Reversal of Mefloquine and Quinine Resistance in *Plasmodium falciparum* with NP30

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Quinoline resistance in malaria is frequently compared with P-glycoprotein-mediated multidrug resistance (*mdr*) in mammalian cells. We have previously reported that nonylphenolethoxylates, such as NP30, are potential *Plasmodium falciparum* P-glycoprotein substrates and drug efflux inhibitors. We used in vitro assays to compare the ability of verapamil and NP30 to sensitize two parasite isolates to four quinolines: chloroquine (CQ), mefloquine (MF), quinine (QN), and quinidine (QD). NP30 was able to sensitize (reversal, >80%) *P. falciparum* to MF, QN, QD, and, to a lesser extent, CQ. The presence of 2 μ M verapamil had no effect on mefloquine resistance; however, the presence of verapamil modulated the activities of QN and QD in a manner parallel to that observed for CQ. Genetic analysis of putative quinoline resistance genes did not suggest an association between known point mutations in *pfcr* and *pfmdr1* and NP30 sensitization activity. We conclude that the sensitization action of NP30 is distinct both phenotypically and genotypically from that of verapamil.

The incidence of malaria is increasing, with an estimated 300 million to 500 million cases of malaria and 3 million deaths from malaria annually (30). Despite optimistic predictions of the eventual control and eradication of malaria made in the past, the spread of drug-resistant parasites, particularly those resistant to chloroquine (CQ), has led to a resurgence of the disease.

New antimalarials have been introduced in recent years (11, 22); however, they are significantly more expensive than CQ and pyrimethamine-sulfadoxine (Fansidar), the treatment options most commonly used in developing nations. The appearance of malaria resistant to CQ was first reported in the late 1950s in South America (33) and Southeast Asia (15) and has since spread to nearly all regions where malaria is endemic (18). The emergence of CQ-resistant *falciparum* malaria, particularly in Africa, has been a global health disaster (18) and is directly responsible for the increased number of deaths from *Plasmodium falciparum* infections (30).

The mechanism by which quinoline-based antimalarials inhibit the growth of blood-stage parasites remains controversial; however, they appear to interfere with the production of the malaria pigment hemozoin (for a review, see reference 28). Quinoline uptake into the food vacuole, where polymerization occurs, may be a passive process (27); and drug-resistant parasites appear to have developed or adapted mechanisms to remove quinolines from this environment (32). Quinoline resistance in *P. falciparum* is frequently compared to multidrug resistance in mammalian cells, a comparison that is supported by the observation that CQ resistance in *P. falciparum* can be reversed by known P-glycoprotein substrates such as verapamil (19), chlorpromazine (3), promethazine (21), chlorpheniramine (4), and citalopram (12). *P. falciparum* contains several

multidrug resistance-like proteins, including Pgh1, which is encoded by *pfmdr1* (14). Site-directed mutagenesis of Pgh1 has demonstrated that Pgh1 is associated with resistance to mefloquine (MF), quinine (QN), quinidine (QD), and halofantrine (26). On the other hand, mutations in Pgh1 were not directly responsible for CQ resistance. A gene on chromosome 7, *pfcr* (9), which encodes a vacuolar transport protein, has been linked to CQ resistance. Parasite transfection studies have demonstrated that mutations in *pfcr* are sufficient to confer CQ resistance in vitro (13). However, in clinical studies *pfcr* mutations appear to be necessary, but not sufficient, to predict CQ treatment outcomes in vivo (10, 24). Other host and, possibly, parasite factors contribute to parasite clearance following CQ therapy. These include host immune response (premunition) and a possible role for *pfmdr1* (26) and other gene products in mediating higher levels of CQ resistance. Furthermore, the observation that the reversal of CQ resistance by verapamil is not uniformly effective (31) is consistent with the hypothesis that multiple gene products modulate the responsiveness of malaria parasites to CQ.

NP30, an uncharged polyethoxylated nonylphenol surfactant containing an average of 30 ethoxylate units (Fig. 1), appears to belong to a novel class of resistance reversal agents since its structure does not contain a nitrogen atom and the surfactant has no ionizable groups (7, 8). We therefore wished to determine if NP30 had quinoline sensitization properties that were similar to or different from those of verapamil, the agent most frequently used to examine CQ resistance in vitro.

MATERIALS AND METHODS

***P. falciparum* cultures.** Human malaria parasites were grown in type A-positive blood obtained by venipuncture of volunteers. Cultures were maintained by the method of Trager and Jensen (29) by using RPMI 1640 supplemented with 10% human serum (either type A or AB [Wisent Inc., St. Bruno, Quebec, Canada]) and 50 μ M hypoxanthine (Gibco). Patient isolates were obtained from pretreatment blood samples from patients enrolled in ethically approved studies at the Tropical Disease Unit, University of Toronto (16, 34). The resistance

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FIG. 1. NP30. NP30 consists of a nonylphenol group onto which an average of 30 ethoxylate residues have been polymerized.

genotypes (point mutations in *pfprt* and *pfmdr1*) of these isolates were determined as described previously (1, 2, 6, 23, 24).

