In Vitro Effect of Fluoroquinolones on Theophylline Metabolism in Human Liver Microsomes

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Received 17 July 1989/Accepted 4 January 1990

Some quinolone antibiotics cause increases in levels of theophylline in plasma that lead to serious adverse effects. We investigated the mechanism of this interaction by developing an in vitro system of human liver microsomes. Theophylline (1,3-dimethylxanthine) was incubated with human liver microsomes in the presence of enoxacin, ciprofloxacin, norfloxacin, or ofloxacin. Theophylline, its demethylated metabolites (3-methylxanthine and 1-methylxanthine), and its hydroxylated metabolite (1,3-dimethyluric acid) were measured by high-pressure liquid chromatography, and $K_m$ and $V_{max}$ values were estimated. Enoxacin and ciprofloxacin selectively blocked the two $N$ demethylations; they significantly inhibited the hydroxylation only at high concentrations. Norfloxacin and ofloxacin caused little or no inhibition of the three metabolites at comparable concentrations. The extent of inhibition was reproducible in five different human livers. Inhibition enzyme kinetics revealed that enoxacin caused competitive and mixed competitive types of inhibition. The o xo metabolite of enoxacin caused little inhibition of theophylline metabolism and was much less potent than the parent compound. Nonspecific inhibition of cytochrome P-450 was ruled out since erythromycin N demethylation (cytochrome P-450 mediated) was unaffected in the presence of enoxacin. These in vitro data correlate with the clinical interaction described for these quinolones and theophylline. We conclude that some quinolones are potent and selective inhibitors of specific isozymes of human cytochrome P-450 that are responsible for theophylline metabolism. This in vitro system may be useful as a model to screen similar compounds for early identification of potential drug interactions.

Acute respiratory infections are relatively common in patients with chronic obstructive pulmonary disease or asthma. Hence, these patients frequently receive theophylline and may require concomitant antimicrobial treatment. Quinolone antibiotics exhibit excellent activity against certain respiratory tract pathogens and have proven useful in the treatment of pulmonary infections (21). However, theophylline plasma levels become elevated in patients treated simultaneously with certain quinolones, such as enoxacin or ciprofloxacin (1, 7, 14, 24, 25), and these elevated levels may result in complaints of nausea, vomiting, tachycardia, or agitation (27). The mechanism of this interaction at the molecular level has not yet been evaluated.

Theophylline is almost entirely (90%) metabolized in the liver by the hepatic mixed-function oxidase system (13) to 3-methylxanthine (3-MX), 1-methylxanthine (1-MX), and 1,3-dimethyluric acid (1,3-DMU) (Fig. 1). Studies in humans indicate that certain quinolones cause a dose-dependent inhibition of theophylline metabolism, resulting in decreased urinary excretion of its metabolites and increased excretion of the parent compound (1, 17). Evidence for this mechanism is further supported by investigations showing that neither protein binding nor renal clearance of theophylline are influenced by coadministration of enoxacin (26). Wijnands et al. suggested (27) that the o xo metabolite of enoxacin, o xo-oxo-oxin, might be responsible for this inhibition. However, subsequent in vitro studies with rat hepatocytes indicate that only the parent compound inhibits theophylline metabolism (9). The aim of the present investigation is to evaluate the use of human liver microsomes as an in vitro system to further investigate the mechanism of the in vivo interaction between theophylline and quinolones.

MATERIALS AND METHODS

Chemicals and reagents. All organic solvents and other chemicals were high-pressure liquid chromatography (HPLC) grade. NADPH, theophylline, 3-MX, 1-MX, 1,3-DMU, and erythromycin were purchased from Sigma Chemical Co., St. Louis, Mo. The quinolones were gifts from the following: enoxacin and oxo-oxo-oxin (Warner Lambert Co., Pharmaceutical Research Div., Ann Arbor, Mich.), ciprofloxacin hydrochloride (Miles Laboratories Inc., Pharmaceutical Div., West Haven, Conn.), ofloxacin (Ortho Diagnostics, Raritan, N.J.), and norfloxacin (Merck Sharp & Dohme, West Point, Pa.).

