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SHORT COMMUNICATION

Reversal of MRP-Mediated Doxorubicin Resistance with Quinoline-Based Drugs

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ABSTRACT. The overexpression of P-glycoprotein (P-gp) and the multidrug resistance-associated protein (MRP) have been shown to confer broad drug resistance in tumor cells. We have demonstrated previously direct binding between MRP and a quinoline-based photoreactive drug (iodo-azido-amino quinoline, IAAQ) (Vezmar *et al.*, *Biochem Biophys Res Commun* 241: 104–111, 1997). In this report, we show the reversal of multidrug resistance in two MRP-overexpressing cell lines, HL60/AR and H69/AR, with four quinoline-based drugs. Non-toxic concentrations (5–20 μM) of chloroquine, quinine, quinidine, and primaquine potentiated the toxicity of doxorubicin in a concentration-dependent manner. These quinoline-based drugs showed a 5- to 10-fold decrease in the IC_{50} of doxorubicin in H69/AR and HL60/AR cells. Primaquine was the most active, with modulation ratios of 10- and 5-fold versus 8- and 3-fold with MK-571 for H69/AR and HL60/AR, respectively. Moreover, using IAAQ, we showed that molar excesses of chloroquine, quinine, quinidine, and MK-571 inhibit the photoaffinity labeling of MRP. Primaquine and vinblastine showed lesser inhibition of MRP photoaffinity labeling by IAAQ. Taken together, the results of this study demonstrated the reversal of doxorubicin resistance with several quinoline-based drugs. Moreover, these drugs have been shown to reverse P-gp-mediated MDR and are clinically well tolerated. BIOCHEM PHARMACOL **59**;10:1245–1252, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. multidrug resistance; chloroquine; multidrug resistance-associated protein (MRP); photoaffinity labeling; quinoline; MDR reversal

Chemotherapeutic drug treatment of cancer patients often leads to the emergence of drug-resistant tumor cells. Similarly, tumor cell lines selected *in vitro* with sublethal concentrations of anticancer drugs acquire a MDR† phenotype and overexpress P-gp (or P-gp1) (for reviews, see Refs. 1 and 2). P-gp1 is an ATP-dependent drug efflux pump [3, 4] and is thought to function as a "flippase" of short-chain lipids, in addition to mediating the transport of normal cell metabolites and xenobiotics [5–7]. The latter function of P-gp1 is supported by experimental evidence whereby disruption of the *P-gp1* gene in mice led to an increase in drug accumulation in normal tissues [8, 9]. Recently, a second membrane transporter, MRP1, was identified in *in vitro* selected H69 small-cell lung carcinoma (SCLC) cells that displayed a MDR phenotype without P-gp1 [10]. Both P-gp1 and MRP1 are members of a large family of ABC (ATP Binding Cassette) trafficking proteins that couple ATP hydrolysis to ligand transport across a lipid membrane [11, 12]. Gene transfer studies of P-gp1 or MRP1, in previously drug-sensitive cells, have been shown to confer largely similar drug resistance profiles [13–16]. MRP1 has been shown to function as a co-transporter of glutathione and natural-product toxins [17]. Furthermore, disruption of the *MRP1* gene in mice leads to increased sensitivity to natural-product toxins and elevated GSH levels in MRP-expressing tissues and organs [18]. The normal function of MRP1 is not certain at present; however, it has been shown to transport GSH-conjugated compounds and other normal cell metabolites, including LTC_4, GSSG, and 17β-estradiol 17-(β-d-glucuronide) [19–22].

P-gp1 expression has been shown in tumors from different cancers. Moreover, in some cancers, the overexpression of P-gp1 correlates with clinical drug resistance or poor prognosis [23–26]. Clinical trials using cyclosporin A and the non-immunosuppressive analog SDZ-PSC 833, which inhibits P-gp-mediated MDR, have been encouraging. However, the use of MDR-reversing drugs has not been effective against tumors that express P-gp from relapsed lymphoma, leukemia, myeloma, or retinoblastoma [25, 27–29]. These findings have led to the speculation that MRP may be responsible for clinical resistance in P-gp-negative tumors. The latter speculation is supported by recent findings whereby the expression of MRP before treatment was correlated with the outcome of treatment in neuroblastoma [30, 31]. Furthermore, a correlation between deletion of the *MRP1* gene and improved outcome of myelomonocytic leukemia has been demonstrated [30].

