located in the second transmembrane spanning domain, and Gly671Val (G671V; rs45511401), near the Walker A motif in the first nucleotide-binding domain. The R433S MRP1 variant showed a 50% decreased transport maximum for leukotriene C4 (LTC_4) [8]; however, cells expressing this variant were more resistant to doxorubicin than those expressing wild-type (WT) MRP1 [8]. The G671V variant showed no difference in in-vitro transport assays using LTC_4 or estradiol-17β-glucuronide ($E_2$17G) as substrates. Wang

antibody (1 : 5000) 1–2 h at room temperature, and finally washed twice with TBS-T buffer for 5 min. Proteins were detected using the enhanced chemiluminescence detection system (ECL Plus; Amersham Biosciences).

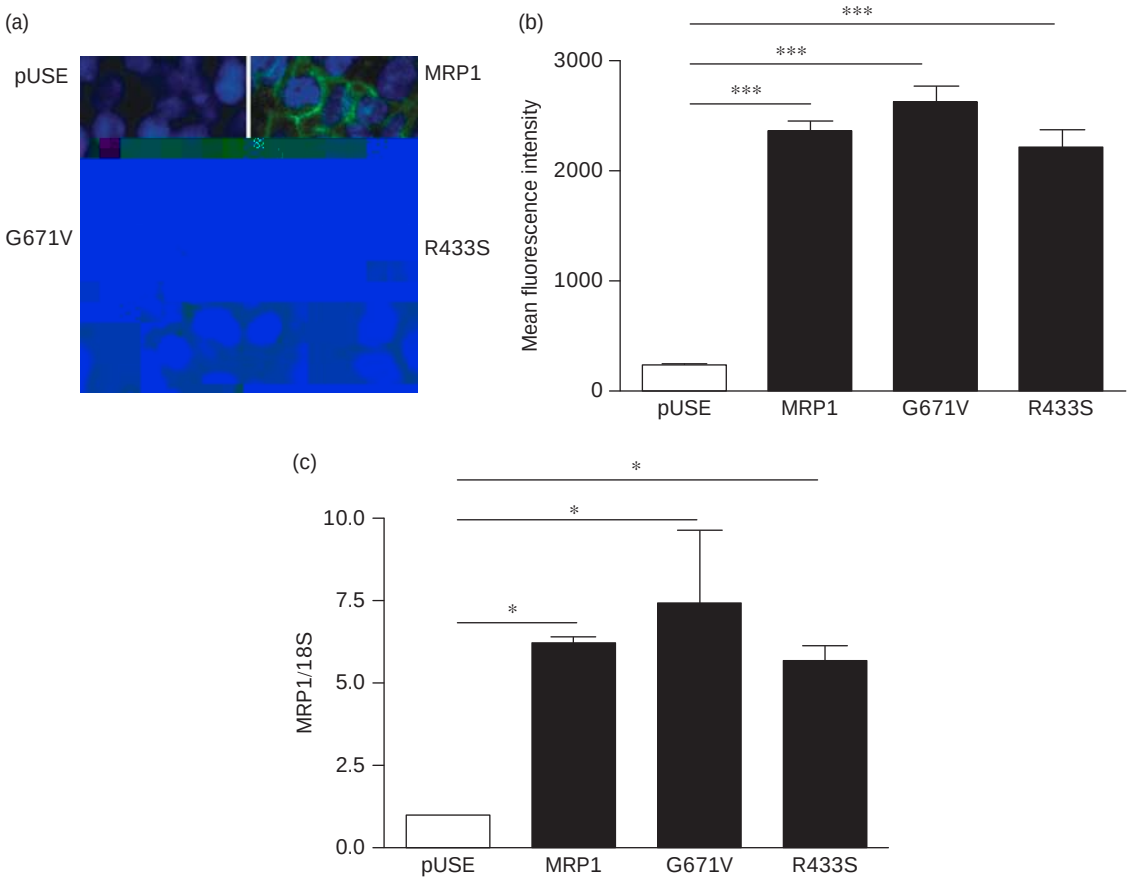
HEK293-transfected cells were cultured to reach 80% confluence. Cell nuclei were stained with Hoescht 33342 and incubated at 37°C for 5 min. Cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked with 1% BSA in PBS, incubated with primary antibody against MRP1 (1 : 2500), and probed with secondary antibodies (AlexaFluor 488 1 : 1000 in 1% BSA in PBS) as described [24]. Cells were washed, rinsed with ddH_2O, air dried, mounting medium was added, and the cells were placed under a cover glass [24]. Images were taken using an Olympus IX71 fluorescent microscope (Olympus America, Melville, New York, USA).

