that are transported in a GSH-independent manner into clusters 5 and 6 (Fig. 6) [30].

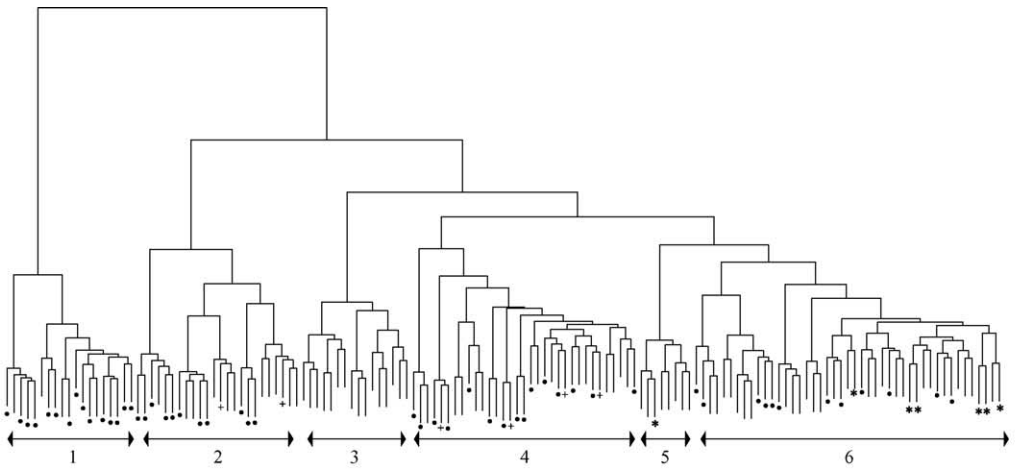
## 4. Discussion

The $L_0$ region of MRP1 has been reported to be essential for $LTC_4$ transport activity of MRP1 [17]. We have previously shown that an MRP1 that has mutations in $L_0$ and that lacks the $TMD_0$ region, is weakly photoaffinity labeled with azidoAG-A in the presence of GSH. This mutant also has a low $LTC_4$ transport activity, although $L_0$ was not the photolabeling site of azidoAG-A [15,31]. In this study, we constructed a new MRP1 mutant designated dmL$_0$MRP1, that had additional mutations in the $L_0$ region. dmL$_0$MRP1 was not photolabeled with azidoAG-A in the presence of GSH and transported neither $LTC_4$ nor $E_2$17β-G. These data confirm and extend the previous findings that specific sequences in the $L_0$ region appear to be indispensable for the GSH- and glucuronate-dependent transport function of MRP1.

However, we have shown that the sequences in $L_0$ in MRP1 that are indispensable for the transport of VCR and VP-16 may not be important for the transport of SN-38 by MRP1. This is consistent with previous data that MRP1 might be able to transport several substrates in the absence of GSH [27]. Thus, we have found that MRP1 expressing cells were resistant to SN-38 in the presence of BSO or AG-A. We have also shown that SN-38 is transported by MRP1 without glucuronization or GSH, as no glucuronized SN-38 could be detected from cells or culture media of MRP1 over-expressing human cancer cells after incubation with SN-38 [19] and SN-38 is transported into MV expressing MRP1 in the absence of GSH (Fig. 4A) [27]. Other groups have reported that MRP1 expressing cells are resistant to calcein and some toxic peptides even in the presence of BSO [18,19]. Rhodamine and quinoline based photoaffinity molecules IAAQ, IACI and IAARh123 can bind MRP1 in the absence of GSH [21–23]. There are two possible mechanisms that might explain GSH-independent transport by MRP1. The first is that the substrates may be able to mimic the interaction of GSH with the $L_0$ region of MRP1 and then be transported through the GSH-dependent pathway. Alternatively, the substrates may interact with MRP1 through different sites than those employed by GSH.

