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Direct Binding of Chloroquine to the Multidrug Resistance Protein (MRP)

POSSIBLE ROLE FOR MRP IN CHLOROQUINE DRUG TRANSPORT AND RESISTANCE IN TUMOR CELLS

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ABSTRACT. Multidrug resistance protein (MRP) transports a range of compounds that include glutathione *S*-conjugates, amphiphilic anionic drugs, and natural-product toxins. However, the mechanism of MRP drug binding and transport is presently unclear. We recently demonstrated the direct binding of a quinoline-based photoactive drug, *N*-{4-[1-hydroxy-2-(dibutylamino)ethyl] quinolin-8-yl}-4-azidosalicylamide (IAAQ), to MRP at a biologically relevant site [Vezmar *et al.*, *Biochem Biophys Res Commun* **241:** 104–111, 1997]. In the present report, we demonstrated that the lysosomotropic or antimalarial drug chloroquine is a substrate for MRP. Specifically, our results showed that chloroquine, similar to leukotriene C_4 (LTC_4) and 3-(3-(2-(7-chloro-2-quinolinyl)ethenyl-phenyl)((3-(dimethyl amino-3-oxo propyl)thio)methyl)thio) propanoic acid (MK 571), inhibits the photoaffinity labeling of MRP by IAAQ. Furthermore, cell growth assays showed MRP-expressing multidrug-resistant cells (H69/AR and HL60/AR) to be more resistant to chloroquine than their parental cells (i.e., IC_{50} of 121 μM versus 28 μM chloroquine for H69/AR and H69, respectively). Moreover, MK 571, an LTD_4 receptor antagonist, reversed the resistance of H69/AR cells to chloroquine. Drug transport studies using [^{14}C]chloroquine demonstrated that MRP-expressing cells accumulate less drug than the parental drug-sensitive cells. The reduced accumulation of [^{14}C]chloroquine in resistant cells was ATP dependent and was due to enhanced drug efflux. Taken together, the results of this study show that MRP modulates the transport of chloroquine by direct binding. BIOCHEM PHARMACOL **56**;6:733–742, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. multidrug resistance; chloroquine; multidrug resistance protein (MRP); drug transport; photoaffinity labeling

Tumor cell lines selected *in vitro* with sublethal concentrations of natural-product drugs respond by overexpressing P-gp1† (for reviews, see Refs. 1 and 2). The overexpression of P-gp1 in response to drug selection occurs frequently, and tumor cells display a pleiotropic resistance towards a diverse group of drugs. Studies with purified P-gp1 reconstituted into defined lipids have confirmed the role of P-gp1 as an ATP-dependent drug efflux pump [3, 4]. The normal function of P-gp1 remains unknown. However, P-gp1 was shown recently to function as a "flipase" of short chain lipids and to mediate the transport of normal cell metabolites and xenobiotics [5–7]. Furthermore, disruption of both P-gp1 and P-gp2 genes in mice led to an increase in drug accumulation in normal tissues [8, 9].

Cole *et al.* [10] described the overexpression of another membrane transporter, the MRP, in *in vitro* selected H69 SCLC cells that displayed an MDR phenotype without P-gp1. Since then, other MDR cell lines have been shown to express MRP without P-gp1 [11]. P-gp1 and MRP are members of a large family of ABC trafficking proteins that couple ATP hydrolysis to ligand transport across a lipid membrane [12, 13]. Although gene transfer studies of P-gp1 or MRP, in previously drug-sensitive cells, were shown to confer largely similar drug resistance profiles, the mechanisms of P-gp1 and MRP-mediated MDR are thought to be different [14–17]. Thus, while P-gp1 binds directly to unmodified drugs and mediates their efflux across the cell membrane in an ATP-dependent manner [18], the mechanism of MRP-mediated MDR is not clear. Several reports have demonstrated increased efflux of unmodified drugs from MRP-overexpressing cells or cells transfected with human MRP cDNA [14, 19, 20]. In contrast, other studies have shown an ATP-dependent efflux of unmodified drugs *only* in the presence of GSH [21, 22]. Hence, an outstanding question concerning the role of GSH in the binding and transport of drugs by MRP remains to be resolved. In a recent report by Rappa *et al.* [23], MRP was shown to function as a co-transporter of GSH and natural-product toxins in embryonic stem cells. Furthermore, disruption of the *MRP* gene in mice led to increased sensitivity to

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† *Abbreviations:* ABC, ATP binding cassette; LT, leukotriene; MDR, multidrug resistance; MRP, multidrug resistance protein; P-gp1, P-glycoprotein; and SCLC, small cell lung cancer.

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natural-product toxins and elevated GSH levels in MRP-expressing tissues [24].

Several studies have now shown MRP to mediate the transport of GSH-conjugated compounds and other normal cell metabolites that include the cysteinyl leukotriene (LTC_4), GSSG, and 17β-estradiol 17-(β-D-glucuronide) [21, 22, 25, 26]. These organic anions are thought to be potential physiological substrates for MRP and are consistent with findings that suggest that MRP is the same or a related protein to the *m*ultispecific *o*rganic *a*nion *t*ransporter (MOAT) [27]. Moreover, direct binding between LTC_4 and MRP has been shown in intact cells and membrane vesicles from MRP-overexpressing cells [26, 28]. Furthermore, LTC_4 binding and transport by MRP were inhibitable with excess (3-(3-(2-(7-chloro-2-quinolinyl) ethenyl)phenyl)((3-dimethyl amino-3-oxo propyl)thio) methyl)thio) propanoic acid (MK 571), an antagonist of the LTD_4 receptor [25]. Interestingly, it has not been possible to demonstrate direct binding between MRP and other substrates of MRP (e.g. natural-product drugs [11]). Using a photoactive quinoline-based drug, (*N*-{4-[1-hydroxy-2-dibutylamino)ethyl] quinolin-8-yl}-4-azidosalicylamide; IAAQ), we recently provided the first evidence for a direct and specific binding of unmodified drug to MRP in the H69/AR SCLC cells [29]. The inhibition of MRP photolabeling by IAAQ with MK 571, also a quinoline-based drug [30], led us to examine the possibility that other quinoline-based drugs may interact directly with MRP. Therefore, it was of interest to determine if the lysosomotropic or antimalarial drug, chloroquine, is a substrate for MRP. An earlier report by Cole *et al.* [31] had shown that MRP-overexpressing cells (H69/AR) are less sensitive to chloroquine. However, it was not known from that study if MRP mediated chloroquine resistance. Moreover, chloroquine has been shown previously to interact with P-gp1 and to reverse P-gp1-mediated MDR [32]. In addition, chloroquine resistance in malaria is thought to be mediated by a P-gp1 homologue, *Pfmdr1* [33–36]. However, the role of *Pfmdr1* in chloroquine resistance in malaria remains controversial [37–39].

In this report, we have used the photoactive quinoline-based drug IAAQ [29] to demonstrate a direct binding between chloroquine and MRP in intact cells and plasma membranes. Moreover, our results implicate MRP in the cross-resistance and transport of chloroquine in MRP-expressing tumor cells.