The transport experiments were performed as described [24]. Sarcolemma or plasma membrane vesicles were prepared by vesiculation through a 25 G needle 15 times before the transport assay. ATP-dependent transport of [^{3}H]GS-HNE into plasma membrane vesicles (5 μg protein/20 μl) was measured in incubations at 37°C for 1 min, whereas that of [^{3}H]LTC_4 was measured at 23°C for 1 min [24,25]. Reactions were terminated, filtered, and radioactivity sequestered within the inside–out vesicles detected as described [24].

To determine cytotoxicity, cells (2.5×10^4/well) were seeded onto a 96-well plate and cultured in the presence of doxorubicin at various concentrations. After 45 h, 20 μl of methylthiazol tetrazolium (5 mg/ml) was added to each well and cultures were continued for an additional 3 h. Supernatants were discarded, 100 μl of dimethylsulfoxide was added to dissolve the formazan crystal, and its concentration was determined by spectrophotometry at A_{540}. The methylthiazol tetrazolium values (absorption expressed as a percentage of control values) obtained after a continuous 48 h exposure of cells to doxorubicin were compared, and the doxorubicin concentrations inhibiting cell growth by 50% (IC_{50}) were calculated from the per cent survival curves.

To determine cellular retention of doxorubicin, cells (5×10^5/well) were plated and cultured in 24-well plates using 6 wells per cell line. After 24 h in culture, cells were treated with 50 μmol/l of doxorubicin for 1 h, the media were replaced with fresh media, and cells were incubated for an additional 30 min in the absence of doxorubicin. Cells were washed twice with PBS, 800 μl of buffer (50 mmol/l of Tris–HCl, pH 8.0, 150 mmol/l of NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS) was added to each well to lyse cells, and 200 μl of cell lysate was assayed for doxorubicin by fluorescence spectroscopy (ex-

Fig. 1



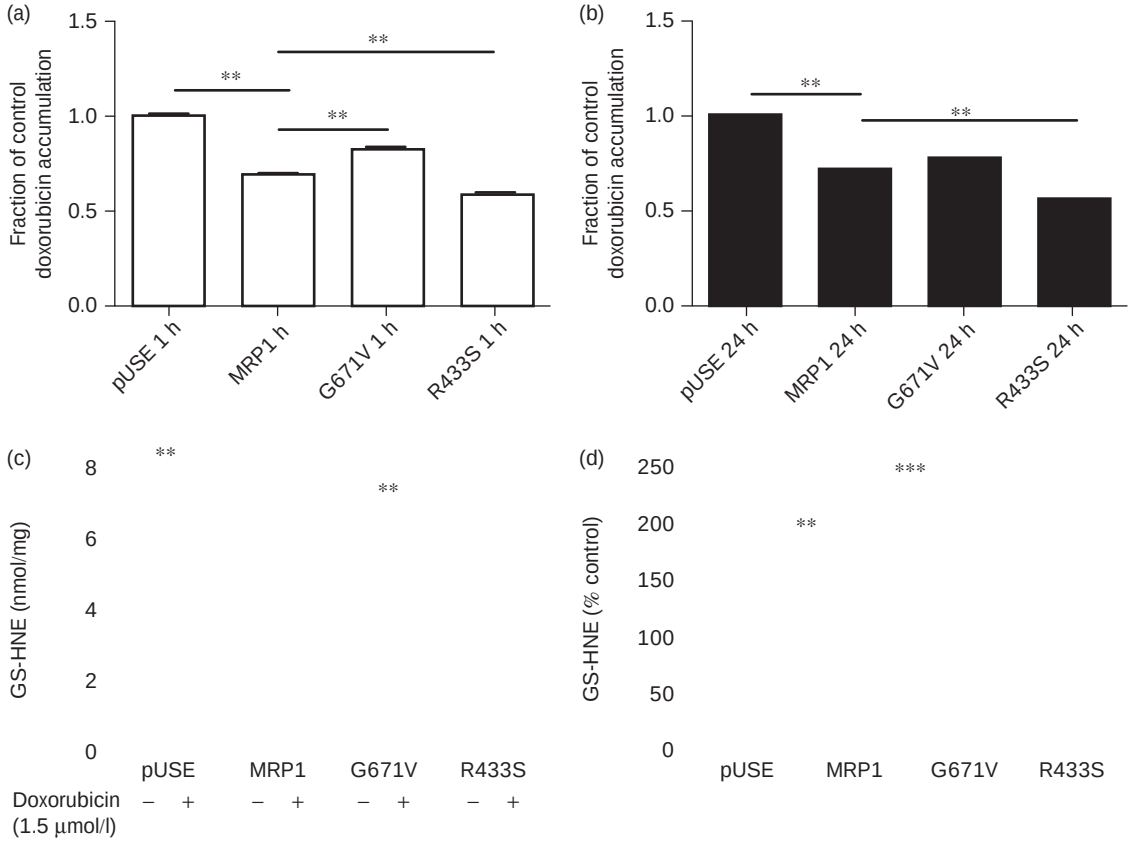
Expression in stable cell lines of multidrug resistance-associated protein 1 (MRP1) and its variants. (a) Cells were immunostained for MRP1 protein; blue = nuclei, green = MRP1 protein. Plasmids containing wild-type MRP1 or the MRP1 variants G761V and R433S were transfected into HEK293 cells to generate stable cell lines. pUSE cells were HEK293 cells transfected with pUSEamp(+) empty vector and used as a control. (b) Cells were stained with MRPr1 primary antibody, followed by fluorescence-labeled secondary antibody (AlexaFlor488) for flow cytometry analyses. Bar graphs show the mean fluorescence intensity ± SE. (c) MRP1 mRNA expression was detected by real-time reverse transcriptase-polymerase chain reaction and normalized by 18S rRNA expression. *P < 0.05, ***P < 0.001.