In this study, we provide evidence to support the latter theory. Using dmL$_0$MRP1, we have shown that SN-38, at least, can be transported in the absence of the specific $L_0$ region that is critical for GSH and GSH-dependent photoanalogs interaction with MRP1 [15,16]. It has been suggested that GSH induces a structural change in MRP1 that is required for the binding of substrates to MRP1 [32]. The ability of SN-38 to be transported in the absence of GSH suggests that either SN-38 might itself induce a structural change in MRP1 or that a structural change might not be required for the transport of SN-38 by MRP1. The fact that SN-38 was transported more slowly than $LTC_4$ by MRP1 might suggest that SN-38 can induce a structural change of MRP1 (Figs. 4A and 5A).

An important question to be determined in future experiments is whether the dmL$_0$MRP1 mutant can confer resistance to SN-38. To date, we have been unable to isolate dmL$_0$MRP1-transfected KB cells that express high amounts of the mutant MRP1 in order to carry out those experiments. We have also been unsuccessful in an attempt to find agents that might reverse MRP1-mediated




**Cluster 1**

- LTC4
- ω-CarboxyNacetyl LTE4
- LTE4
- LTD4
- NAcetyl LTE4
- NAc-DNP-Cys
- S-(2,4dinitrophenyl) glutathione
- S-(pazidophenacyl) glutathione
- S-(PGA1) glutathione
- Glutathione prostaglandin A conjugates
- GSSG
- Nethylmaleimide glutathione
- Glutathione
- S-Methyl GSH
- S-Hexyl GSH
- S-Octyl GSH
- S-Decyl GSH
- 4-Glutathionyl cyclophosphamide
- Monochloromonoglutathionyl melphalan

**Cluster 2**

- 3-α-sulfatolithocholyl taurine
- Glycolithocholate-3-sulfate
- 6-α-glucuronosylhydeoxycholate
- Sodium deoxycholate
- Glycocholate
- Taurocholate
- 17β-Estradiol-3-sulfato-17(β-D-glucuronide)
- 17β-Estradiol-3(β-D-glucuronide)
- 16α,17β-Estriol-3(β-D-glucuronide)
- 17β-Estradiol-17(β-D-glucuronide)
- 16α,17β-Estriol-17(β-D-glucuronide)
- Estramustin
- +Estrone sulfate
- 17β-Estradiol
- 17α-Ethinyl-17β-estradiol
- Glucuronosyl etoposide
- Etoposide
- Teniposide
- Grepafloxacin glucuronide
- Nafenopin glucuronide
- Glucuronate
- NNAL-O-glucuronide
- 4-Methylumbelliferone glucuronide
- α-Naphthyl-β-D-glucuronide

**Cluster 3**

- Acyclovir
- PMEG
- PMEA
- 6-mercaptopurine
- Thioguanine
- Azathioprine
- ACNU
- Cordycepin
- 8-azaadenosine
- 8-bromoaenosine
- 3TC
- AZT
- d4T
- Caffeine
- Uric acid
- 5-fluorouracil

**Cluster 4**

- Arsenite
- Arsenate
- Vanadate
- Daunomycin
- +Doxorubicin
- Epirubicin
- BQ123
- BQ518
- Actinomycin D
- Cyclosporin A
- SDZ PSC833
- Colchicine
- Colcemid
- Vinblastine
- +Vincristine
- Monoglucuronosylbilirubin
- Bisglucuronosylbilirubin
- Cadmium
- Cisplatin
- Potassium antimonyl tartrate
- Mitomycin C
- Novobiocin
- +Aflatoxin B1
- NIK250
- Diethyl pyrocarbonate
- Pravastatin
- Paclitaxel
- +AG-A
- Neomycin
- Hygromycin B
- DTT
- Dipyridamole
- Mitoxantrone

**Cluster 5**

- Camptothecin
- Topotecan
- *SN-38
- CPT-11
- CPT-11 carboxylate form
- SN38 carboxylate form
- SN38-Glu carboxylate form
- SN38-Glu lactone form