MATERIALS AND METHODS

Materials

Iodine-125 (100.7 mCi/mL) was purchased from Amersham Biochemical Inc. The LTD_4 receptor antagonist MK 571 was provided by Dr. A. W. Ford–Hutchinson (Merck–Frosst Centre for Therapeutic Research [30]). LTC_4 was purchased from the Cayman Chemical Co. The SCLC cells (H69 and H69/AR) were gifts from Dr. Susan P. C. Cole (Cancer Research Laboratories). The HL60 and HL60/AR cells were a gift from Dr. M. Center at Kansas State University. All other chemicals were of the highest commercial grade available.

Cell Culture and Plasma Membrane Preparation

Drug-sensitive (H69 and HL60) and -resistant (H69/AR and HL60/AR) cells were grown in RPMI 1640 medium containing 4 mM glutamine and 5–10% fetal bovine serum (HyClone). Resistant cells were cultured continuously in the presence of doxorubicin; however, cells used for drug transport studies were grown in drug-free medium for 10 days prior to the date of the experiment. Plasma membranes from H69 and H69/AR cells were prepared essentially as described by Lin *et al.* [40]. Membrane fractions were stored at −80° if not used immediately. Protein concentrations were determined by the method of Lowry *et al.* [41].

Cytotoxicity Assays

Cells were harvested and plated in 96-well plates at 0.5 to 1.0×10^4 cells/well. After allowing the cells to recover for 24 hr, cells were grown in increasing concentrations of chloroquine in the absence or presence of non-toxic concentrations of MK 571. Cells were grown for 4 days at 37° before the addition of the MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]. The plates were processed as previously described [42]. The effects of drugs on the viability of cells were expressed as the means ± SD of three independent experiments in which triplicates were assayed.

Drug Transport

Drug-sensitive and -resistant cells (1×10^6 cells/sample) were washed three times with PBS (pH 7.2) containing 1.0 μM [^{14}C]chloroquine (52 mCi/mmol) and 5 mM D-glucose or 2-deoxy-glucose and 100 nM sodium azide. Drug accumulation in cells was stopped at different time points (0–60 min) with the addition of 1 mL of ice-cold PBS, and then cells were washed three times with the same solution. The cell pellets were lysed in 100 μL of 1 M NaOH followed by neutralization with equal volumes of 1 M HCl, and the accumulated radiolabel was determined by fluorography using 1219 Racbeta model counters (LKB, Wallace). For drug efflux, cells were loaded with 10 μM [^{14}C]chloroquine in the presence of 100 nM sodium azide and 10 mM 2-deoxy-glucose for 30 min at 37°. Then cells were washed with PBS containing 5 mM D-glucose, and the incubation was continued for 60 min at 37°. Samples were collected at different times, up to 60 min. Cells were washed in ice-cold PBS, and the cell pellets were lysed and processed as indicated above.

Photoaffinity Labeling

For photoaffinity labeling of cells, H69 or H69/AR cells were washed with PBS and preincubated for 20 min at 37°

in the presence of excess vinblastine, chloroquine, LTC_4, or MK 571 before the addition of 0.1 to 0.25 μM IAAQ [29]. Cells were incubated at room temperature in the dark for 30 min and then were transferred to ice for 10 min. Following the latter incubation on ice, cells were irradiated for 10 min on ice with a UV source at 254 nm (Stratagene UV crosslinker, Stratagene) and lysed in 20 μL of 50 mM Tris (pH 7.4) containing 1% NP-40, 5 mM $MgCl_2$, and protease inhibitors (3 μg/mL of leupeptin and 2 mM phenylmethylsulfonyl fluoride). IAAQ-photolabeled proteins were isolated by a brief centrifugation and processed for SDS–PAGE. For photoaffinity labeling of plasma membranes, 15- to 30-μg aliquots of plasma membranes prepared from H69 and H69/AR cells were photoaffinity labeled as described above.

SDS–PAGE and Western Blotting

Samples containing IAAQ-labeled proteins were mixed with 1:5 (v/v) of buffer I [10 mM Tris–HCl, pH 8.0, containing 2% SDS, 50 mM dithiothreitol (DTT), 1 mM EDTA] and an equal volume of buffer II [2x buffer I and 9 M urea]. The solubilized proteins were then resolved by SDS–PAGE using the Fairbanks gel system with some modifications [43]. Gel slabs containing the resolved proteins were fixed in 50% methanol, dried, and exposed to XAR Kodak film at −70°. For Western blotting, proteins were transferred to nitrocellulose membrane and probed with the MRP-specific monoclonal antibody QCRL-1 (0.5 μg/mL) essentially as outlined by Towbin *et al.* [44]. Immunoreactive proteins were visualized by chemiluminescence using the ECL method (Amersham Inc.).

RESULTS

MRP has been shown to confer resistance to a broad spectrum of dissimilar drugs [11]. Our recent demonstration that the photoactive drug IAAQ and MK 571, both quinoline-based drugs (Fig. 1), bind directly to MRP led us to examine the possibility that other quinoline-based drugs also may be substrates for MRP [29]. Of several possible quinoline-based drugs, we were interested to know if the antimalarial drug chloroquine (Fig. 1) is a substrate for MRP. To determine if chloroquine interacts directly with MRP, IAAQ was used to photoaffinity label MRP in the absence and in the presence of excess chloroquine, MK 571, LTC_4, and vinblastine (Fig. 2). The results in Fig. 2A (lanes 1 and 2) show the photoaffinity labeling of MRP with IAAQ in H69/AR but not in H69 cells. Further, a molar excess of chloroquine, MK 571, LTC_4, and, to a lesser extent, vinblastine inhibited MRP photoaffinity labeling in intact H69/AR cells (Fig. 2A). Similar photoaffinity labeling results of MRP were also obtained using plasma membranes from H69 and H69/AR cells (Fig. 2B). In intact cells, LTC_4 at 75-fold molar excess completely inhibited the photoaffinity labeling of MRP with IAAQ (lane 9, Fig. 2A). Chloroquine and MK 571 were equal in

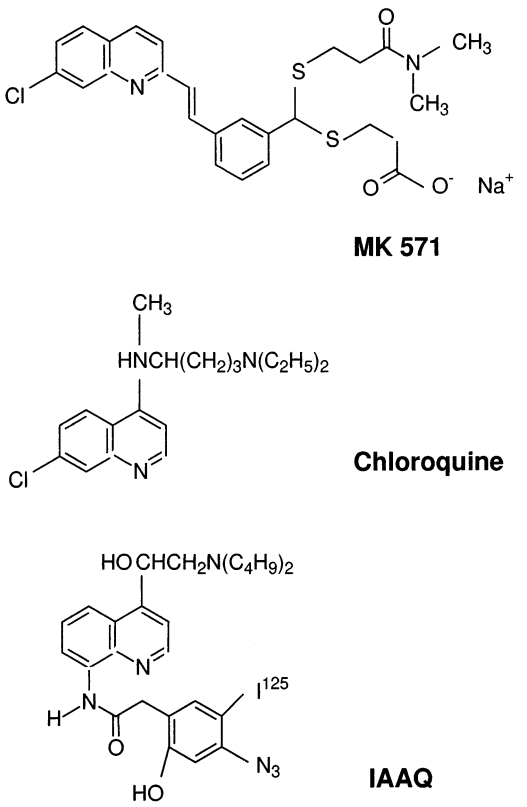

FIG. 1. Organic structures of quinoline-based drugs: MK 571, chloroquine, and IAAQ (*N*-{4-[1-hydroxy-2-(dibutylamino)ethyl] quinolin-8-yl}-4-azidosalicylamide).

their ability to inhibit IAAQ photolabeling of MRP (Fig. 2A). Vinblastine at 300- to 1000-fold excess of IAAQ also inhibited MRP photoaffinity labeling but to a much lesser extent than LTC_4, MK 571, or chloroquine (lanes 3 and 4 of Fig. 2, A and B). Together, these results show that chloroquine, LTC_4, and MK 571 interact directly with MRP.