Human liver samples. Specimens of liver were obtained during surgery under the protocol approved by the Committee for the Conduct of Human Research at the Medical College of Virginia. All patients had normal serum transaminase and bilirubin levels. The ages, genders, and medications of patients are listed in detail elsewhere (23, 28). All liver samples were transported from the operating room on ice. A total of 22 livers was used, including the liver used for preparing microsomes for the method development and standard curves. The samples were mixed and homogenized, and microsomes were prepared by differential centrifugation (22). Aliquots (1.0 ml) of the prepared microsomes were stored at −70°C. Protein concentrations of the microsomal samples were determined colorimetrically (6). Theophylline metabolism by human liver microsomes. The
incubation procedure was a modification of previous work (15). The incubation mixture (0.5 ml) consisted of 0.5 mg of total human microsomal protein, along with theophylline at a final concentration of 20 mM in 0.1 M phosphate buffer, pH 7.4. The reaction was started by adding NADPH (1.2 mM) and was carried out in air at 37°C in a metabolic shaker for 30 min. The incubation was stopped by cooling in an ice bath and adding 400 µl of 2% zinc sulfate. The precipitated proteins were removed by centrifugation at 15,000 x g for 10 min. The aqueous filtrate was saturated with 300 mg of ammonium sulfate, and 25 µl of a 5-µg/ml solution of beta-hydroxypropyl theophylline was added as an internal standard. The mixture was extracted with two 5-ml portions of methylene chloride-isopropyl alcohol (80:20, vol/vol). The organic phase of the mixture was evaporated to dryness and reconstituted in 200 µl of the mobile phase of the mixture.

The three metabolites of theophylline, 3-MX, 1-MX, and 1.3-DMU, were analyzed by using a modification of the HPLC procedure of Robson et al. (16). The reconstituted sample (50 µl) was injected onto a C-18 Econosphere column (4.6 by 15.0 mm, 5 µm; Alltech Associates, Applied Science Div., State College, Pa.). The HPLC gradient system consisted of two Gilson model 302 pumps, a model 811 Dynamic mixer, a model 802B manometric module, and a model 231 sample injector. The flow rate was 1 ml/min. The A_290 was monitored by a Shimadzu SPD-6A UV spectrophotometric detector at 0.001 absorbance units full scale. The analyte peaks were quantitated by using a Gilson 714 V1.1B HPLC data acquisition and control system. Standards diluted in human liver microsomes were prepared freshly and found to be linear over the concentration ranges of 10 to 250 ng/ml for 3-MX, 20 to 250 ng/ml for 1-MX, and 250 to 4,000 ng/ml for 1.3-DMU. The mean absolute recovery evaluated at eight concentrations and expressed as a percentage of direct injection was 75 to 85% for the three metabolites. The accuracy (percentage of error of assayed samples relative to their spiked concentrations) was within ±10%. The precision of the method was within ±15% at all concentrations. The limits of detection (defined as the concentrations that would provide a signal equivalent to twice the noise level) were 10 ng/ml for 3-MX, 20 ng/ml for 1-MX, and 10 ng/ml for 1.3-DMU. Adequate selectivity was demonstrated by the absence of interfering peaks in blank human liver microsome chromatograms.

Erythromycin N demethylation. Microsomal samples (0.5 mg) were incubated with 0.4 mM erythromycin in a 0.5-ml reaction mixture made up to volume in 0.1 M potassium phosphate buffer (pH 7.4). NADPH (1.2 mM) was added to initiate the reaction. After 30 min of incubation at 37°C in a metabolic shaker, 25% trichloroacetic acid was added and the mixture was set on ice for 10 min to stop the reaction. The methyl group liberated due to the N demethylation is eventually converted to formaldehyde, which was assayed by the method of Nash (10).

Data analysis. K_m and V_max values (rate constant and maximum rate for metabolite formation, respectively) were calculated by using NONLIN, a least-squares nonlinear curve-fitting program (8), with initial parameter estimates obtained from Hanes plots (3). Analysis of variance by SAS (Statistical Analysis Package System [19]) was used to compare the K_m and V_max values of the three metabolites. Dunnett's multiple comparison of means (4) was used to evaluate the effect of various quinolones on the rate of

![Image](http://aac.asm.org/Downloaded_from/http://aac.asm.org/on_June_21_2017_by_guest)
metabolite formation. A probability value of <0.05 was considered statistically significant.

RESULTS

The major route of theophylline metabolism is via 8-hydroxylation to 1,3-DMU, which accounts for 45 to 55% of total theophylline clearance. The other metabolites are 3-MX and 1-methyluric acid, which represent 10 to 13 and 20 to 25% of theophylline clearance, respectively (13). 1-Methyluric acid is formed from 1-MX in vivo by xanthine oxidase (2). However, since xanthine oxidase is a cytoplasmic enzyme and is not present in the microsomal fraction, we could quantify only 1-MX and not 1-methyluric acid. Microsomes from all human livers metabolized theophylline by 1- and 3-N demethylation to form 3-MX and 1-MX and by 8-hydroxylation to form 1,3-DMU. Under the condition described, the rates of formation of 3-MX, 1-MX, and 1,3-DMU were linear, occurred within a period of 5 to 30 min, and were directly dependent on protein concentrations between 0.1 and 2.0 mg/ml (data not shown).

