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Quinine Specifically Inhibits the Proteolipid Subunit of the F_0F_1 H^+-ATPase of *Streptococcus pneumoniae*

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***Streptococcus pneumoniae* is uniquely sensitive to quinine and its derivatives, but only those alkaloids having antimalarial properties, i.e., those in the *erythro* configuration, also possess antipneumococcal activity. Quinine and related compounds inhibit the pneumococcal H^+-ATPase. Quinine- and optochin-resistant pneumococci showed mutations that change amino acid residues located in one of the two transmembrane α-helices of the *c* subunit of the F_0F_1 H^+-ATPase.**

Plasmodium falciparum is the causative agent of malaria, one of the most serious widespread infectious diseases (14). Although chloroquine has been the mainstay of antimalarial treatment for the past 40 years, resistance in *P. falciparum* is now widespread, resulting in difficulties in selecting appropriate drugs for both prophylaxis and treatment (22). Quinine and quinidine are the only drugs available for the parenteral treatment of severe chloroquine-resistant *P. falciparum* malaria (13, 24). It should be emphasized, however, that despite years of intensive research, the mechanism of action of antimalarial compounds is still controversial.

Since *Streptococcus pneumoniae* is particularly sensitive to quinine (Qin^s) and optochin (Opt^s [ethylhydrocuprein hydrochloride]) (12), we have selected this bacterium as a possible alternative model system for a better understanding of the mechanism of action of antimalarial agents. We have recently demonstrated that optochin specifically inhibits the membrane-associated F_0F_1 H^+-ATPase of *S. pneumoniae* and that Opt^r pneumococcal mutants have point mutations in the *atpC* gene encoding the *c* subunit of the F_0 complex (4). The H^+-ATPases utilize the transmembrane proton gradient to drive ATP synthesis or, in reverse, the hydrolysis of ATP to extrude protons from the cell (5, 19).

Given the chemical similarities among the *Cinchona* alkaloids (20) and the unique sensitivity of *S. pneumoniae* to these drugs, we have looked for the primary target of quinine by using a combined genetic and biochemical approach.

The MICs for the wild-type R6 strain of *S. pneumoniae* of the *erythro*-cinchona alkaloids quinine, quinidine, cinchonine, and cinchonidine (Sigma) were determined (Fig. 1). The MIC of quinine was 80 μM (28.8 μg/ml) (Table 1), and the MIC of the others (not shown), as well as that of the mixture epiquinine-epiquinidine (a generous gift from A. C. de Wit, DSM Andeno, Maarssen, Holland), was 1 mM. Identical values were found for the pneumococcal M22 strain (not shown). P_i was assayed as previously described (2). The effect of these alkaloids on the membrane-associated H^+-ATPase activity was assayed as described previously (4), except that membranes were first incubated with the drug for 20 min at room temperature and then for 8 min at 37°C before the addition of ATP. All compounds inhibited this activity to different degrees that parallel their MICs (described below), suggesting a common mechanism of action. Interestingly, a mixture of 9-epiquinine and 9-epiquinidine, the *threo*-cinchona alkaloids, did not inhibit the ATPase activity of R6 even at the highest concentration used (1 mM) (not shown). The activity of the amino alcohol antimalarial agents does have structural requirements, as exemplified by the active *erythro*-cinchona alkaloids and the inactive *threo*-cinchona alkaloids (20). Of primary importance is the intramolecular N-O distance (Fig. 1). This distance is larger in the *erythro* forms (intermolecular interactions are favored) than in the *threo* forms, in which intramolecular interactions are preferred. The same three-dimensional requirements appear to be displayed by these compounds in *S. pneumoniae* (Table 1). In addition, a different susceptibility to quinine and quinidine has been found (Table 1) that agrees with that previously reported for *P. falciparum* (17, 23). Moreover, membranes of Opt^r (MJ11 and MJ2) and Qin^r strains (MJQ3 and MJQ4) showed different levels of resistance to inhibition by optochin and quinine (Fig. 2) corresponding to the different MICs of optochin and quinine.