To determine if MRP confers resistance to chloroquine, we measured the effect of increasing concentrations of chloroquine on the growth of H69/AR and HL60/AR MDR cells versus the parental H69 and HL60 cells (Fig. 3A). The results show H69/AR cells to be less sensitive to chloroquine than the parental H69 cells (IC_{50} of 121 μM vs 28 μM chloroquine, respectively). Similar results were also observed with another MRP-expressing cell line [HL60/AR; Fig. 3A (b)]. However, HL60/AR cells were more sensitive to chloroquine than the H69/AR cells (IC_{50} 12 μM vs 121 μM, respectively). Differences in the sensitivity of HL60/AR and H69/AR to chloroquine and other cytotoxic drugs (data not shown) are likely due to differences in MRP expression between the two cell lines (Fig. 3C). To further confirm the role of MRP in the cross-resistance of H69/AR cells to chloroquine, cells were grown in increasing concentrations of chloroquine plus non-toxic concentrations of MK 571. The results in Fig. 3B show that MK 571 (at 10 or 30 μM) increased the sensitivity of H69/AR cells to chloroquine (from IC_{50} of 121 μM in the absence of

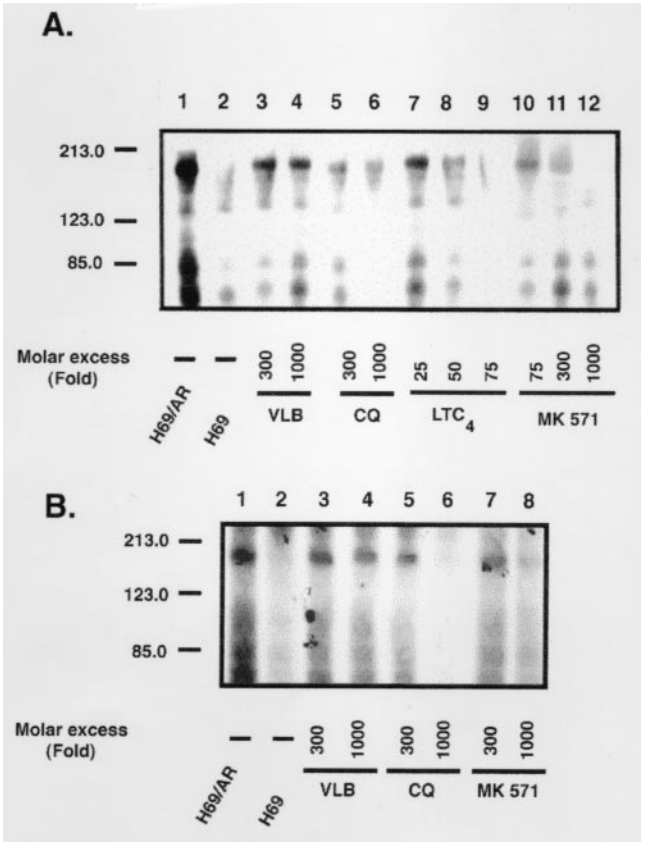

FIG. 2. Effects of chloroquine, vinblastine, MK 571, and LTC_4 on photoaffinity labeling of MRP in intact cells (A) and in plasma membranes (B). H69 or H69/AR cells were photoaffinity labeled with 0.25 μM IAAQ in the absence (lanes 2A and 1A, respectively) or presence of 300- and 1000-fold molar excess of vinblastine (VLB), chloroquine (CQ), or 25- to 75-fold of LTC_4 or 75- to 1000-fold MK 571 (lanes 3A–12A, respectively). Similarly, plasma membranes from H69 and H69/AR cells were photoaffinity labeled with 0.25 μM IAAQ in the absence (lanes 2B and 1B, respectively) and the presence of 300- to 1000-fold molar excess of vinblastine (VLB), chloroquine (CQ), or MK 571 (lanes 3B–8B, respectively). The numbers on the left of each panel are M_r standards in kilodaltons.

MK 571 to IC_{50} of 89 or 73 μM in the presence of 10 or 30 μM of MK 571, respectively). Similar concentrations of MK 571 did not affect the sensitivity of H69 cells to chloroquine [Fig. 3B (a)].

The above results show a correlation between MRP expression and reduced sensitivity to chloroquine in H69/AR and HL60/AR cells. However, it was not clear if the observed effects were due to differences in chloroquine transport or metabolism. Panels A and C of Fig. 4 show the accumulation of [^{14}C]chloroquine in drug-resistant (H69/AR and HL60/AR) and -sensitive (H69 and HL60) cells. Both H69/AR and HL60/AR cells accumulated less [^{14}C]chloroquine than the H69 and HL60 parental cells (Fig. 4, A and C). Moreover, the measured decrease in [^{14}C]chloroquine accumulation in resistant cells was reversed completely when cells were preincubated for 30 min with sodium azide and 2-deoxy-glucose (Fig. 4, B and D, respectively). Interestingly, and in view of the differences in chloroquine cross-resistance and MRP expression, H69/AR and HL60/AR showed similar levels of chloroquine accumulation (Fig. 4). However, it should be mentioned that differences in the subcellular distribution of MRP have been described previously between these two cell lines [45, 46].

To determine if the reduced chloroquine accumulation in resistant cells was due to enhanced efflux, cells were loaded with [^{14}C]chloroquine (see Materials and Methods), and drug efflux was measured in the absence and in the presence of sodium azide and 2-deoxy-glucose. The results in Fig. 5A show a more rapid efflux of [^{14}C]chloroquine from HL60/AR than from HL60 cells. By contrast, there was no enhanced efflux of [^{14}C]chloroquine from H69/AR cells compared with the parental H69 cells (Fig. 5B). However, depletion of ATP levels with sodium azide and 2-deoxy-glucose inhibited the efflux of chloroquine from both sensitive (HL60 or H69) and resistant (HL60/AR or H69/AR) cells (Fig. 5, A and B). To determine if the reduced accumulation, without enhanced efflux, of [^{14}C]chloroquine in H69/AR cells is due to an enhanced metabolism of the drug, cells were incubated with [^{14}C]chloroquine, and the fate of the [^{14}C]radiolabel was monitored by HPLC, as previously described [47]. The mobility of the extracted radiolabeled material from H69/AR or H69 cells was compared with the unaltered [^{14}C]chloroquine by HPLC using a C_{18} reverse-phase column [47]. Analysis of the HPLC results showed the elution time of the extracted radiolabel to be identical to that of unmodified [^{14}C]chloroquine (data not shown). Thus, differences in [^{14}C]chloroquine transport between H69/AR and HL60/AR are not due to the metabolism or organic modification of the drug.