By varying the concentrations of theophylline from 0 to 30 mM, typical Michaelis-Menten plots for all three metabolites were obtained (Fig. 2). By using liver samples from three patients, estimates of \( K_m \) and \( V_{max} \) values were obtained from Hanes plots (3). These estimates were used in NONLIN to calculate the reported \( K_m \) and \( V_{max} \) values. The mean \( K_m \) values of the N demethylations were similar, namely \( 1.76 \pm 0.26 \text{mM} \) for 3-MX and \( 1.68 \pm 0.18 \text{mM} \) for 1-MX formation, and the mean \( K_m \) for 8-hydroxylation was \( 4.50 \pm 1.16 \text{mM} \). \( V_{max} \)s were estimated to be \( 2.24 \pm 0.12 \) and \( 2.44 \pm 0.06 \text{pmol/mg of microsomal protein per min for 3-MX and 1-MX, respectively, and 22.61 \pm 1.65 \text{pmol/mg per min for 1,3-DMU.} \) The \( K_m \) and \( V_{max} \) values for the two demethylations were not significantly different from each other but were different from the values for 8-hydroxylation (\( P < 0.05 \)).

Theophylline (10 mM; 900 \( \mu \)g/ml) was incubated with 10, 100, 1,000, and 2,000 \( \mu \)g of enoxacin, ciprofloxacin, norfloxacin, and ofloxacin per ml in microsomes prepared from liver obtained from a single patient who had been receiving no medications. After a rough approximation of the molecular weights of the four quinolones, the concentrations can be represented as 0.3 mM (or 1,000 \( \mu \)g/ml). Controls involved incubating theophylline with an equal volume of buffer instead of the quinolone solution. The pattern of inhibition was clearly dose related and was more selective for 3-MX and 1-MX formation than for 1,3-DMU formation (Fig. 3). At a concentration of 1,000 \( \mu \)g/ml, enoxacin resulted in nearly complete inhibition of both demethylations. A similar pattern was observed with ciprofloxacin, although the extent of inhibition was not as great (Fig. 3a and b). In contrast to the effect on N demethylation, at 1,000 \( \mu \)g/ml, enoxacin and ciprofloxacin caused, respectively, approximately 55 and 45% less inhibition of 1,3-DMU formation (Fig. 3c). Norfloxacin and ofloxacin caused no significant inhibition of formation of any of the metabolites at 1,000 \( \mu \)g/ml, although there was slight inhibition for the two N demethylations at 2,000 \( \mu \)g/ml. The 8-hydroxylation pathway was not significantly altered by norfloxacin or ofloxacin even at the highest concentration (Fig. 3c).

In order to allow statistical interpretation, the preceding experiment was replicated in microsomes from five different human livers with enoxacin, ciprofloxacin, and norfloxacin (but not ofloxacin) at the same concentrations as described before. Analysis of covariance using the uninhibited theophylline metabolite formation as the covariant indicated that the degree of inhibition was directly related to the extent of uninhibited theophylline metabolism (\( P < 0.05 \)). The extent of inhibition of the two N demethylations was dependent on the initial rate of metabolite formation in the control (Fig. 4). The percentages of inhibition of the two N demethylations were higher in liver samples which had higher rates of metabolite formation. Therefore, the percentages of inhibition were compared between the quinolones after adjusting for the control, which was considered to be 100%. The pattern of inhibition was similar to that previously described. Dunnett's test for comparison of means showed that enoxacin caused significant inhibition compared with the control at 100, 1,000, and 2,000 \( \mu \)g/ml for both N demethylations (\( P < 0.05 \)). The 8-hydroxylation, however, was significantly inhibited by enoxacin only at concentrations of 1,000 and 2,000 \( \mu \)g/ml (\( P < 0.05 \)). Both ciprofloxacin and norfloxacin caused significant inhibition of the two N demethylations only at 1,000 and 2,000 \( \mu \)g/ml (\( P < 0.05 \)). Inhibition of 1,3-DMU formation was significant for ciprofloxacin (but not norfloxacin) only at 2,000 \( \mu \)g/ml when compared with the control. Table 1 represents the percentages of inhibition of theophylline metabolism caused by the three quinolones at
at 1,000 μg/ml. At 1,000 μg/ml, norfloxacin did not cause any significant inhibition of the three theophylline metabolites, unlike enoxacin and ciprofloxacin. At 100 μg/ml, only enoxacin caused inhibition of both N demethylations and ciprofloxacin and norfloxacin did not cause any inhibition. Although the inhibition by the three quinolones of the two N demethylations at 2,000 μg/ml did reach statistical significance, a clear trend was observed in which enoxacin was the most potent inhibitor, followed by ciprofloxacin and norfloxacin, both of which caused a relatively small inhibition.