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† *Abbreviations*: MDR, multidrug resistance/resistant; MRP, multidrug resistance-associated protein; P-gp, P-glycoprotein; IAAQ, iodo-azido-amino quinoline; and LTC_4, cysteinyl leukotriene, leukotriene C.

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The initial finding that buthionine sulfoximine, an inhibitor of GSH synthesis, reverses MRP-mediated MDR in some tumor cell lines was followed by the identification of other drugs that also affect GSH levels and reverse MRP-mediated MDR in tumor cells [32, 33]. Moreover, several reports have demonstrated that verapamil can reverse both P-gp- and MRP-mediated MDR [34, 35]. However, the latter findings have been controversial [36–38]. More recently, structurally diverse compounds have been shown to reverse MRP-mediated MDR in tumor cell lines. These compounds include the LTD_4 receptor inhibitor MK-571 [39], the pyridine analog PAK-104P [40], the dihydropyridine derivative NIK250 [41], imizothiazole derivatives (for example, N276–12; [42]), and the pipecolinate derivative VX-710 [43]. However, with the exception of the pipecolinate derivative VX-710 [43], it is not known if the above MRP-mediated MDR-reversing drugs interact with MRP. Using a photoactive quinoline-based drug (IAAQ), we recently provided evidence for a direct and specific binding of unmodified drug to MRP in H69/AR SCLC cells [44]. In the present study, it was of interest to examine the effects of several quinoline-based drugs on MRP-mediated drug resistance. The results showed that quinoline-based drugs reverse MRP-mediated doxorubicin resistance by interacting directly with MRP.

MATERIALS AND METHODS

Materials

Iodine-125 (100.7 mCi/mL) was purchased from Amersham Biochemical Inc. 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 gifts 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 as described by Lin *et al.* [45]. Membrane fractions were stored at −80° if not used immediately. Protein concentrations were determined by the method of Lowry *et al.* [46].

Cytotoxicity Assays

Cells were harvested and seeded in 96-well plates at 0.5 to 1.0×10^4 cells/well. Following a 24-hr recovery period, cells were grown in increasing concentrations of doxorubicin in the absence or presence of non-toxic concentrations of chloroquine, quinine, quinidine, and primaquine. Cells were allowed to grow for 4 days at 37° before the addition of the MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. The plates were processed as previously described [47].

Photoaffinity Labeling and SDS–PAGE

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, MK-571, quinine, primaquine, and quinidine before the addition of 0.25 μM IAAQ [44]. Then cells were incubated at room temperature in the dark for 30 min and transferred to ice for 10 min. Following the incubation on ice, cells were irradiated for 10 min on ice with a UV source at 254 nm (Stratagene UV crosslinker, Stratagene). Cells were centrifuged for 5 min at 2500 rpm in an Eppendorf microfuge. The supernatant containing the radiolabel was removed, and cells were lysed in 20 μL of 50 mM Tris (pH 7.4) containing 1% Nonidet P-40, 5 mM $MgCl_2$, and protease inhibitors (3 μg/mL of leupeptin and 2 mM phenylmethylsulfonyl fluoride). IAAQ-photolabeled proteins were isolated by brief centrifugation and processed for SDS–PAGE. Samples containing IAAQ-labeled proteins were mixed with buffer I [10 mM Tris–HCl, pH 8.0, containing 2% SDS, 50 mM dithiothreitol, 1 mM EDTA] at a ratio of 1:5 (v/v) and then with an equal volume of buffer II [2x buffer I and 9 M urea]. The solubilized proteins then were resolved by SDS–PAGE using the Fairbanks gel system with some modification [48]. Gel slabs containing the resolved proteins were fixed in 50% methanol, dried, and exposed to Kodak XAR film at −70°.