DISCUSSION

P-gp1 and MRP are members of a large family of evolutionarily conserved ABC trafficking proteins [12, 13]. Although a certain overlap in P-gp1 and MRP substrate specificity exists, the two proteins are thought to differ in their mechanisms of drug transport [11]. For example, unlike P-gp1, MRP binds to and mediates the transport of GSH-conjugated drugs [28, 48, 49]. In addition, a GSH-dependent vincristine transport was shown in membranes from cells transfected with MRP [21]. However, it was not clear if GSH was required for drug binding and transport by MRP. Our previous demonstration that a quinoline-based drug binds directly to MRP in intact cells and in membrane fractions suggests that GSH is not required for drug binding [29]. In the present study, we showed that chloroquine, another quinoline-based drug, also binds directly to MRP. This is based on the ability of chloroquine to inhibit the photoaffinity labeling of MRP by IAAQ in purified membranes. In addition, we also found that the binding of chloroquine to MRP did not require direct modification of the drug (data not shown). Similar inhibition of MRP

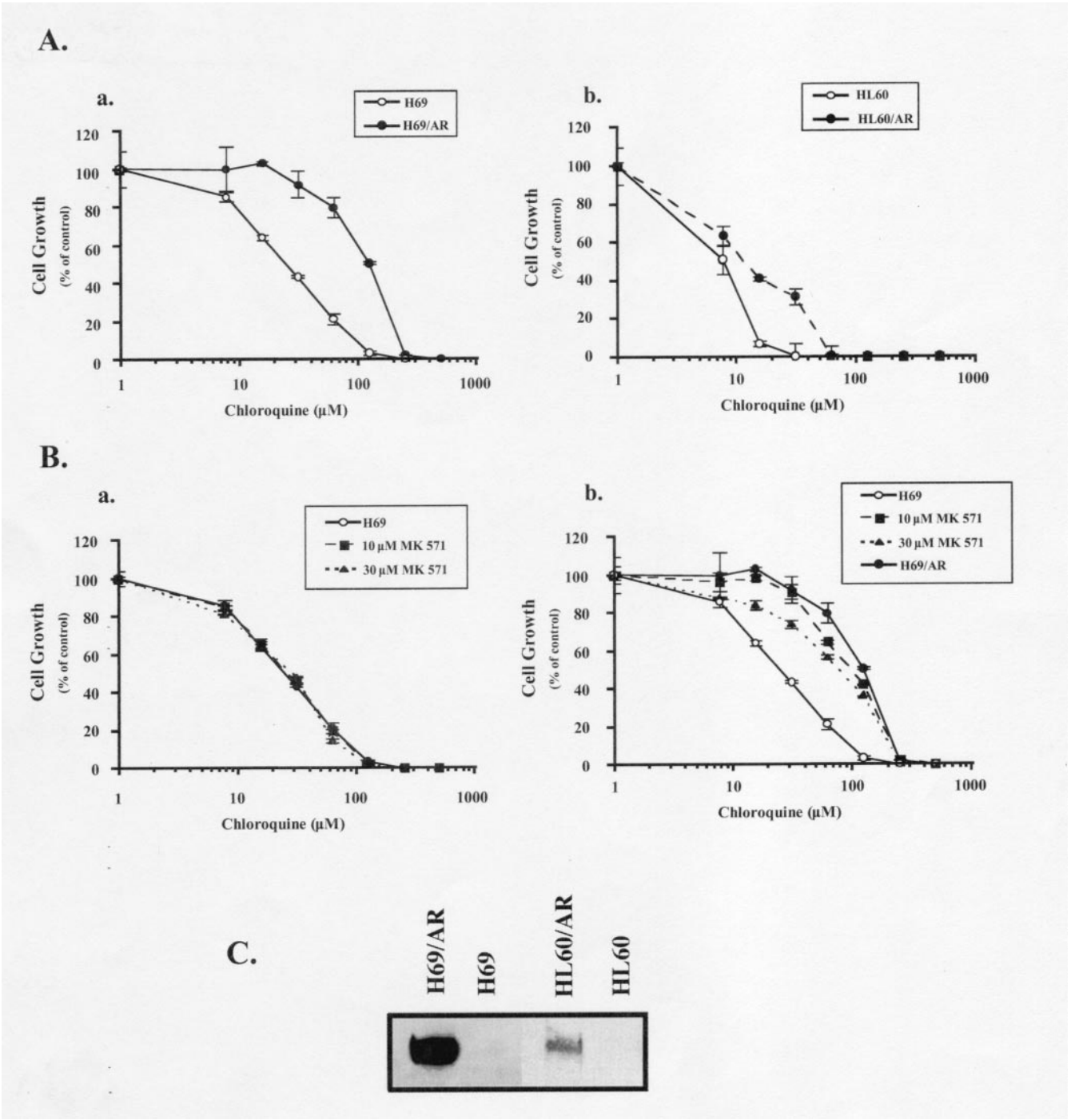

FIG. 3. Chloroquine cross-resistance and reversal with MK 571 in tumor cell lines. Drug-sensitive (H69 or HL60) and -resistant (H69/AR or HL60/AR) cells were plated at 0.5 to 1.0 × 10^4 and incubated in increasing concentrations of chloroquine (1–500 μM) (Fig. 3A, a and b, respectively). The effects of 10 and 30 μM MK 571 on the chloroquine cross-resistance of H69 and H69/AR are shown in Fig. 3B, a and b, respectively. The IC_{50} values were determined from the graph. Each value is the mean ± SD of three experiments in which triplicates were assayed. Figure 3C shows a Western blot of total cell lysates from MRP-overexpressing cells (H69/AR and HL60/AR) and their parental cells (H69 and HL60) probed with QCRL-1 monoclonal antibody.

photolabeling with IAAQ in intact cells and plasma membranes was also observed in the presence of LTC_4, MK 571, and, to a lesser extent, vinblastine (Fig. 2). Thus, although the above results are consistent with a direct binding between MRP and quinoline-based drugs (IAAQ, MK 571, and chloroquine), a catalytic role of GSH in MRP drug binding cannot be ruled out. Nevertheless, based on these results, we speculate about the ability of MRP to recognize compounds that contain a quinoline moiety. It is interesting in this respect that earlier studies [50] with P-gp1 have suggested that most drugs that bind to P-gp1 contain a planar benzene ring and a cationic charge (i.e. a quinoline