Fig. 2

(a)

(b)

Genotype	Doxorubicin IC_{50} nmol/l (95% CI)



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In view of the cellular GS-HNE retention in HEK_{G671V} cells, we examined further whether the MRP1 polymorphisms would have an impact on their substrate specificity or transport capacity. We first characterized transport of the classic MRP1 substrate, LTC_4, in plasma membrane vesicles after normalization for expression of MRP1 (Fig. 6a). The G671V variant was comparable with WT MRP1 with respect to LTC_4 transport, whereas LTC_4 transport was decreased by 75% in the R433S variant compared with WT MRP1 (Fig. 6b), consistent with previous reports [7,8]. To determine whether the G671V variant might have an altered dependence on glutathione for transport, we examined the effects of glutathione; 0.5 mmol/l of glutathione had no effect on transport, whereas 5 mmol/l of glutathione completely inhibited LTC_4 transport by MRP1 and both its variants (Fig. 6b).

We next determined the kinetic parameters for GS-HNE transport in plasma membrane vesicles. GS-HNE was transported by Michaelis–Menten kinetics (Fig. 6c), and showed a markedly reduced transport by the G671V variant such that the $_{max}$ was decreased to about 15% of that by WT MRP1 (Fig. 6c). In contrast, the $_{max}$ of the R433S variant was increased over two-fold relative to WT MRP1. The K_m value of the G671V variants did not differ significantly from that of WT MRP1 (Fig. 6c), and agreed well with the K_m of 1.6 µmol/l reported previously for MRP1 [20]. The estimate of the K_m for the R433S variant was higher ($\geq$ 6 µmol/l), consistent with its increased $_{max}$ (Fig. 6c). The $_{max}/K_m$ of the G671V variant was only about 10% (0.025 mg/l/min) of that of WT MRP1 (0.24 mg/l/min), indicating a markedly decreased transport efficiency of the G671V variant.

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The loss of MRP1-mediated GS-HNE transport could be significant if this transporter were the main mechanism for the heart to eliminate GS-HNE. To investigate whether the cardiac sarcolemma can transport GS-HNE, and the

importance of Mrp1, we isolated sarcolemma membranes from FVB WT and Mrp1$^{-/-}$ mice that were treated with doxorubicin (20 mg/kg, intraperitoneally) and killed 24 h later. As shown in Fig. 7, sarcolemma from FVB mice transported GS-HNE in a saturable manner, with a K_m and $_{max}$ of 2.5 ± 1.1 μmol/l and 911 ± 166 pmol/min/mg protein,

found that HEK_{G671V} cells retained the highest amount of this MRP1 substrate. We also measured the redox status of glutathione and GSSG in each of the cell lines in response to doxorubicin treatment. Glutathione and GSSG were the highest in HEK_{pUSE} cells, consistent with the known glutathione-efflux and GSSG-efflux activities of MRP1. Glutathione and GSSG levels decreased across time following doxorubicin exposure in all cell lines, and were the lowest at all time points in HEK

MRP3, Grant . [30] substituted amino acids 425-516 of MRP1 in the region spanning transmembrane helices 8 and 9 with those of amino acids 411-502 of MRP3, and found complete loss of LTC_4 transport, but a modest enhancement in $E_2$17βG transport, with minimal effects on transport of methotrexate, a substrate common to both MRP1 and MRP3 [30]. A cluster of three amino acids (Tyr440, Ile441, and Met443) in MRP1 and Phe426, Leu427, and Leu429 of MRP3 made major contributions to these differences. The conclusion of these authors that amino acids in this region of MRP1/MRP3 make significant contributions to substrate specificity is consistent with the current findings that the Arg433 of MRP1 also selectively influenced MRP1 substrate specificity. Interestingly, alignment of this region of MRP1 (Fig. 2 of [29]) shows that Arg433 of MRP1 is conserved in MRP3, MRP5, MRP6, and MRP7, and is replaced with a lysine in MRP2 and MRP4, suggesting the importance of a cationic amino acid in this position.

