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Piperine, a Phytochemical Potentiator of Ciprofloxacin against *Staphylococcus aureus*

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Piperine, a trans-trans isomer of 1-piperoyl-piperidine, in combination with ciprofloxacin markedly reduced the MICs and mutation prevention concentration of ciprofloxacin for *Staphylococcus aureus*, including methicillin-resistant *S. aureus*. The enhanced accumulation and decreased efflux of ethidium bromide in the wild-type and mutant (CIP^r-1) strains in the presence of piperine suggest its involvement in the inhibition of bacterial efflux pumps.

Ciprofloxacin, the most frequently used fluoroquinolone, is less effective against gram-positive bacteria, including *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Enterococcus faecalis* (8). Fluoroquinolones inhibit DNA synthesis by inhibiting the closely related enzymes gyrase and topoisomerase IV. The resistance to this class of antibiotics is caused by changes in the genes coding for DNA gyrase (*gyrA* or *gyrB*) or for topoisomerase IV (*parC* or *parE*) (11). The regions in which these mutations occur are termed quinolone resistance-determining regions (16, 17). However multidrug efflux transporters also play a major role in contributing to the resistance of gram-positive organisms to fluoroquinolones by actively extruding fluoroquinolones and multiple other drugs from cells (4, 12, 21).

Piperine, the major plant alkaloid present in black pepper (*Piper nigrum*) and long pepper (*Piper longum*), is reported to have bioavailability-enhancing activity for some nutritional substances and for some drugs (1, 3). Piperine has previously been shown to inhibit several cytochrome P450-mediated pathways and phase II reactions in animal models (2, 19). It has also been proven to be an inhibitor of human P-glycoprotein (5). In this report, we describe for the first time the potentiating effect of piperine with ciprofloxacin in in vitro combination studies against *S. aureus* and its suggestive role as an efflux pump inhibitor.

S. aureus ATCC 29213 was obtained from the American Type Culture Collection (Manassas, Va.). Methicillin-resistant *S. aureus* (MRSA) 33, MRSA 450, and MRSA 15187 were obtained as a kind gift from Ranbaxy Research Laboratories (New Delhi, India). Ciprofloxacin powder was obtained from Cadila Pharmaceuticals, Gujarat, India. Piperine of 99% purity was provided by the Pharmacology Division, Regional Research Laboratory, Jammu Tawi, India. Reserpine was obtained from Sigma Aldrich, St. Louis, Mo. Mueller-Hinton broth (Becton Dickinson, Cockeysville, Md.) supplemented with calcium (25 mg/liter) and magnesium (12.5 mg/liter) was used for all susceptibility and killing curve experiments. Mueller-Hinton agar (Becton Dickinson) was used for mutation studies. Trypticase soy agar (Becton Dickinson) was used for colony counts.

In vitro combination studies. Combination studies were performed by a broth checkerboard method (9). The final concentrations ranged from 0.03 μg/ml to 64 μg/ml for ciprofloxacin and from 0.8 μg/ml to 50 μg/ml for piperine. The final bacterial inoculum in each well was 5×10^5 CFU/ml. The plates were incubated at 37°C for 24 h. Piperine did not show any antibacterial activity when tested up to 100 μg/ml (data not shown). However, there was a twofold reduction in the MIC of ciprofloxacin (from 0.25 μg/ml to 0.12 μg/ml) for *S. aureus* ATCC 29213 when tested in combination with piperine at 12.5 and 25 μg/ml (Table 1). The MIC was further reduced fourfold with 50 μg/ml piperine. This combination of ciprofloxacin with piperine was also effective in reducing the MIC for one of the MRSA isolates.

Time-kill studies. Time-kill studies were carried out by following the principles defined by the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) (14). *S. aureus* ATCC 29213 was used as the test bacterium in this study. Ciprofloxacin was tested at 0.25, 0.5, and 1 μg/ml, respectively. Piperine at a concentration of 50 μg/ml was combined with ciprofloxacin at concentrations of 0.25 and 0.5 μg/ml. Ciprofloxacin brought about 99.9% killing or a 3-log reduction at 1 μg/ml, whereas in combination with piperine at 50 μg/ml, ciprofloxacin could bring about the same level of killing at half the concentration, i.e., 0.5 μg/ml (data not shown).