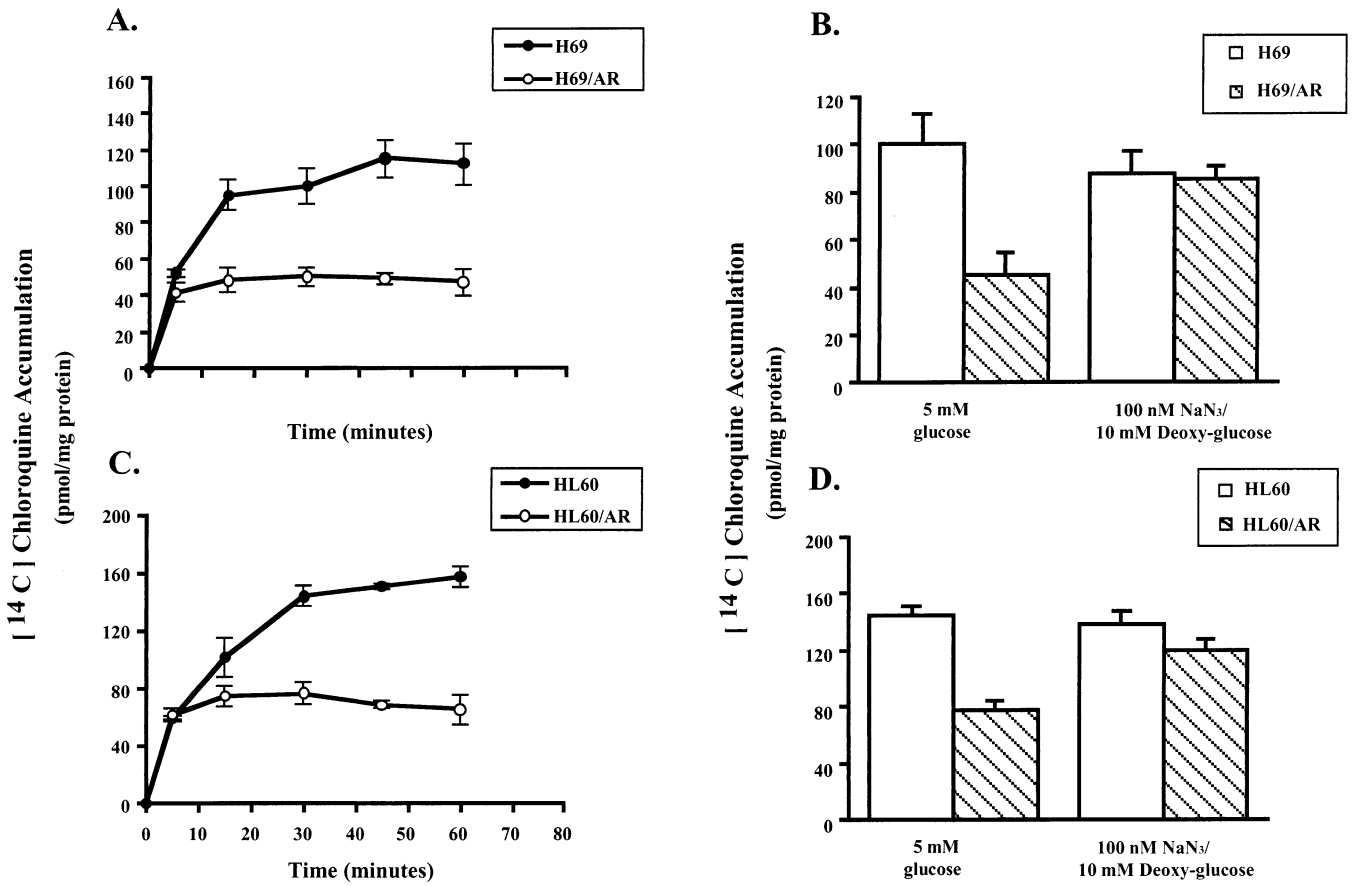

FIG. 4. [^{14}C]Chloroquine accumulation in MRP-expressing cells and their parental cells. Drug sensitive (H69 or HL60) and -resistant (H69/AR and HL60/AR) cells were incubated in the presence of 1 μM [^{14}C]chloroquine at 37°. Samples were removed, and the associated radioactivity was determined at 5–60 min by fluorography (Fig. 4, A and C). Similarly, cells were incubated in the presence of 1 μM [^{14}C]chloroquine in the absence and in the presence of 100 nM sodium azide and 10 mM 2-deoxy-glucose (Fig. 4, B and D). Each value is the mean ± SD of three experiments in which triplicates were assayed.

moiety). Moreover, both P-gp1 and MRP bind to chloroquine, MK 571, and IAAQ.* Thus, it is likely that P-gp1 and MRP recognize certain structural features in quinoline-based drugs.

The finding that MRP-expressing cells (H69/AR and HL60/AR) are less sensitive to chloroquine than their parental (H69 and HL60) cells is consistent with our photoaffinity labeling results in Fig. 2. Furthermore, incubation of resistant cells with increasing concentrations of chloroquine plus MK 571 reversed the sensitivity of H69/AR, but not H69 cells, to chloroquine. Although MK 571 was shown previously to reverse MRP-mediated drug resistance [51], its inability to reverse completely the cross-resistance of H69/AR cells to chloroquine may be due to its poor drug reversal capacity. Alternatively, it is possible that more than one mechanism mediates the resistance of H69/AR cells to chloroquine.

The mechanism by which MRP mediates resistance to structurally dissimilar drugs is not well understood. It has been suggested that, similar to P-gp1, MRP functions as an energy-dependent drug efflux pump [16, 20, 21, 28, 45, 52]. Consistent with this function, our drug transport studies with [^{14}C]chloroquine showed an energy-dependent decrease in the accumulation of drug in H69/AR and HL60/AR compared with H69 and HL60 cells. The decrease in [^{14}C]chloroquine accumulation in H69/AR versus H69 cells was not due to differences in the intracellular pH since the addition of 10 mM NH_4Cl to both cell lines did not alter their drug accumulation (data not shown). In addition, the intracellular pH of both cell lines was determined previously to be identical, with a pH of 7.55 [53]. Furthermore, we show that in HL60/AR cells [^{14}C]chloroquine efflux is energy dependent. In this study, chloroquine efflux from HL60 and HL60/AR cells was 30 and 60%, respectively. The latter results are consistent with those from an earlier study [54] showing a 15 and 40% efflux of [^{3}H]daunorubicin from HL60 and HL60/AR cells, respectively. The absence of enhanced chloroquine efflux from H69/AR cells relative to the parental H69 cells is not clear. However, a similar decrease in drug accumulation without detectable drug efflux was also observed in other MRP-expressing cells (e.g. MCF7/VP; [55]). Moreover, previous drug transport studies with H69/AR cells showed both drug

*Vezmar M and Georges E, manuscript in preparation.