RESULTS AND DISCUSSION

Reversal of MRP-Mediated MDR with Quinoline-Based Drugs

Gene transfer studies have now confirmed the role of MRP in MDR *in vitro* [13, 15]. Furthermore, there is increasing evidence for the role of MRP in drug-resistant cancers [25, 27–29]. However, as MRP-mediated MDR is not reversed effectively by P-gp inhibitors, there is a need to identify drugs that can reverse MRP-mediated MDR [36, 37]. We have demonstrated previously direct interaction between MRP and a quinoline-based photoreactive drug (IAAQ) [44]. The photoaffinity labeling of MRP with IAAQ was inhibited by other quinoline-based drugs such as MK-571 and chloroquine, in addition to other physiologically relevant ligands such as LTC_4. In this report, we showed the ability of several drugs that contain the quinoline moiety (Fig. 1) to reverse MRP-mediated resistance to doxorubicin in two *in vitro* selected MDR cell lines (H69/AR, Fig. 2A, and HL60/AR, Fig. 2B). Figure 2 shows the ability of chloroquine, quinine, quinidine, MK-571, and primaquine (at 5–20 μM) to reverse the resistance of H69/AR and HL60/AR to doxorubicin in a concentration-dependent

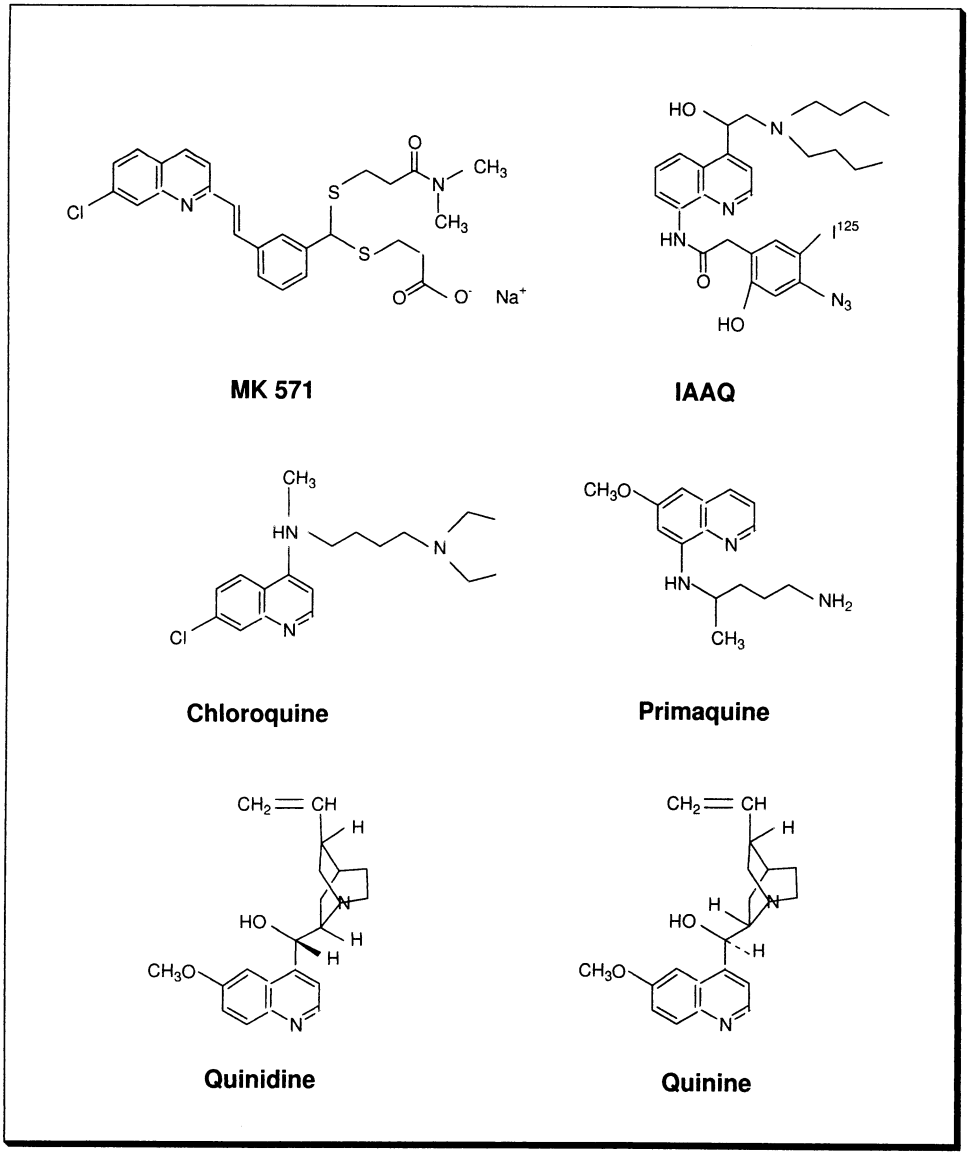

FIG. 1. Organic structures of quinoline-based drugs: MK-571, IAAQ, chloroquine, primaquine, quinidine, and quinine.