Seven Qin^r mutants (MJQ1 to MJQ7) were selected by plating R6 cells in Mueller-Hinton agar plates (Difco) supplemented with 5% defibrinated sheep blood and containing quinine (60 μg/ml). It is noteworthy that chromosomal DNA (prepared as described in reference 4) from the Qin^r mutants transformed with high efficiency the Qin^s M22 strain not only to Qin^r but also to Opt^r (Table 2). Furthermore, chromosomal DNAs from two Opt^r strains, MJ11 and MJ2, that carried mutations producing amino acid changes in the proton-ATPase *c* subunit (Ala-49→Thr in MJ11 and Val-48→Leu in MJ2) (4) were also able to transform the M22 strain to both Opt^r and Qin^r. The optochin concentration used to select transformants was 8 μM (3 μg/ml), except when the donor DNA was prepared from MJQ1, -2, -3, -5, -6, or -7, in which case transformants were scored at 4 μM, in accordance with the MIC of optochin for these strains (8 μM). Also in agreement with the MIC of quinine for MJ11 (Table 1), no Qin^r M22 transformants were obtained when scored at 100 μM (Table 2). These results strongly suggested that the Opt^r and Qin^r phenotypes were determined by the same mutation. A 1,438-bp DNA fragment containing *atpC*, *atpA*, and the 3′ end of *atpB* was obtained by PCR amplification (4) of MJQ1-to-MJQ7 DNAs with oligonucleotides 594 (5′-CCAGCGAGACTAACGGT CAAATCTGCCACC-3′) and 660 (5′-CGCTTCCAATAGC GGTTAAAAGTTGACAAA-3′) (Fig. 3). This fragment was

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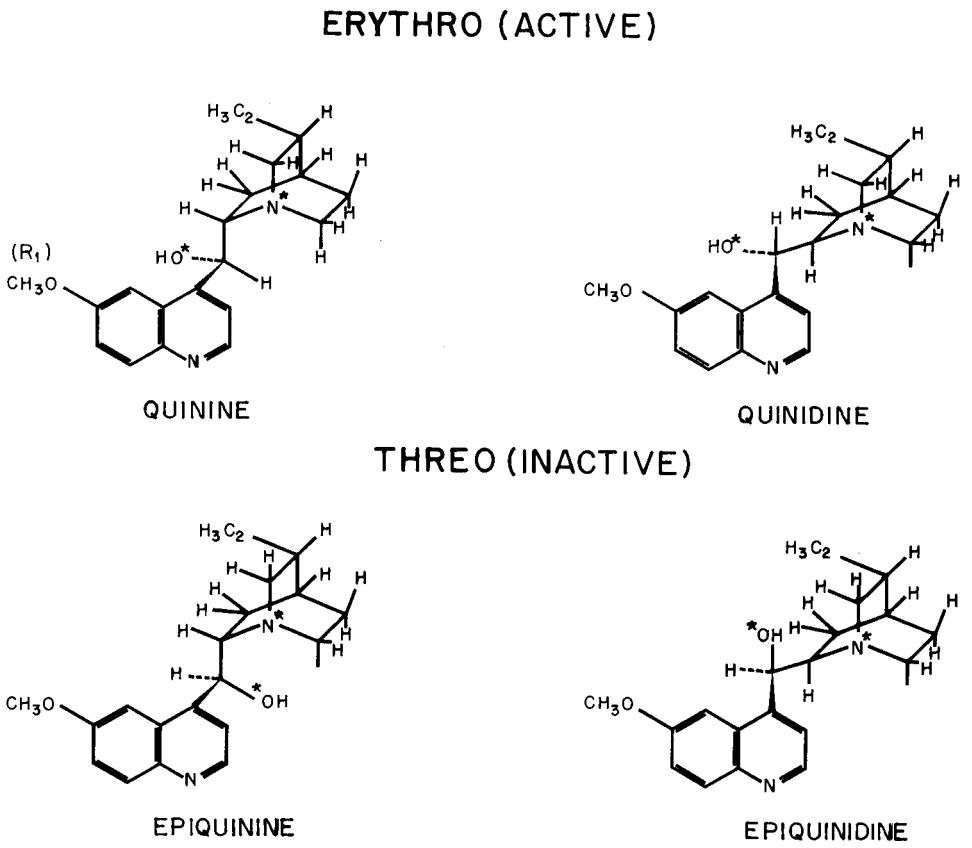