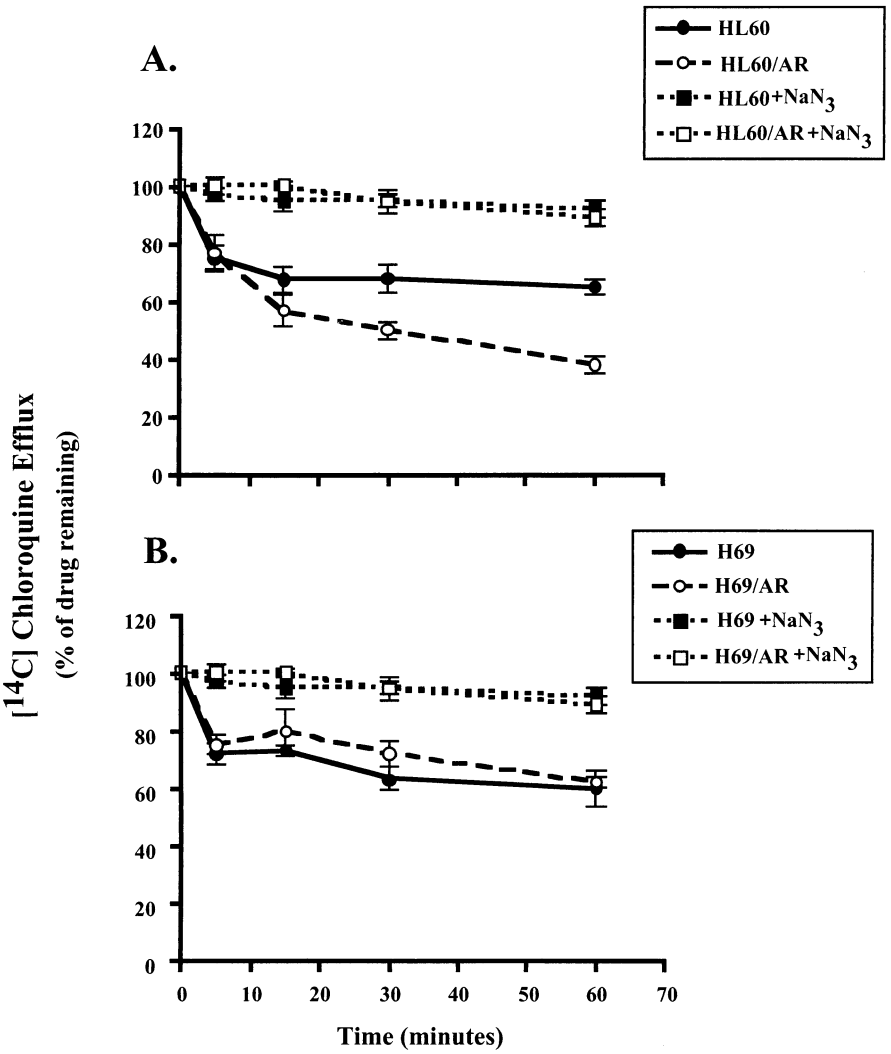

FIG. 5. [^{14}C]Chloroquine efflux from MRP-overexpressing cells and their parental cells. Drug-sensitive (H69 or HL60) and -resistant (H69/AR or HL60/AR) cells were loaded with 10 μM [^{14}C]chloroquine in the absence or presence of 10 mM sodium azide for 30 min after which drug extrusion was potentiated with the administration of 5 mM D-glucose to one half of the samples. For the remaining half of the samples, [^{14}C]chloroquine levels were determined in the presence of sodium azide. Samples were collected, and the amount of drug remaining in the cells was measured by fluorography. Each value is the mean ± SD of two experiments in which triplicates were assayed.

efflux and accumulation to be unchanged relative to H69 drug-sensitive cells [56]. However, cells transfected with MRP cDNA isolated from H69/AR cells showed a large decrease in vincristine accumulation but only a slight difference in drug efflux [16]. Although the latter findings remain unclear, the localization of MRP to the cell membrane and endosomal vesicles was suggested to be responsible for differences in drug accumulation versus efflux [16]. Taken together, although differences in chloroquine transport in H69/AR and HL60/AR are not clear, these differences may be due to differences in the subcellular distribution of MRP in these two cell lines [45, 46]. Alternatively, other cellular changes in HL60/AR and H69/AR [57–59], in addition to MRP, may be responsible for the differences in chloroquine transport between these two cell lines. Work is underway to confirm these results using cells transfected with human MRP cDNA.

Chloroquine has been extremely effective in the control and prophylaxis of malaria. Unfortunately, resistance to chloroquine and other quinoline-related drugs has rendered these drugs ineffective in many regions where malaria is endemic [60, 61]. Although the mechanism of chloroquine resistance in the parasite is not well understood, it has been known for some time that resistant malaria accumulates less chloroquine than drug-sensitive malaria [62]. Furthermore, the reduced accumulation of chloroquine is energy dependent [63]. Given the latter characteristics of chloroquine-resistant malaria, which are similar to P-gp1-mediated MDR, it was suggested earlier that a P-glycoprotein homologue (*Pfmdr1*) could mediate chloroquine resistance in malaria [34–36]. However, the role of *Pfmdr1* in chloroquine resistance in *Plasmodium falciparum* remains controversial [37, 64–66]. Given the overlap in MRP and P-gp1 substrate specificity, we speculate about the possible role of an MRP homologue in chloroquine resistance in *falciparum* malaria. MRP homologues have been isolated from *Leishmania tarentolae* [67, 68], yeast [69], and *Caenorhabditis elegans* [70]. Moreover, these MRP homologues were shown to confer resistance to metal oxyanions and antimony [67, 68, 70].