Selection of resistant mutants in vitro. The frequency of ciprofloxacin-resistant mutants was determined as previously described (7). A bacterial suspension containing 10^9 CFU (100 μl) of *S. aureus* ATCC 29213 was plated onto Mueller-Hinton agar containing ciprofloxacin concentrations equal to 4, 8, and 16 times the MIC. The same concentrations of ciprofloxacin were also tested in combination with piperine at 25 and 50 μg/ml, respectively. Mutation frequencies were calculated by dividing the total number of colonies appearing after 48 h of incubation at 37°C on the drug-containing plate by the total number of CFU plated. Mutation frequencies are presented in Table 2. The concentration of ciprofloxacin at which no mutant was selected, 4 μg/ml (16 times the MIC), has been defined as

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TABLE 1. MIC of ciprofloxacin in combination with piperine

Piperine concn (μg/ml)	MIC (μg) for *S. aureus* strain:			
	ATCC 29213	MRSA 15187	MRSA 33	MRSA 450
0	0.25	>16	>16	>16
12.5	0.12	>16	>16	>16
25	0.12	16	>16	>16
50	0.06	8	16	16

the mutation prevention concentration (MPC). Ciprofloxacin in combination with piperine resulted in a lower mutation frequency (Table 2), whereas with ciprofloxacin at 2 μg/ml (8 times the MIC) in combination with 25 μg/ml piperine, no mutant was selected, thereby reducing the MPC of ciprofloxacin from 4 μg/ml to 2 μg/ml in the combination. When the concentration of piperine was further increased to 50 μg/ml, there was no mutant detected, even at 1 μg/ml ciprofloxacin. The MPC has been proposed as a new measurement of antibiotic potency by which the ability to restrict selection of resistant mutants is evaluated. To be clinically useful, the MPC must be below the maximum concentration of the drug in serum (C_{max}) or tissue at the site of infection. Thus, use of a combination of the MPC and pharmacokinetic parameters provides a way to compare antibacterial agents for the potential ability to restrict the selection of resistant mutants (18). The MPC of ciprofloxacin in our study was 4 μg/ml, which is achievable in the human body only when ciprofloxacin reaches its C_{max}. However, the combination of ciprofloxacin with piperine at 25 μg/ml brings down the MPC to 2 μg/ml, thereby ensuring that resistant mutants will not be selected even when the ciprofloxacin concentration drops below the C_{max} in the human body. A detailed pharmacokinetic study of piperine is required in order to find out the plasma levels of piperine.

Selection and susceptibility of resistant mutants. Ciprofloxacin-resistant *S. aureus* mutants were selected by plating 10^8 CFU of *S. aureus* ATCC 29213 on Mueller-Hinton agar medium. Twenty colonies were picked and individually passaged on Mueller-Hinton agar medium with increasing concentrations of ciprofloxacin (up to 128 μg/ml). The susceptibilities of these mutants to ciprofloxacin (in the presence or absence of 25 μg/ml reserpine and piperine) were determined by the agar dilution method as recommended by the Clinical and Laboratory Standards Institute (15). Twenty resistant mutants for which the MIC of ciprofloxacin was 128 or >128 μg/ml were selected (data not shown). Table 3 describes the MICs of ciprofloxacin and ethidium bromide for wild-type *S. aureus* ATCC 29213 and one of the above-mentioned mutants (CIP^r-1). The reversal of the MICs of ciprofloxacin and ethidium bromide by piperine was studied by using reserpine

TABLE 2. Frequency of mutation of *S. aureus* ATCC 29213

Piperine concn (μg/ml)	Mutation frequency with ciprofloxacin at:		
	4× MIC (1 μg/ml)	8× MIC (2 μg/ml)	16× MIC (4 μg/ml)
0	$>10^{-6}$	1.2×10^{-8}	$<10^{-9}$
25	1.3×10^{-8}	$<10^{-9}$	$<10^{-9}$
50	$<10^{-9}$	$<10^{-9}$	$<10^{-9}$

TABLE 3. Drug susceptibilities of *S. aureus* ATCC 29213 and the ciprofloxacin-selected mutant CIP^r-1

Compound(s) (concn [μg/ml])	MIC (μg/ml)	
	S. aureus ATCC 29213	CIP^r-1
Ethidium bromide	4	8
Ethidium bromide + reserpine (25)	2	2
Ethidium bromide + piperine (25)	1	2
Ciprofloxacin	0.25	128
Ciprofloxacin + reserpine (25)	0.12	64
Ciprofloxacin + piperine (25)	0.12	8

(a known efflux pump blocker) as a control. For the CIP^r-1 mutant, there was a 512-fold increase in the MIC of ciprofloxacin and a twofold increase in the MIC of ethidium bromide compared with those for the wild type. The increase in the MICs of ciprofloxacin and ethidium bromide for both isolates (wild type and CIP^r-1 mutant) was reversed by piperine and reserpine. Since efflux is the only known mechanism for ethidium bromide resistance, the reversal of its MIC for the CIP^r-1 mutant to a value similar to that for the wild type indicates piperine's role as an efflux inhibitor.