To evaluate the mechanism of inhibition, enoxacin at 0, 0.00326, 0.0326, 0.326, 3.26, and 6.52 mM was incubated with 2, 5, 10, 20, and 30 mM theophylline in human liver microsomes from the patient previously described. Analysis of double-reciprocal plots (20), Dixon plots (3), and Cornish-Bowden plots (3) showed that enoxacin blocked 3-MX and 1-MX formation by mixed competitive inhibition. Typical Dixon plots of 1/v versus [i] are shown in Fig. 5 for 3-MX and 1-MX formation. The 1,3-DMU formation was blocked by a competitive type of inhibition. The inhibition constants (K_i) for each of the metabolites were evaluated from the above-mentioned plots and were 0.3, 0.1, and 3.4 mM for 3-MX, 1-MX, and 1,3-DMU, respectively.

Further evaluation of the mechanism of inhibition was carried out by investigating the effect of enoxacin on erythromycin N demethylation. Erythromycin N demethylation proceeds through different isozymes than those responsible for N demethylation of theophylline (23). Microsomes from three different human livers were incubated with erythromycin (0.4 mM) in buffer (control) and in the presence of enoxacin at concentrations of 0.0326, 0.326, 3.26, and 6.52 mM. Enoxacin caused no significant inhibition of the rate of N demethylation of erythromycin. The average rate in the presence of enoxacin at all the concentrations (0.230 ± 0.06 nmol/mg per min) was not significantly different from that of the control (0.249 ± 0.09 nmol/mg per min).

The mechanism of inhibition was also investigated to determine whether the parent compound or the metabolite of enoxacin was responsible for the observed inhibition of theophylline metabolism. In humans, oxo-enoxacin is present in plasma at a concentration that is approximately 10% that of the parent compound (11). Incubation of 100, 500, and 1000 μg of oxo-enoxacin per ml with theophylline was performed in four different human liver microsomal preparations. For comparison, 500 and 1,000 μg of enoxacin

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**FIG. 3.** Effect of quinolones on theophylline metabolism. Theophylline (10 mM), with NADPH (1.2 mM), was incubated alone and in the presence of 10, 100, 1,000, and 2,000 μg of norfloxacin (□), ofloxacin (△), ciprofloxacin (○), and enoxacin (▽) per ml at 37°C for 30 min in microsomes prepared from human liver. Results are expressed as percent inhibition of rate of metabolite formation with respect to control. Effect on 3-MX (a), 1-MX (b), and 3-DMU (c) formation.

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**FIG. 4.** Correlation of uninhibited theophylline N demethylation with the extent of inhibition by enoxacin. The x axis represents the rate of metabolite formation in the absence of enoxacin by each of the five liver microsomes. The dependent variable is the percent inhibition by enoxacin at 1,000 μg/ml with respect to control.

**TABLE 1.** Inhibition of theophylline metabolism by some quinolonesa

<table>
<thead>
<tr>
<th>Quinolone</th>
<th>% Inhibition (±SD, n = 5) of:</th>
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<tbody>
<tr>
<td>3-MX</td>
<td>1-MX</td>
</tr>
<tr>
<td>Enoxacin</td>
<td>76.94 ± 10.19</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>54.12 ± 14.59</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>34.53 ± 10.07</td>
</tr>
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a Quinolones, 1,000 μg/ml, were incubated with 900 μg of theophylline per ml. Other concentrations of quinolines evaluated were 10, 100, and 2,000 μg/ml (for details, see text).
SARKAR and 3.4 ml highest concentration, the oxo-enoxacin caused slight (20% contrast, olites of potent inhibitor in microsomes, the results of which correlate ofloxacin comparing the theophylline. In the demethylations than microsomes, the results of which correlate ofloxacin in vivo interactions of quinolones investigated an anithosis of theophylline metabolite Nonspecific inhibition of theophylline metabolism and comparison of inhibition by enoxacin (Eno) and oxo-enoxacin (Oxoeno). Four different human liver microsomal preparations were incubated with various concentrations of enoxacin or oxo-enoxacin in the presence of theophylline (10 mM) and NADPH (1.2 mM) at 37°C for 30 min.