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References

1. Endicott JA and Ling V, The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu Rev Biochem* **58:** 137–171, 1989.
2. Gottesman MM and Pastan I, Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62:** 385–427, 1993.
3. Shapiro AB and Ling V, Reconstitution of drug transport by purified P-glycoprotein. *J Biol Chem* **270:** 16167–16175, 1995.
4. Doige CA, Yu X and Sharom FJ, ATPase activity of partially purified P-glycoprotein from multidrug-resistant Chinese hamster ovary cells. *Biochim Biophys Acta* **1109:** 149–160, 1992.
5. van Helvoort A, Smitt JH, Sprong A, Fritzsche I, Schinkel AH, Borst P and Meer G-V, MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. *Cell* **87:** 507–517, 1996.
6. Metherall JE, Li H and Waugh K, Role of multidrug resistance P-glycoprotein in cholesterol biosynthesis. *J Biol Chem* **271:** 2634–2640, 1996.
7. Borst P, Schinkel AH, Smit JJM, Wagenaar E, Vandeemte RL, Smith AJ, Eijdems EWHM, Baas F and Zaman JGR, Classical and novel forms of multidrug-resistance and the physiological functions of P-glycoproteins in mammals. *Pharmacol Ther* **60:** 289–299, 1993.
8. Schinkel AH, Smit JJM, van Tellingen O, Beijnen JH, Wagenaar E, van Deemter L, Mol CAAM, van der Valk MA, Robanus-Maandag EC, te Riele HPJ, Berns AJM and Borst P, Disruption of the mouse *mdr1a* P-glycoprotein gene leads to a deficiency in the blood–brain barrier and to increased sensitivity to drugs. *Cell* **77:** 491–502, 1994.
9. Schinkel AH, Mayer U, Wagenaar E, Mol CAAM, van Deemter L, Smit JJM, van der Valk MA, Voordouw AC, Spits H, van Tellingen O, Zijlmans JMJM, Fibbe WE and Borst P, Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. *Proc Natl Acad Sci USA* **94:** 4028–4033, 1997.
10. Cole SPC, Bharswaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AMV and Deeley RG, Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* **258:** 1650–1654, 1992.
11. Cole SP and Deeley RG, Multidrug resistance associated with overexpression of MRP. *Cancer Treat Res* **87:** 39–62, 1996.
12. Hyde SC, Emsley P, Hartshorn MJ, Mimmack MM, Gileadi U, Pearce SR, Gallagher MP, Gill DR, Hubbard RE and Higgins CF, Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature* **346:** 362–365, 1990.
13. Higgins CF, ABC transporters: From microorganisms to man. *Annu Rev Cell Biol* **8:** 67–113, 1992.
14. Zaman GJR, Flens MJ, Vanleusden MR, Dehaas M, Mulder HS, Lankelma J, Pinedo HM, Scheper RJ, Baas F, Broxterman HJ and Borst P, The human multidrug resistance-associated protein MRP is a plasma-membrane drug-efflux pump. *Proc Natl Acad Sci USA* **91:** 8822–8826, 1994.
15. Gros P, Neriah YB, Croop JM and Housman DE, Isolation and expression of a complementary DNA that confers multidrug resistance. *Nature* **323:** 728–731, 1986.
16. Cole SP, Sparks KE, Fraser K, Loe DW, Grant CE, Wilson GM and Deeley RG, Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. *Cancer Res* **54:** 5902–5910, 1994.
17. Ueda K, Cardarelli C, Gottesman MM and Pastan I, Expression of a full-length cDNA for the human "*MDR1*" gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proc Natl Acad Sci USA* **84:** 3004–3008, 1987.
18. Nielsen D, Maare C and Skovsgaard T, Kinetics of daunorubicin transport in Ehrlich ascites tumor cells with different expression of P-glycoprotein. *Biochem Pharmacol* **47:** 2125–2135, 1994.
19. Borst P, Kool M and Evers R, Do cMOATS (MRP2), other MRP homologues, and LRP play a role in MDR? *Semin Cancer Biol* **8:** 205–213, 1997.
20. Zaman GJR, Versantvoort CHM, Smit JJM, Eijdems EWHM, de Haas M, Smith AJ, Broxterman HJ, Mulder NH, de Vries EGE, Baas F and Borst P, Analysis of the expression of MRP, the gene for a new putative transmembrane drug transporter, in human multidrug resistant lung cancer cell lines. *Cancer Res* **53:** 1747–1750, 1993.
21. Loe DW, Almquist KC, Deeley RG and Cole SPC, Multidrug resistance protein (MRP)-mediated transport of leukotriene C_4 and chemotherapeutic agents in membrane vesicles. Demonstration of glutathione-dependent vincristine transport. *J Biol Chem* **271:** 9675–9682, 1996.
22. Loe DW, Almquist KC, Cole SPC and Deeley RG, ATP-dependent 17β-estradiol 17-(β-D-glucuronide) transport by multidrug resistance protein (MRP). Inhibition by cholestatic steroids. *J Biol Chem* **271:** 9683–9689, 1996.
23. Rappa G, Lorico A, Flavell RA and Sartorelli AC, Evidence that multidrug resistance protein (MRP) functions as a co-transporter of glutathione and natural product toxins. *Cancer Res* **57:** 5232–5237, 1997.
24. Lorico A, Rappa G, Finch RA, Yang D, Flavell RA and Sartorelli AC, Disruption of the murine *MRP* (multidrug resistance protein) gene leads to increased sensitivity to etoposide (VP-16) and increased levels of glutathione. *Cancer Res* **57:** 5238–5242, 1997.
25. Jedlitschky G, Leier I, Buchholz U, Barnouin K, Kurz G and Keppler D, Transport of glutathione, glucuronate, and sulfate conjugates by the *MRP* gene-encoded conjugate export pump. *Cancer Res* **56:** 988–994, 1996.
26. Leier I, Jedlitschky G, Buchholz U, Center M, Cole SPC, Deeley RG and Keppler D, ATP-dependent glutathione disulphide transport mediated by the *MRP* gene-encoded conjugate export pump. *Biochem J* **314:** 433–437, 1996.
27. Muller M, Meijer C, Zaman GJ, Borst P, Scheper RJ, Mulder NH, de Vries EGE and Jansen PLM, Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione *S*-conjugate transport. *Proc Natl Acad Sci USA* **91:** 13033–13037, 1994.
28. Leier I, Jedlitschky G, Buchholz U, Cole SPC, Deeley RG and Keppler D, The *MRP* gene encodes an ATP-dependent export pump for leukotriene C_4 and structurally related conjugates. *J Biol Chem* **269:** 27807–27810, 1994.
29. Vezmar M, Deady LW, Tilley L and Georges E, The quinoline-based drug, *N*-{4-[1-hydroxy-2-(dibutylamino)ethyl] quinolin-8-yl}-4-azidosalicylamide, photoaffinity labels the multidrug resistance protein (MRP) at a biologically relevant site. *Biochem Biophys Res Commun* **241:** 104–111, 1997.
30. Jones TR, Zamboni R, Belley M, Champion E, Charette L, Ford-Hutchinson AW, Frenette R, Gauthier J-Y, Leger S, Masson P, McFarlane CS, Piechuta H, Rokach J, Williams H,