Accumulation and efflux of ethidium bromide. Measurement of the levels of ethidium bromide accumulation and efflux in *S. aureus* ATCC 29213 (wild strain) and strain CIP^r-1 (ciprofloxacin-selected mutant) was based on a previously described method (6). The increase in fluorescence as ethidium bromide accumulated in the cells was recorded fluorometrically with a Perkin-Elmer LS50 spectrofluorimeter (excitation wavelength, 530 nm; emission wavelength, 600 nm) at 30°C. The effect of piperine on drug accumulation was determined in a similar way, except that piperine was added to the uptake buffer at a concentration of 25 μg/ml. To determine ethidium bromide loss, bacterial suspensions were prepared as described above and exposed to ethidium bromide (2 μg/ml) in the presence of piperine (25 μg/ml) for 30 min at 37°C. The loss of ethidium bromide from the cells was measured as a decrease in fluorescence. Figure 1A compares the levels of accumulation of ethidium bromide in wild-type *S. aureus* ATCC 29213 and mutant CIP^r-1. The rate of accumulation in the mutant was significantly lower. However, with the addition of piperine (25 μg/ml) after 30 min, this difference in accumulation was abolished, although the overall level of ethidium bromide accumulation increased in both strains. The rate of ethidium bromide loss from the mutant (CIP^r-1) was significantly increased compared with that from the wild-type strain. Again, addition of piperine dramatically decreased the efflux rate in the CIP^r-1 mutant (Fig. 1B). Similar results were obtained when reserpine was used as an efflux pump blocker (data not shown).

The reversal of the MIC of ciprofloxacin, enhanced accumulation of ethidium bromide, and inhibition of efflux from mutant (CIP^r-1) cells preloaded with ethidium bromide suggest that the potentiating activity of piperine may occur through inhibition of ciprofloxacin efflux from *S. aureus*. The role of the membrane transporter NorA in the efflux of fluoroquinolones in *S. aureus* has been demonstrated (13, 20). Ethidium bromide is a substrate for many gram-positive multidrug resistance pumps, including NorA. The efficiency of the efflux pumps for

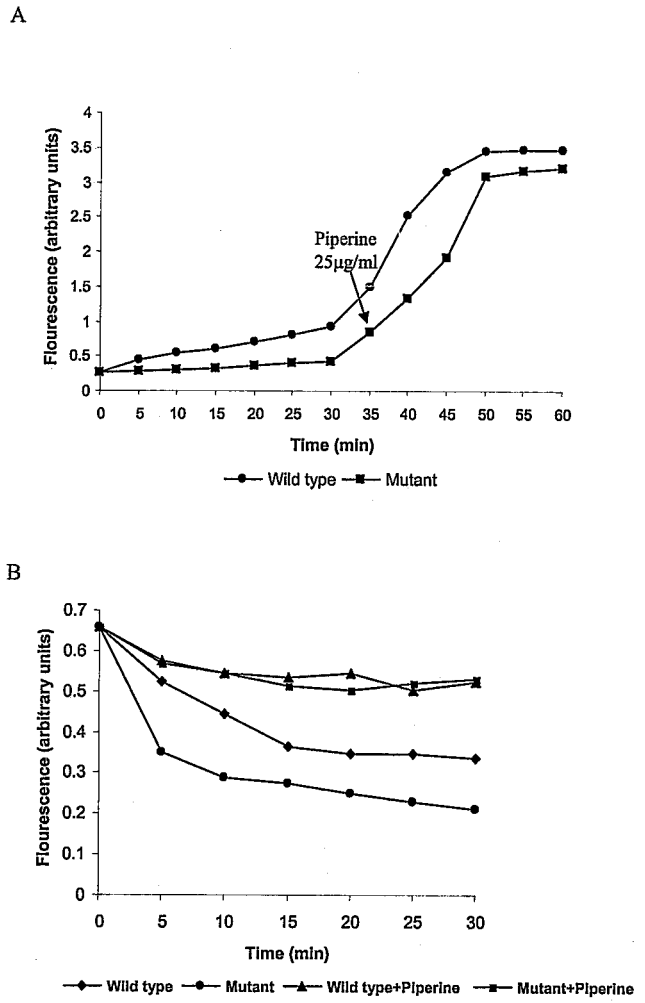

FIG. 1. Accumulation (A) and efflux (B) of ethidium bromide in wild-type and mutant (CIP^r-1) strains and effect of piperine (25 μg/ml) thereon.

which ethidium bromide is a substrate can be accessed fluorometrically by the loss of fluorescence over time from cells loaded with ethidium bromide (10). The enhanced accumulation of ethidium bromide and blockage of its efflux from mutant (CIP^r-1) cells preloaded with ethidium bromide by piperine suggests that piperine inhibits the efflux of ethidium bromide in the same manner as that of reserpine (a known inhibitor of major bacterial efflux pumps, including NorA). GG918, a known P-glycoprotein inhibitor, was also reported to have ciprofloxacin-enhancing activity against *S. aureus* by inhibiting efflux (10). Our study thus reveals that piperine is yet another P-glycoprotein inhibitor that inhibits ciprofloxacin efflux from bacterial cells.

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