FIG. 1. Chemical structure of the amino alcohol antimalarial agents used in this study. Optochin is as quinine is, but R1 is CH_3—CH_2—. Important N and O atoms for the reactivity of the molecules are indicated by asterisks.

electrophoresed in agarose (18), isolated from gel slices with the Geneclean kit (BIO 101), and used to transform *S. pneumoniae* M22. Both Opt^r and Qin^r transformants were obtained, indicating that the mutations responsible for these phenotypes were included in the amplified fragments. To determine if the *atpC* gene was responsible for the Qin^r phenotype, as it was for the Opt^r phenotype (4), this gene was cloned from strain MJQ4 DNA into *Escherichia coli* (Fig. 3). For this determination, PCR products from an amplification of MJQ4 DNA with oligonucleotides 660 and 594 were cut with *Ase*I (Fig. 3), and the 335-bp fragment containing *atpC* was treated with T4 DNA polymerase and ligated to *Sma*I-digested pUC18 (25). The ligation mixture was used to transform *E. coli* DH5α as previously described (8), and transformants were selected in Luria-Bertani medium containing ampicillin at 100 μg/ml. The recombinant plasmid was able to transform M22 to Qin^r and Opt^r with high efficiency (Table 2), indicating that the mutation responsible was included in *atpC*. The nucleotide sequence of the complete *atpC* gene was determined directly from the PCR products with the *fmol* DNA sequencing system (Promega) with oligonucleotide 660 as a primer, and point mutations were observed in the seven Qin^r mutants. Six of them (MJQ1, -2, -3, -5, -6, and -7) had the same mutation: a G-to-A transition that would produce a Met-23 (ATG)-to-Ile (ATA) change. In one strain (MJQ4), the mutation (a G-to-C transversion) would produce a Gly-20 (GGT)-to-Ala (GCT) change in the amino acid sequence of the ATPase *c* subunit.

TABLE 1. Susceptibility of *S. pneumoniae* strains to several *Cinchona* alkaloids

Strain	MIC (μM) of[a]:			
	Optochin	Quinine	Quinidine	Epiquinine-epiquinidine
R6	3	80	1,000	1,000
MJ11	63	100	500	1,000
MJ2	63	320	500	ND[b]
MJQ3[c]	8	200	1,000	ND
MJQ4	31	250	500	ND

[a] For the determination of MICs, cells were grown in Todd-Hewitt broth (Difco) supplemented with 0.5% yeast extract (Difco) containing different drug concentrations.
[b] ND, not determined.
[c] Same as MJQ1, MJQ2, MJQ5, MJQ6, and MJQ7.

Combined genetic and chemical modification studies support a hairpin-like structure for the *c* subunit with two α-helices that transverse the membrane in an antiparallel fashion, separated by a conserved polar loop region that possibly forms the F_1 binding region (5, 19). The mutations that confer both Opt^r and Qin^r phenotypes would produce changes in Gly-20 or Met-23 of helix 1 (those selected via Qin^r) and Val-48 or Ala-49 of helix 2 (those selected via Opt^r) (Fig. 4). These four residues would be closely juxtaposed within the membrane bilayer when the protein is folded and would be associated with the *a* and *b* subunits to form the F_0 complex of the H^+-ATPase. Independently of how the mutants were selected, cross-resistance between optochin and quinine was found. The two strains obtained via Opt^r (MJ11 and MJ2) have the highest value for optochin (63 μM). With respect to Qin^r, the MIC of quinine for strain MJ11 is 100 μM (1.25 times the MIC for R6),