Young RN, DeHaven RN and Pong SS, Pharmacology of L-660,711 (MK-571): A novel potent and selective leukotriene D_4 receptor antagonist. *Can J Physiol Pharmacol* **67:** 17–28, 1989.
31. Cole SPC, Downes HF and Slovak ML, Effect of calcium antagonists on the chemosensitivity of two multidrug-resistant human tumour cell lines which do not overexpress P-glycoprotein. *Br J Cancer* **59:** 42–46, 1989.
32. Beck WT, Cirtain MC, Danks MK, Felsted RL, Safa AR, Wolverton JS, Suttle DP and Trent JM, Pharmacological, molecular, and cytogenetic analysis of "atypical" multidrug-resistant human leukemic cells. *Cancer Res* **47:** 5455–5460, 1987.
33. Foote SJ, Thompson JK, Cowman AF and Kemp DJ, Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P. falciparum*. *Cell* **57:** 921–930, 1989.
34. Ruetz S, Delling U, Brault M, Schurr E and Gros P, The *pfmdr1* gene of *Plasmodium falciparum* confers cellular resistance to antimalarial drugs in yeast cells. *Proc Natl Acad Sci USA* **93:** 9942–9947, 1996.
35. van Es HHG, Karcz S, Chu F, Cowman AF, Vidal S, Gros P and Schurr E, Expression of the plasmodial *pfmdr1* gene in mammalian cells is associated with increased susceptibility to chloroquine. *Mol Cell Biol* **14:** 2419–2428, 1994.
36. Cowman AF, Galatis D and Thompson JK, Selection for mefloquine resistance in *Plasmodium falciparum* is linked to amplification of the *pfmdr1* gene and cross-resistance to halofantrine and quinine. *Proc Natl Acad Sci USA* **91:** 1143–1147, 1994.
37. Wellems TE, Walker-Jonah A and Panton LJ, Genetic mapping of the chloroquine-resistance locus on *Plasmodium falciparum* chromosome 7. *Proc Natl Acad Sci USA* **88:** 3382–3386, 1991.
38. Awad-el-Kariem FM, Miles MA and Warhurst DC, Chloroquine-resistant *plasmodium falciparum* isolates from the Sudan lack two mutations in the *pfmdr1* gene thought to be associated with chloroquine resistance. *Trans R Soc Trop Med Hyg* **86:** 587–589, 1992.
39. Basco LK, de Pecoulas PE, Le Bras J and Wilson CM, *Plasmodium falciparum*: Molecular characterization of multidrug-resistant Cambodian isolates. *Exp Parasitol* **82:** 97–103, 1996.
40. Lin PH, Selinfreund R, Wakshull E and Wharton W, Rapid and efficient purification of plasma membrane from cultured cells: Characterization of epidermal growth factor binding. *Biochemistry* **26:** 731–736, 1987.
41. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
42. Nare B, Prichard RK and Georges E, Characterization of rhodamine 123 binding to P-glycoprotein in human multidrug-resistant cells. *Mol Pharmacol* **45:** 1145–1152, 1994.
43. Fairbanks G, Steck TL and Wallach DFH, Electrophoretic analysis of major polypeptides of the human erythrocyte membrane. *Biochemistry* **10:** 2606–2617, 1971.
44. Towbin H, Staehelin T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* **76:** 4350–4354, 1979.
45. Almquist KC, Loe DW, Hipfner DR, Mackie JE, Cole SPC and Deeley RG, Characterization of the M_r 190,000 multidrug resistance protein (MRP) in drug-selected and transfected human tumor cells. *Cancer Res* **55:** 102–110, 1995.
46. Marquardt D, McCrone S and Center MS, Mechanisms of multidrug resistance in HL60 cells: Detection of resistance-associated proteins with antibodies against synthetic peptides that correspond to the deduced sequence of P-glycoprotein. *Cancer Res* **50:** 1426–1430, 1990.
47. Nare B, Liu Z, Prichard RK and Georges E, Benzimidazoles, potent anti-mitotic drugs: Substrates for the P-glycoprotein transporter in multidrug-resistant cells. *Biochem Pharmacol* **48:** 2215–2222, 1994.
48. Loe DW, Stewart RK, Massey TE, Deeley RG and Cole SPC, ATP-dependent transport of aflatoxin B_1 and its glutathione conjugates by the product of the multidrug resistance protein (*MRP*) gene. *Mol Pharmacol* **51:** 1034–1041, 1997.
49. Asakura T, Takahashi N, Takada K, Inoue T and Ohkawa K, Drug conjugate of doxorubicin with glutathione is a potent reverser of multidrug resistance in rat hepatoma cells. *Anticancer Drugs* **8:** 199–203, 1997.
50. Zamora JM, Pearce HL and Beck WT, Physical–chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. *Mol Pharmacol* **33:** 454–462, 1988.
51. Gekeler V, Ise W, Sanders KH, Ulrich WR and Beck J, The leukotriene LTD_4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. *Biochem Biophys Res Commun* **208:** 345–352, 1995.
52. Feller N, Kuiper CM, Lankelma J, Ruhdal JK, Scheper RJ, Pinedo HM and Broxterman HJ, Functional detection of MDR1/P170 and MRP/P190-mediated multidrug resistance in tumour cells by flow cytometry. *Br J Cancer* **72:** 543–549, 1995.
53. Lautier D, Canitrot Y, Deeley RG and Cole SPC, Multidrug resistance mediated by the multidrug resistance protein (*MRP*) gene. *Biochem Pharmacol* **52:** 967–977, 1996.
54. Marsh W, Sicheri D and Center MS, Isolation and characterization of adriamycin-resistant HL-60 cells which are not defective in the initial intracellular accumulation of drug. *Cancer Res* **46:** 4053–4057, 1986.
55. Schneider E, Horton JK, Yang CH, Nakagawa M and Cowan KH, Multidrug resistance-associated protein gene overexpression and reduced drug sensitivity of topoisomerase II in a human breast carcinoma MCF7 cell line selected for etoposide resistance. *Cancer Res* **54:** 152–158, 1994.
56. Cole SP, Chanda ER, Dicke FP, Gerlach JH and Mirski SE, Non-P-glycoprotein-mediated multidrug resistance in a small cell lung cancer cell line: Evidence for decreased susceptibility to drug-induced DNA damage and reduced levels of topoisomerase II. *Cancer Res* **51:** 3345–3352, 1991.
57. Mirski SEL and Cole SPC, Multidrug resistance-associated antigens on drug-sensitive and -resistant human tumour cell lines. *Br J Cancer* **64:** 15–22, 1991.
58. Marquardt D and Center MS, Involvement of vacuolar $H(^+)$-adenosine triphosphatase activity in multidrug resistance in HL60 cells. *J Natl Cancer Inst* **83:** 1098–1102, 1991.
59. Wang Y, Pan X-Q, Lheureux F and Georges E, Overexpression of 40-kDa protein in human multidrug resistant cells. *Biochem Biophys Res Commun* **236:** 483–488, 1997.
60. Cowman AF, Mechanisms of drug resistance in malaria. *Aust N Z J Med* **25:** 837–844, 1995.
61. Foley M and Tilley L, Quinoline antimalarials: Mechanisms of action and resistance. *Int J Parasitol* **27:** 231–240, 1997.
62. Yayon A, Cabantchik ZI and Ginsburg H, Susceptibility of human malaria parasites to chloroquine is pH dependent. *Proc Natl Acad Sci USA* **82:** 2784–2788, 1985.
63. Krogstad DJ, Gluzman IY, Herwaldt BL, Schlesinger PH and Wellems TE, Energy dependence of chloroquine accumulation and chloroquine efflux in *Plasmodium falciparum*. *Biochem Pharmacol* **43:** 57–62, 1992.
64. Wellems TE, Panton LJ, Gluzman IY, do Rosario VE, Gwadz RW, Walker-Jonah A and Krogstad DJ, Chloroquine resis-

tance not linked to *mdr*-like genes in a *Plasmodium falciparum* cross. *Nature* **345:** 253–255, 1990.
65. Lim AS and Cowman AF, *Plasmodium falciparum*: Chloroquine selection of a cloned line and DNA rearrangements. *Exp Parasitol* **83:** 283–294, 1996.
66. Lim ASY, Galatis D and Cowman AF, *Plasmodium falciparum*: Amplification and overexpression of *pfmdr1* is not necessary for increased mefloquine resistance. *Exp Parasitol* **83:** 295–303, 1996.
67. Papadopoulou B, Roy G, Dey S, Rosen BP and Ouellette M, Contribution of the *Leishmania* P-glycoprotein-related gene *1tpgpA* to oxyanion resistance. *J Biol Chem* **269:** 11980–11986, 1994.
68. Callahan HL and Beverley SM, Heavy metal resistance: A new role of P-glycoproteins in *Leishmania*. *J Biol Chem* **266:** 18427–18430, 1991.
69. Szczypka MS, Wemmie JA, Moye-Rowley WS and Thiele DJ, A yeast metal resistance protein similar to human cystic fibrosis transmembrane conductance regulator (CFTR) and multidrug resistance-associated protein. *J Biol Chem* **269:** 22853–22857, 1994.
70. Broeks A, Gerrard B, Allikmets R, Dean M and Plasterk RHA, Homologues of the human multidrug resistance genes *MRP* and *MDR* contribute to heavy metal resistance in the soil nematode *Caenorhabditis elegans*. *EMBO J* **15:** 6132–6143, 1996.