In vitro drug susceptibility testing. In vitro drug susceptibility testing was performed by using a lactate dehydrogenase assay modified to be specific for the presence of *P. falciparum* lactate dehydrogenase (5, 17, 25). The concentration of drug that resulted in a 50% inhibition of viability of the parasite cultures (IC_{50}) was determined by a nonlinear regression analysis using the computer program Sigma Plot 2000 (Jandel Scientific). The IC_{50} s represent the means of four determinations.

NP30. Samples of NP30 were the kind gift of Union Carbide and were made up as 1% (wt/vol) stock solutions in water after extensive drying by lyophilization to obtain accurate weight measurements. Checkerboard assays (two-dimensional arrays of the surfactant concentration versus the quinoline concentration) were initially undertaken to determine the IC_{50} s of NP30 and the quinolines alone and in combination. A single concentration of NP30 (0.003% [wt/vol]; 21 μ M) was used in assays for comparison of the activity of NP30 with that of verapamil on the basis of the fact that this concentration of NP30 offered a favorable degree of sensitization with minimal direct antiparasitic effect.

RESULTS

We have previously determined that NP30 both has anti-*P. falciparum* effects and can reverse CQ resistance in some isolates (8). To test the hypothesis that NP30 and verapamil interacted with a common site, we determined whether the presence of 2 μ M verapamil enhanced the anti-*P. falciparum* effects of NP30. The presence of verapamil did not modify the anti-*P. falciparum* activity of NP30 (Fig. 2), suggesting that verapamil and NP30 have distinct sites of action. Examination of the capacity of verapamil to sensitize two *P. falciparum* isolates, designated isolate 1 (which originated in West Africa) and isolate 2 (which originated in the Indian subcontinent), to CQ indicated that a high degree of sensitization could be obtained in isolate 1 but that only a limited degree of sensi-

FIG. 2. Effect of verapamil on the anti-*Plasmodium* action of NP30. The IC_{50} of NP30 for *Plasmodium* cultures was determined in the absence (closed bars) and presence (open bars) of 2 μ M verapamil. The values represent the means $\pm$ standard errors of the means of three independent assays.

TABLE 1. Genotype sequences of putative drug resistance genes in *P. falciparum* patient isolates^a

Parasite isolate	Mutation				
	<i>pfprt</i> (K76T)	<i>pfmdr</i> (N86Y)	<i>pfmdr</i> (C1034S)	<i>pfmdr</i> (D1042N)	<i>pfmdr</i> (Y1246D)
1	T	Y	S	N	D
2	T	Y	S	N	D

^a The mutation at position 86 (N, wild type; Y, mutant) within the P-glycoprotein efflux pump and putative resistance gene, *pfmdr1*, has been associated with CQ resistance. The presence or absence of this mutation was determined by PCR and restriction fragment length polymorphism analysis. Additional mutations within *pfmdr1*, C1034S (C, wild type; S, mutant), D1042N (D, wild type; N, mutant), and Y1246D (Y, wild type; D, mutant), have been associated with MF resistance (26). The presence or absence of these mutations was determined by PCR and sequencing. The mutation at position 76 (K, wild type; T, mutant) within the putative resistance gene *pfprt* has been associated with CQ resistance.

zation could be obtained in isolate 2 (Fig. 3). When 21 μ M NP30 was present, the IC_{50} of CQ for isolate 1 was nearly identical to that for the untreated sample; however, the IC_{50} of CQ for isolate 2 was reduced to a value less than that for the verapamil-treated sample. This finding suggests that fully verapamil-sensitive CQ resistance is independent of the site of action of NP30.

Previous work has indicated that some forms of quinoline resistance may be unique to CQ and has suggested that resistance to MF, QN, and QD may result from mutations in a common efflux protein, such as Pgh1 (26). We therefore determined what effect the presence of verapamil and NP30 had on the IC_{50} s of MF, QN, and QD in the two isolates. The IC_{50} s of MF for isolates 1 and 2 decreased significantly in the presence of NP30; however, verapamil had no effect (Fig. 3). NP30 resulted in a marked reversal of QN and QD resistance; however, the effect of verapamil in the presence of QN and QD was variable and not as dramatic as that of NP30 (Fig. 3). We therefore conclude that (i) NP30 and verapamil have dissimilar sensitization properties, (ii) MF resistance results from a verapamil-insensitive but NP30-sensitive mechanism, and (iii) QN and QD resistance can be reversed by either verapamil or NP30.