manner. Similar concentrations of the latter drugs did not potentiate doxorubicin toxicity significantly in drug-sensitive cells (HL60 and H69) (data not shown). Estimates of the IC_{50} values for H69/AR and HL60/AR for doxorubicin in the presence of the quinoline-based drugs showed primaquine to be the most effective in potentiating the toxicity of doxorubicin in both MRP-overexpressing cell lines (Table 1). Earlier studies have shown MK-571 to reverse MRP-mediated drug resistance in several MRP-expressing cell lines [49–51]. The results in Table 1 also showed chloroquine and quinidine to be less potent than MK-571 [39] and quinine, with a modulation ratio of 5-fold versus 7-fold in H69/AR cells, respectively. Primaquine was a better reversing drug than MK-571 and the other quinoline-based drugs, with a modulation ratio of 10-fold in H69/AR cells (Table 1). A similar trend was also observed in HL60/AR cells, with primaquine being the most effective in potentiating doxorubicin toxicity. However, the modulation ratios of drug resistance reversal in HL60/AR cells were less than those seen with H69/AR cells (Table 1). Moreover, unlike the situation in H69/AR cells, quinidine was a better reversing drug than MK-571 in HL60/AR cells, whereas chloroquine and quinine were as effective as MK-571.

The incomplete reversal of doxorubicin resistance in H69/AR and HL60/AR cells with the quinoline-based drugs mentioned above is likely due to other mechanisms of resistance that are not inhibited by these drugs. Similar results were observed with other MRP-expressing cell lines and the MRP-reversing drug VX-710 [43]. Although it is not entirely clear why the quinoline-based drugs and VX-710 do not completely reverse doxorubicin resistance,