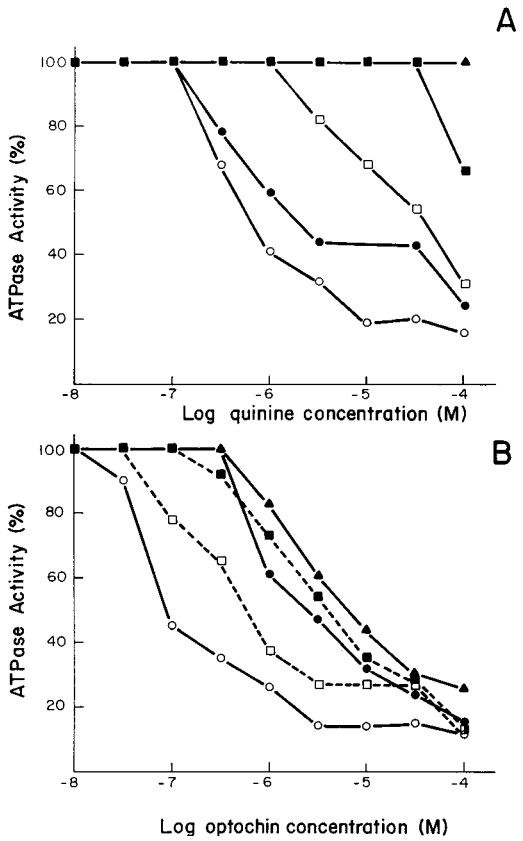

FIG. 2. Effects of quinine (A) and optochin (B) on ATPase activity in membranes from the wild type (R6 [○]) and mutants (MJ2 [▲], MJ11 [●], MJQ3 [□], and MJQ4 [■]). Values are the averages of at least three independent determinations.

TABLE 2. Transformation of *S. pneumoniae* M22 with chromosomal DNAs, PCR products, and cloned *atpC* genes

Donor DNA	Efficiency of transformation (10^4 transformants/ml) with[a]:			
	Optochin (μM)		Quinine (μM)	
	8	4	80	100
R6				
Chromosomal	<0.01	<0.01	<0.01	<0.01
PCR product	<0.05	<0.05	<0.05	<0.05
MJ11				
Chromosomal	50	ND[b]	4	<0.01
PCR product	45	ND	15	<0.05
Cloned *atpC*	10	ND	25	<0.01
MJ2				
Chromosomal	20	ND	19	24
PCR product	37	ND	50	60
MJQ3[c]				
Chromosomal	ND	1	25	1
PCR product	ND	2	40	1
MJQ4				
Chromosomal	26	ND	70	31
PCR product	20	ND	150	100
Cloned *atpC*	23	ND	100	100

[a] Transformation was performed as described elsewhere (21) and transformants were selected in C medium (21) containing 0.08% yeast extract and the indicated drug concentration.
[b] ND, not determined.
[c] Identical results were obtained with MJQ1, MJQ2, MJQ5, MJQ6, and MJQ7.

whereas the MIC for MJ2 is 320 μM (four times the MIC for R6), which represents the largest increase among the strains studied here. Even when the selection was carried out through Qin^r (strains MJQ3 and MJQ4), the increases were smaller (Table 1). These results support the finding that the interaction of the chemically similar drugs optochin and quinine with the *c* subunit occurs in the same region of the protein and that mutants isolated through optochin or quinine selection would have mutations in either of the two α-helices of the *c* subunit at random. Analysis of more Qin^r and Opt^r mutants would clarify this point.

Mutations that eliminate the carboxyl group of Asp-61 in the *E. coli c* subunit (equivalent to Glu-52 in *S. pneumoniae*) in helix 2 inhibit H^+ translocation (5, 19). Glu-52 was not altered in the Opt^r Qin^r pneumococcal mutants, although inhibition of H^+-ATPase activity was observed (Fig. 2). A functional ATPase from a double mutant *E. coli* strain in which the Asp-61 carboxyl group was eliminated and the carboxyl function was replaced by an Asp residue at position 24 in helix 1 (equivalent to Val-15 of *S. pneumoniae*) has been isolated and characterized (11). The functioning of the double mutant suggests that both helices may interact as a unit to present the essential carboxyl group during H^+ translocation. Hence, the interaction of quinine and optochin with the *c* subunit would also cause a conformational change in F_0, hindering the proper presentation of the essential carboxyl group of Glu-52 for H^+ translocation.