Gly671 is seven amino acids upstream of the Walker A motif of NBD1 in MRP1, and is conserved in CFTR, TAP1, YCF1, and some bacterial ABC transporters [33], again implying an important function. Despite being located close to the Walker A motif, the substitution of a valine must not have affected the rate of ATP hydrolysis, on the basis of the retention of LTC_4 transport.

In conclusion, cells expressing the G671V MRP1 variant were more sensitive to doxorubicin than cells expressing WT MRP1, most likely due to an increase in the accumu-

likely that the G671V variant alters MRP1 recognition of doxorubicin. The decreased GS-HNE transport capacity of the G671V variant further indicates that MRP1 polymorphisms can play a significant role in MRP1 activity, and that these findings may be clinically important in patients receiving chemotherapy, particularly doxorubicin. Close monitoring for cardiac toxicity may therefore be beneficial in patients with the MRP1 G761V polymorphism who are receiving doxorubicin chemotherapy.

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1 Shan K, Lincoff AM, Young JB. Anthracycline-induced cardiotoxicity. Ann Intern Med 1996; **125**:47–58.
2 Henderson IC, Allegra JC, Woodcock T, Wolff S, Bryan S, Cartwright K, et al. Randomized clinical trial comparing mitoxantrone with doxorubicin in previously treated patients with metastatic breast cancer. J Clin Oncol 1989; **7**:560–571.
3 Allen A. The cardiotoxicity of chemotherapeutic drugs. Semin Oncol 1992; **19**:529–542.
4 Wojtacki J, Lewicka-Nowak E, Lesniewski-Kmak K. Anthracycline-induced cardiotoxicity: clinical course, risk factors, pathogenesis, detection and prevention–review of the literature. Med Sci Monit 2000; **6**:411–420.
5 Ozdemir V, Shear NH, Kalow W. What will be the role of pharmacogenetics in evaluating drug safety and minimising adverse effects? Drug Saf 2001; **24**:75–85.
6 Pirmohamed M, Park BK. Genetic susceptibility to adverse drug reactions. Trends Pharmacol Sci 2001; **22**:298–305.
7 Conrad S, Kauffmann HM, Ito K, Deeley RG, Cole SP, Schrenk D. Identification of human multidrug resistance protein 1 (MRP1) mutations and characterization of a G671V substitution. J Hum Genet 2001; **46**:656–663.
8 Conrad S, Kauffmann HM, Ito K, Leslie EM, Deeley RG, Schrenk D, et al. A naturally occurring mutation in MRP1 results in a selective decrease in organic anion transport and in increased doxorubicin resistance. Pharmacogenetics 2002; **12**:321–330.
9 Wang Z, Sew PH, Ambrose H, Ryan S, Chong SS, Lee EJ, et al. Nucleotide sequence analyses of the MRP1 gene in four populations suggest negative selection on its coding region. BMC Genomics 2006; **7**:111.
10 Wojnowski L, Kulle B, Schirmer M, Schluter G, Schmidt A, Rosenberger A, et al. NAD(P)H oxidase and multidrug resistance protein genetic polymorphisms are associated with doxorubicin-induced cardiotoxicity. Circulation 2005; **112**:3754–3762.
11 Thornalley PJ, Dodd NJ. Free radical production from normal and adriamycin-treated rat cardiac sarcosomes. Biochem Pharmacol 1985; **34**:669–674.

resistance protein (MRP) 1 (ABCC1) and MRP3 (ABCC3). Drug Metab Dispos 2008; **36**:2571–2581.
31 Hubatsch I, Ridderstrom M, Mannervik B. Human glutathione transferase A4-4: an alpha class enzyme with high catalytic efficiency in the conjugation of 4-hydroxynonenal and other genotoxic products of lipid peroxidation. Biochem J 1998; **330 (Pt 1):**175–179.
32 Ishikawa T, Esterbauer H, Sies H. Role of cardiac glutathione transferase and of the glutathione S-conjugate export system in biotransformation of 4-hydroxynonenal in the heart. J Biol Chem 1986; **261**: 1576–1581.
33 Deeley RG, Westlake C, Cole SP. Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. Physiol Rev 2006; **86**:849–899.
34 Jungsuwadee P, Vore M. Efflux transporters in comprehensive toxicology, Editor-in-Chief, Charlene A. McQueen. In: Guengerich FP, editor. Biotransformation, Chapter 26. 2nd ed. Boston, USA: Elsevier; 2010. pp. 557–601.
35 Stride BD, Cole SP, Deeley RG. Localization of a substrate specificity domain in the multidrug resistance protein. J Biol Chem 1999; **274**: 22877–22883.