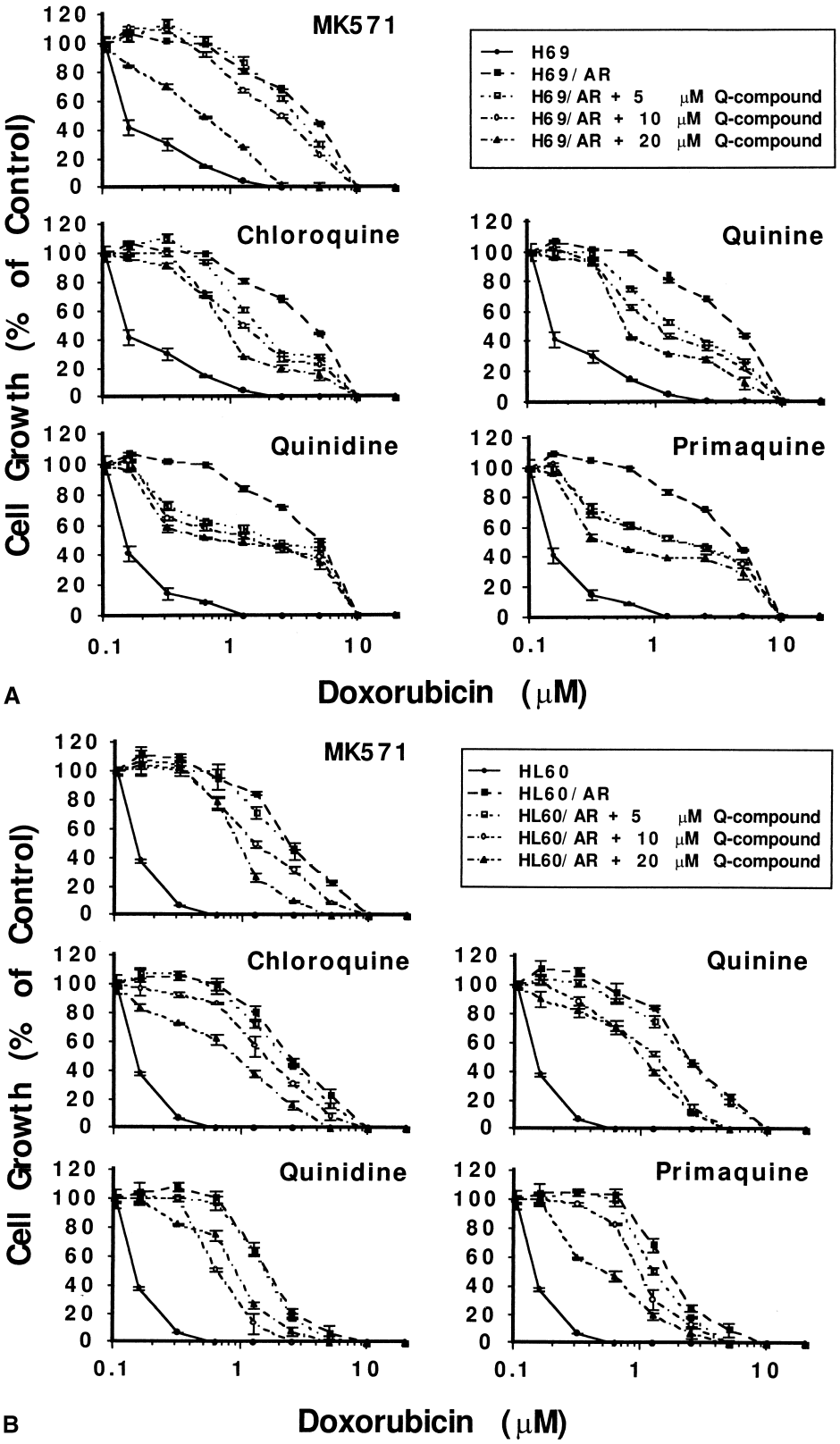

FIG. 2. Reversal of doxorubicin resistance by quinoline-based drugs. Drug-sensitive (H69 and HL60) and -resistant (H69/AR and HL60/AR) cells were seeded into 96-well plates (at 0.5 to 1.0 × 10^4 cells/well) and incubated with increasing concentrations of doxorubicin in the absence and presence of 5–20 μM concentrations of quinoline-based drugs (MK-571, chloroquine, quinine, primaquine, and quinidine). Panels A and B show the effects of quinoline-based drugs on the viability of H69/AR and HL60/AR cells, respectively. The viability of cells was estimated by measuring the absorbance at 450 nm. Each point is the mean (± SD) of three independent experiments.

TABLE 1. Modulation of doxorubicin resistance of H69/AR and HL60/AR cells with quinoline-based drugs

Reversing agents		IC_{50}* (μM)	Modulation ratio†
H69/AR		4.25	
Doxorubicin alone			
+ Chloroquine	(5.0 μM)	1.63	2.60
	(10 μM)	1.21	3.50
	(20 μM)	0.87	4.90
+ Quinine	(5.0 μM)	1.50	2.83
	(10 μM)	0.93	4.56
	(20 μM)	0.60	7.08
+ MK-571	(5.0 μM)	3.00	1.41
	(10 μM)	2.40	1.77
	(20 μM)	0.57	7.50
+ Primaquine	(5.0 μM)	1.97	2.15
	(10 μM)	1.79	2.37
	(20 μM)	0.42	10.11
+ Quinidine	(5.0 μM)	1.97	2.15
	(10 μM)	1.54	2.76
	(20 μM)	0.81	5.25
HL60/AR		2.35	
Doxorubicin alone			
+ Chloroquine	(5.0 μM)	2.00	1.18
	(10 μM)	1.48	1.60
	(20 μM)	0.85	2.80
+ Quinine	(5.0 μM)	2.20	1.07
	(10 μM)	1.31	1.80
	(20 μM)	0.97	2.42
+ MK-571	(5.0 μM)	2.06	1.14
	(10 μM)	1.21	1.94
	(20 μM)	0.87	2.70
+ Primaquine	(5.0 μM)	1.25	1.88
	(10 μM)	0.95	2.47
	(20 μM)	0.52	4.52
+ Quindine	(5.0 μM)	1.55	1.51
	(10 μM)	0.89	2.64
	(20 μM)	0.64	3.70