Since it is well documented that the proton-ATPases of bacteria, chloroplasts, and mitochondria are similar in structure and function, possibly reflecting the endosymbiotic hypothesis of evolution (7), the results reported here invite speculation that the primary target for quinine in *P. falciparum* would also be the subunit equivalent to the bacterial *c* protein of the mitochondrial F_0F_1 H^+-ATPase. In fact, it has been reported that an appropriate functioning of mitochondria is critical for survival and growth of erythrocytic stages of malarial parasites (1, 6). The vacuolar H^+-ATPase (16) might also be considered an alternative target since its proteolipid subunit and the eubacterial proteolipid are evolutionarily related (10, 15). It is well known that antimalarial agents increase the pH of the food vacuole of *P. falciparum* and that this increase is

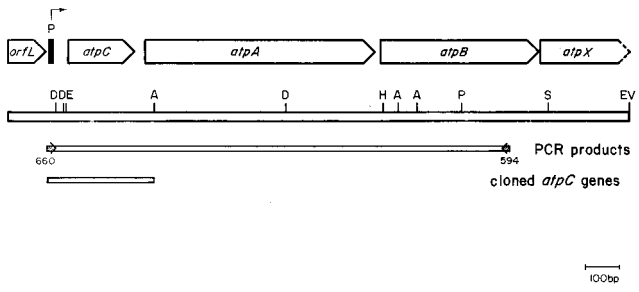

FIG. 3. Genetic structure and physical map of the *atp* region of *S. pneumoniae* R6 DNA. P indicates the putative promoter; genes are indicated by arrows. A, *Ase*I; D, *Dra*I; E, *Eco*RI; EV, *Eco*RV; H, *Hph*I; S, *Sph*I. Chromosomal regions amplified by PCR with oligonucleotides 594 and 660 (shaded arrows) are indicated. The regions including *atpC* genes cloned in pUC18 are also indicated.

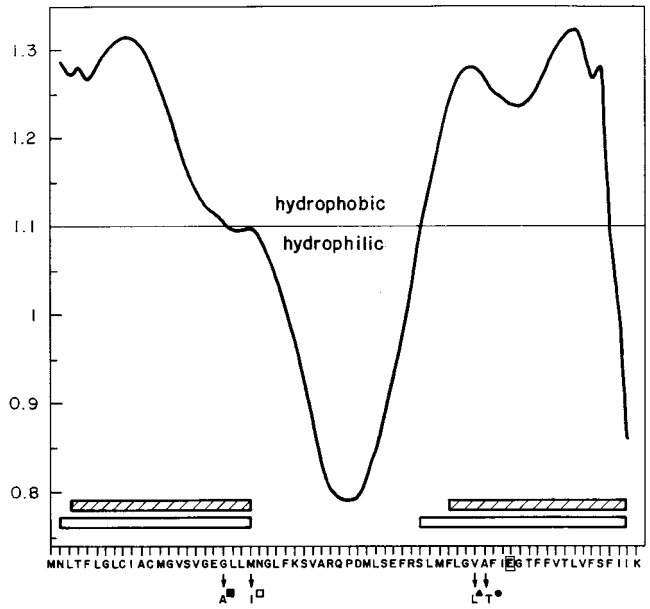

FIG. 4. Prediction of the secondary structure of the *c* subunit. Software from PCGene 6.0 was used. Bars indicate the transmembrane α-helices predicted by the RAOARGOS (open bars) and HELIXMEM (hatched bars) programs. The amino acid sequence of the *c* subunit is indicated, together with the changes observed in the mutants (MJ2 [▲], MJ11 [●], MJQ3 [□], and MJQ4 [■]). The Glu-52 residue, thought to be involved in proton translocation, is boxed.

accompanied by inhibition of parasite growth, possibly because the acid proteases of the parasite can no longer degrade hemoglobin (9). Quinine and quinidine might bind to the proteolipid subunit of the vacuolar ATPase of the sensitive parasite and inhibit the proton pump, which would result in an additional increase in the vesicle pH. In agreement with this hypothesis, it has been observed that quinine strongly inhibits the vacuolar membrane ATPase activity of *P. falciparum* (3).

Finally, notwithstanding that further experiments with *P. falciparum* will be necessary to demonstrate that the primary target of quinine in *S. pneumoniae* is similar to that in the malarial parasite, the pneumococcal system described here appears to be an interesting model strategy for testing the putative antimalarial activity of new compounds, either natural or synthetic, structurally related to the *Cinchona* alkaloids.

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