**Cluster 6**

- BSO
- Probenecid
- Chlorambucil
- Melphalan
- Carbenicillin
- Temocaprilat
- 5,10-Methylenetetrahydorofolate
- Tetrahydrofolate
- 5-Methyltetrahydrofolate
- GW1843
- Methotrexate
- ZD1694
- Difloxacin
- Rufloxacin
- Ciprofloxacin
- Norfloxacin
- E3040-glucuronide
- E3040
- E3040 sulfate
- GF109203X
- Tamoxifen
- Sulfinpyrazone
- BQ485
- *4A6 synthetic peptide
- Verapamil
- Podophyllotoxin
- Trimetrexate
- 4-Methylumbelliferone sulfate
- mAMSA
- BSP
- DIDS
- *IACI
- *IAAQ
- Quinidine
- Pyrimethamine
- Ethacrynic acid
- Indomethacin
- MK571
- Acetaminophen
- Genistein
- Benzbromarone
- *Rhodamine 123
- *IAARh123
- Fluo-3
- *Calcein

Fig. 6. Clustering of MRP1 substrates. In the dendrogram and the substrates lists, the dots indicate compounds that have been reported to be transported by MRP1. The plus (+) and the asterisk (*) indicate substrates that are transported by GSH-dependent and -independent manner, respectively. All other chemicals have been reported to be substrates of MRP family proteins. Leukotriene $E_4$, $LTE_4$; Leukotriene $D_4$, $LTD_4$; 9-(2-phosphonylmethoxyethyl) guanine, PMEG; 9-(2-phosphonylmethoxyethyl) adenine, PMEA; 3-[(4-amino-2-methyl-5-pyrimidinyl) methyl]-1-(2-chloroethyl)-1-nitrosourea, ACNU; 2′,3′-dideoxy-3′-thiacytidine, 3TC; azidothymidine, AZT; 2′,3′-didehydro-3′-deoxythymidine, d4T; agosterol A, AG-A; dithiothreitol, DTT; 4′-(9-acridinylamino)methanesulfon-*m*-anisidide, mAMSA; sulfobromophthalein, BSP; 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid, DIDS.

SN-38 resistance. Several agents such as quinolin have been reported to be transported by MRP1 without GSH. However, it was not possible to use high enough concentrations of these reagents in order to reverse resistance as these compounds are more toxic than previously reported reversing agents.

Since the difference between GSH-independent and -dependent transport of substrates must lie in the chemical structure of the substrates, we calculated the CLogP values of the substrates. However, the CLogP values did not strongly correlate with the mechanism of transport of the substrates. Nevertheless, substrates that were transported in a GSH-independent manner were clustered in the same clusters when a different analysis was used (Fig. 6). This analysis involved the measurement of the Tanimoto coefficient between all pairwise chemical structures and their clustering using the Ward hierarchical method [33]. The Ward hierarchical clustering is one of the major classification methods that attempts to minimize the loss associated with each grouping. The information loss is defined by Ward in terms of an error sum of squares criterion. In other words, this method can generate the clusters with very high classification sensitivity by comparing the compounds similarities based on pairwise similarities of chemical 2D structures and minimization within-cluster variances. The fact that the substrates that are transported in a GSH-independent manner were classified to two similar clusters by this analysis suggests that these compounds share a common feature(s), most likely based on their chemical structures.

In summary, in this study, the use of the $dmL_0$MRP1 mutant has provided evidence that SN-38 can be transported by MRP1 in a different way from GSH- and glucuronate-dependent transport. The $dmL_0$MRP1 mutant could transport SN-38, even though the mutant MRP1 could not be photolabeled with azidoAG-A and could not transport $LTC_4$ or $E_2$17β-G. An understanding of this GSH-independent transport mechanism of MRP1 at the molecular level will be important for understanding pathways of MRP1-mediated drug transport.

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