*An IC_{50} drug concentration was obtained from the graphs and represents 50% inhibition of MTT dye formation. Each value is the mean of at least three determinations.

†The modulation ratio was calculated from the IC_{50} for drug alone (Dox) versus the IC_{50} in the presence of the modulating agents.

it is likely that changes other than MRP or P-gp in these *in vitro* selected cells mediate the resistance to doxorubicin. For example, changes in topoisomerase II have been shown to occur following selection of cells with DNA-damaging drugs such as doxorubicin [52–56]; H69/AR cells have been shown previously to contain reduced levels of topoisomerase II [57]. In addition, we recently demonstrated the overexpression of a 40-kDa protein in H69/AR and HL60/AR that was not detected in their parental cells [58]. Alternatively, more potent MRP-reversing drugs may be required to observe full drug reversal. Taken together, these results show the reversal of doxorubicin resistance by several quinoline-based drugs. The ability of these compounds to reverse MRP-mediated MDR is interesting, since some of these drugs have been shown previously to reverse P-gp-mediated MDR [59, 60] and are well tolerated in the clinic for the treatment of other diseases, such as malaria [61].

Effects of Quinoline-Based Drugs on MRP Binding to IAAQ

The mechanism of reversal of MRP-mediated MDR is not well understood. Several studies have shown MRP to mediate the transport of glutathione-conjugated compounds and other normal cell metabolites that include LTC_4, GSSG, and 17β-estradiol 17-(β-D-glucuronide) [19–22]. Direct binding between LTC_4 and MRP has been shown in intact cells and membrane vesicles from MRP-overexpressing cells [22, 62]. Furthermore, LTC_4 binding and transport by MRP are inhibitable by excess MK-571, an antagonist of the LTD_4 receptor [21]. Given the above findings in this study, it was of interest to know if these quinoline-based drugs reverse MRP-mediated MDR by interacting directly with MRP. Figure 3 shows the photoaffinity labeling of plasma membranes from H69 and H69/AR cells with IAAQ in the absence and presence of a molar excess (300- to 1000-fold) of chloroquine, vinblastine, quinidine, primaquine, quinine, and MK-571. The results in Fig. 3 show that molar excesses of MK-571, chloroquine, quinine, and quinidine inhibited MRP photolabeling with IAAQ in a concentration-dependent manner. However, a molar excess of vinblastine or primaquine showed a lesser decrease in MRP photoaffinity labeling. The ability of chloroquine, quinine, MK-571, and quinidine to reverse MRP-mediated MDR and inhibit its photoaffinity labeling with IAAQ suggests a mechanism of reversal similar to that seen for P-gp [63]. However, unlike P-gp, MRP-mediated drug transport is thought to require GSH [20, 22]. To determine if drug binding to MRP is modulated by GSH, plasma membranes from H69/AR cells were photoaffinity labeled with IAAQ in the presence of increasing concentrations of GSH. Our results showed no change in MRP photolabeling in the presence of 0.05 to 1 mM GSH (results not shown). Thus, the presence or absence of GSH does not appear to modulate MRP photoaffinity labeling with IAAQ.