The use of two isolates with different CQ resistance phenotypes suggested that isolate 1 and isolate 2 might not share common point mutations in putative quinoline resistance genes. We therefore determined if previously characterized point mutations in the *pfprt* and *pfmdr* genes were present (Table 1). The point mutation patterns seen in the *pfprt* and *pfmdr* genes were not able to differentiate between the two distinct CQ resistance phenotypes (NP30 sensitive versus NP30 insensitive) observed.

DISCUSSION

Quinoline resistance in the erythrocytic stages of *P. falciparum* is frequently compared to multidrug resistance in mammalian cells, in part because of the initial observation that chloroquine resistance could be reversed by verapamil. NP30 is also able to reverse some forms of quinoline resistance in *P. falciparum* cultures; however, the results obtained in this study suggest that its resistance reversal properties are different from those of verapamil. This is seen most clearly in the case of

FIG. 3. Effect of verapamil or NP30 on the IC₅₀s of four quinolines in two *P. falciparum* patient isolates. The IC₅₀s of CQ, MF, QN, and QD were determined in the presence of drug alone, 2 μ M verapamil, or 21 μ M NP30 for two isolates. The values represent the means $\pm$ standard errors of the means of four independent assays. Asterisks indicate that the values were significantly different ($P < 0.05$) from the control values.

isolate 1, in which CQ resistance could be reversed only by verapamil, while MF resistance could be reversed only by NP30. While this suggests that MF and CQ resistance is due to the expression of independent NP30-inhibitable and verapamil-inhibitable mechanisms, the results observed for QN and QD do not support this hypothesis. The QN and QD IC₅₀s for isolate 1 can be dramatically decreased by the presence of either NP30 or verapamil. This suggests that both the verapamil-sensitive and the NP30-sensitive resistance elements are a necessary part of the QN and QD resistance process, since disruption of either makes the parasites sensitive to the drug's presence. If the two resistance mechanisms ran in a parallel fashion, we would expect that the presence of either agent alone would have little effect because of redundancy. Both agents are effective; therefore, it appears that they have a serial arrangement (e.g., they may facilitate transport between different compartments within the parasite). The same relationship between QN and QD resistance and the MF and CQ resistance patterns was also seen in isolate 2, in which the degree of reversal seen for QN and QD in isolate 2 supports the hypothesis that QD and QN efflux requires both the CQ and MF efflux mechanisms to be functioning simultaneously.

While the results obtained with CQ and MF for isolate 1 strongly suggest that the actions of verapamil and NP30 can be independent of each other, the results obtained with CQ for isolate 2 suggest that there can also be some overlap in their actions. The presence of either verapamil or NP30 results in a moderate, but significant, drop in the CQ IC₅₀ for isolate 2. When both verapamil and NP30 were added to the same assay, their effects did not appear to be additive (data not shown), suggesting that this result is not due to the presence of a mixed population of verapamil- and NP30-sensitive parasites. CQ resistance can be either fully or partially sensitive to the presence of verapamil, and recent reports suggest that this behavior may be associated with the presence of specific sequences in the *pfert* gene (20). It would be of interest to know if these *pfert* polymorphisms were related to or were independent of the NP30 CQ sensitization potential.

It would appear that in our original report of the sensitization properties of NP30 (8) we used a group of parasite isolates that were sensitized by the presence of NP30. Our present findings indicate that there is a subset of *P. falciparum* isolates that display CQ resistance that is insensitive to NP30. The difference between the NP30 sensitivities of these isolates cannot be explained by previously reported point mutations in *pfert* and *pfmdr1* associated with quinoline resistance, since these mutations were present in the two isolates used in this study. Further work is required to characterize the interaction of both verapamil and NP30 with resistance elements in *P. falciparum*.

NP30 has both anti-*P. falciparum* and sensitization abilities. The concentration of NP30 chosen for the evaluation whose results are presented in Fig. 2 (21 μ M) was chosen because it permitted us to observe a high degree of sensitization, despite the presence of the direct anti-*P. falciparum* action of the agent. Higher levels of NP30 in the assays may result in a higher degree of CQ resistance reversal in isolate 2 than that shown in Fig. 2; however, it is of interest that QN and QD resistance reversal was nearly complete at this concentration. This implies that CQ resistance in this isolate is sensitive to the

presence of NP30; however, the concentration of NP30 required for CQ resistance reversal may be higher than that required for QN and QD resistance reversal.

The finding that NP30 can be used to reverse MF, QN, and QD resistance in vitro may prove to be clinically useful, since this agent does not have the pharmacological effects on the human host that previously described agents, such as verapamil and promethazine, have. Furthermore, the low cost of this material may make its use in developing countries an economically viable proposition.

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