vitro human liver microsomal system, we found that the extent of inhibition can be graded as enoxacin > ciprofloxacin > norfloxacin. Sano et al. (18) found that the mean cumulative urine recoveries of 3-MX, 1-MU, and 1,3-DMU in humans were decreased from that of control by 64, 63, and 28%, respectively, after administration of enoxacin. These results correlate well with those from our human liver microsomal system (Table 1). We found that enoxacin inhibits 3-MX, 1-MX, and 1,3-DMU formation from control by 76, 68, and 31%, respectively.

The results from the quinolones were compared as percentages of inhibition relative to that of the control. This was necessary because data analysis revealed that the extent of inhibition was related to the magnitude of the uninhibited theophylline metabolism. Although high concentrations of the quinolones were used in our in vitro system, they were necessary to allow quantitation of the metabolites of theophylline, and concentrations of this magnitude are common in microsomal metabolism studies (16). The ratios of theophylline and the quinolone concentrations in this model are quite similar to those observed in a clinical situation (12). Previous investigators have speculated on the mechanism of this interaction. The role of the oxo metabolite of enoxacin, initially proposed to be responsible (27), is unlikely for a number of reasons. Studies in rat hepatocytes showed that oxo-oxo-enoxacin did not cause inhibition of theophylline metabolism (9). In the present study, oxo-oxo-enoxacin caused slight inhibition of theophylline metabolism only at the highest concentrations. At 1/10 the concentration of enoxacin (the expected ration in serum), oxo-oxo-enoxacin causes no significant inhibition. Our observation that the extent of inhibition of the two N demethylations is dependent on the initial rate of metabolite formation in the control indicates that patients with lower initial theophylline levels probably metabolize theophylline faster and are also prone to higher inhibition due to some of the quinolones. The theophylline levels in such patients would be significantly higher than in those patients with normal levels of theophylline in serum.

Evidence from in vitro studies (5) and a recent immunoinhibition study (16) has shown that more than 90% of theophylline metabolism is mediated by the oxidative phase I cytochrome P-450 system. Nonspecific inhibition of cytochrome P-450 enzymes by the quinolones is not likely
since increasing concentrations of enoxacin did not cause inhibition of erythromycin N demethylation. Immuno-inhibition studies in our laboratory have shown that the polycyclic aromatic-hydrocarbon-inducible cytochrome P-450 is mainly involved in the formation of the two N-demethylated metabolites. Since enoxacin and ciprofloxacin profoundly inhibited the N demethylations and not the hydroxylation, one can conclude that quinolones selectively inhibit only certain isozymes in this system. On the basis of our enzyme kinetics data, we speculate that an inhibitory complex is being formed between certain quinolones and the enzymes involved in theophylline metabolism. This complexation appears to be reversible, at least for enoxacin, since the type of inhibition was found to be mixed competitive. Since these quinolones cause a more potent inhibition to the two N demethylations of theophylline than of the 8-hydroxylation, these data suggest that a similar isozyme may be mediating the theophylline N demethylation and the metabolism of quinolones. It could also be possible that enoxacin is a noncatalyzing substrate which interferes with the enzymes involved in theophylline N demethylations. The smaller inhibition constants for the two N demethylations confirm a stronger interaction of enoxacin with the cytochrome P-450 isozymes involved with 3-MX and 1-MX formation. A weaker interaction of enoxacin with 1,3-DMU is evidenced by its larger $K_i$.

This in vitro system allows study of the mechanisms of drug-drug interactions which may not be possible in humans and may also provide a valuable screening tool for other interactions of potential clinical importance. Since our results closely parallel clinical observations in patients, we propose that such an in vitro model of human liver microsomes could be used both to screen new or existing drugs for potential interactions and to evaluate the mechanism of inhibition at the molecular level pending additional investigations. The use of such a model may eventually save time and expense involved with clinical trials and may allow detailed study of drug interactions when studies in humans cannot be conducted because of ethical considerations.

ACKNOWLEDGMENT

This work was supported by a grant from Warner-Lambert Co., Pharmaceuticals Division, Ann Arbor, Mich.

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