The modest inhibition of MRP photolabeling by IAAQ in the presence of primaquine is surprising given the ability of this drug to potentiate the toxicity of doxorubicin in H69/AR and HL60/AR cells (Table 1). A possible explanation for the observed results may be that primaquine interacts with a different binding domain in MRP. This possibility is supported by our unpublished results using a photoactive analogue of Rhodamine 123, which photolabels MRP specifically but interacts with a different site than IAAQ.* Alternatively, primaquine may reverse MRP-mediated MDR through another mechanism in addition to direct binding to MRP, for example through oxidative stress. Indeed, primaquine was one of the first agents recognized to produce oxidative stress on red blood cells [64–66]. This effect of primaquine was shown later to be due to its hydroxylated metabolites, which lead to the production of hydrogen peroxides and the subsequent

* Daoud R, Deady LW, Tilley L, Scheper RJ and Georges E, Manuscript submitted for publication.

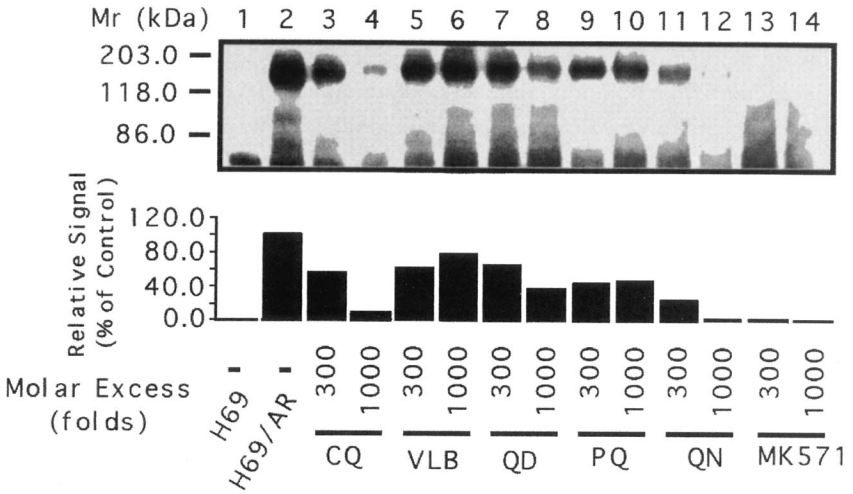

FIG. 3. Effect of quinoline drugs on the photoaffinity labeling of MRP with [^{125}I]IAAQ. H69 (lane 1) or H69/AR (lane 2) cells were photoaffinity labeled with 0.25 μM [^{125}I]IAAQ in the absence or presence of 300- and 1000-fold molar excess of chloroquine (CQ), vinblastine (VLB), quinidine (QD), primaquine (PQ), quinine (QN), and MK-571 (lanes 2–14, respectively).

depletion of normal anti-oxidants (NADPH or GSH) [67]. Interestingly, both H69/AR and HL60/AR cells were shown to contain lower levels of GSH than their parental cells (H69 and HL60) [33, 68–70]. Moreover, we and others have shown H69/AR and HL60/AR cells to be collaterally sensitive to buthionine sulfoximine ([70]; Lincoln M and Georges E, unpublished results). The latter findings are consistent with results from recent studies that show that MRP1 mediates low-affinity transport of GSH in an ATP-dependent manner [71]. Thus, given the role of MRP as a co-transporter of GSH and drugs [17], further decrease in GSH levels due to primaquine-induced oxidative stress may be responsible for the potency of primaquine in reversing MRP-mediated MDR (Table 1).

In conclusion, our results showed the reversal of MRP-mediated doxorubicin resistance by several quinoline drugs in MRP-overexpressing cells. Moreover, the mechanism of reversal appeared to be mediated through direct binding to MRP. Furthermore, these results were consistent with findings from a recent study that demonstrated the reversal of MRP-mediated MDR by a quinoline derivative, MS-209, through direct interaction with MRP [72]. However, unlike the MS-209 quinoline derivatives, the quinoline-based drugs described in this study are more potent reversing drugs. More importantly, all of the quinoline-based compounds described in this study are currently used in the treatment of other diseases and are well tolerated. Ongoing studies will determine their efficacy in reversing MRP-mediated MDR *in vivo* in animal model systems.

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