Cytochrome P-450 Complex Formation by Dirithromycin and Other Macrolides in Rat and Human Livers

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Some macrolide antibiotics cause clinical drug interactions, resulting in altered metabolism of concomitantly administered drugs, via formation of an inactive cytochrome P-450 complex. In the present study, the formation of a cytochrome P-450 type I binding spectrum and a metabolic intermediate complex by troleandomycin and dirithromycin was assessed in liver microsomes obtained from untreated rats and phenobarbital- or dexamethasone-pretreated rats. Troleandomycin produced a type I binding spectrum and metabolic intermediate complex in microsomes from dexamethasone- and phenobarbital-pretreated rats. Dirithromycin did not produce a detectable type I binding spectrum but formed a small cytochrome P-450 metabolic intermediate complex (6% of that formed by troleandomycin) in microsomes from dexamethasone-pretreated rats only. The formation of a cytochrome P-450 type I binding spectrum and a metabolic intermediate complex by troleandomycin, erythromycin, dirithromycin, and erythromycylamine was also assessed in microsomes prepared from human livers. Troleandomycin and erythromycin formed a type I binding spectrum and a metabolic intermediate complex which were larger in microsomes from subjects on barbiturate therapy than in microsomes from subjects with no recent barbiturate exposure. Erythromycylamine did not form a detectable type I binding spectrum with any of the human microsomal samples, but a small metabolic intermediate complex was formed with microsomes from a subject on phenobarbital, phenytoin, and propranolol therapy. Dirithromycin did not form a detectable type I binding spectrum or a metabolic intermediate complex in any human liver sample. Preclinical quantitation of the human metabolic intermediate complex may be helpful in predicting the possibility of clinical drug interactions of new drug candidates.

A number of classes of xenobiotic compounds such as amphetamines, arylamines, methylenedioxybenzenes, dioxolanes, and hydrazines interact with cytochrome P-450, resulting in the formation of a cytochrome P-450 type I binding spectrum and, subsequent to reduction of the drug enzyme complex with NADPH, a characteristic metabolic intermediate (MI) complex (10). Among those compounds producing cytochrome P-450 MI complexes are the macrolide antibiotics erythromycin, oleandomycin, troleandomycin, clarithromycin, and roxithromycin, although the latter two macrolides are weak formers of cytochrome P-450 MI complexes (13). Three additional macrolide antibiotics, josamycin, midecamycin, and spiramycin, do not form cytochrome P-450 MI complexes (4, 13). Cytochrome P-450 MI complex formation by erythromycin and troleandomycin results in the formation of a metabolically inactive cytochrome P-450 species (3, 11). Furthermore, administration of these macrolide antibiotics results in clinical pharmacokinetic interactions with steroids (8), theophylline (2), and cyclosporin (5). It was, therefore, of interest to determine if dirithromycin, a new macrolide antibiotic, and erythromycylamine, a metabolite of dirithromycin, would form a cytochrome P-450 MI complex similar to those of troleandomycin, erythromycin, and other macrolide antibiotics. In this article, we report on the relative abilities of troleandomycin and dirithromycin to form a cytochrome P-450 type I binding spectrum and an MI complex in vitro with hepatic microsomes from untreated and phenobarbital- or dexamethasone-pretreated rats. In addition, the relative abilities of troleandomycin, erythromycin, erythromycylamine, and dirithromycin to form a cytochrome P-450 type I binding spectrum and an MI complex in vitro with hepatic microsomes from patients with and without recent barbiturate and/or phenytoin drug exposure are reported.

MATERIALS AND METHODS

Dirithromycin (90.3% pure), erythromycin (>95% pure), erythromycylamine (92% pure), and phenobarbital sodium (>98% pure) were obtained from Eli Lilly & Co., and troleandomycin (>95% pure) was obtained from Pfizer. Dexamethasone (98% pure) and NADPH (tetrasodium salt, 98% pure) were purchased from Sigma Chemical Co., and dimethyl sulfoxide (high-performance liquid chromatography grade) was purchased from Burdick and Jackson. All other chemicals were of the highest grade available.

Male Sprague-Dawley rats (250 ± 10 g) were obtained from Harlan Industries, Indianapolis, Ind. Rats were administered phenobarbital sodium (60 mg/kg of body weight per day, intraperitoneally) in saline for 4 days prior to euthanasia or dexamethasone (150 mg/kg/day, intraperitoneally) in corn oil for 3 days prior to euthanasia. Animals were euthanatized by decapitation and allowed to exsanguinate. Hepatic microsomes were prepared by combining the livers from two untreated, two phenobarbital-pretreated, and two dexamethasone-pretreated rats. Livers were removed and perfused with ice-cold 1.15% potassium chloride and minced in 4 volumes of 0.25 M sucrose. The tissue was homogenized with a Polytron tissue homogenizer, and the homogenate was centrifuged at 5°C for 20 min at 9,000 × g in a Sorvall RC5-B centrifuge with an SS-34 rotor. The resultant super-

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nated fraction was then centrifuged at 5°C for 60 min at 105,000 x g in a Beckman L8-60M ultracentrifuge with a 50.2TI rotor. The subsequent microsomal pellet was resuspended in 1.15% potassium chloride and centrifuged at 5°C for 30 min at 105,000 x g. The washed microsomal pellet was resuspended in ice-cold 0.1 M potassium phosphate buffer (pH 7.4). Microsomal protein was assayed by the method of Lowry et al. (7), and microsomal fractions were diluted to 1 mg of protein per ml in 0.1 M potassium phosphate buffer (pH 7.4).

Human liver samples were obtained at surgery in accordance with protocols approved by the Committee for the Conduct of Human Research at The Medical College of Wisconsin, Milwaukee. All subjects had normal plasma bilirubin and transaminase levels. The ages, genders, smoking habits, and recent drug histories of the subjects are reported in Table 1. Hepatic microsomes were prepared as previously reported (17) and stored at -70°C in 100 mM potassium phosphate storage buffer (pH 7.4) containing 1.0 mM EDTA, 1 mM dithiothreitol, 20 μM butylated hydroxytoluene, and 20% (vol/vol) glycerol, with no intervening freeze-thaw cycles between preparation and use.

Hepatic microsomal fractions were assayed for formation of drug-cytochrome P-450 type I binding spectra and cytochrome P-450 MI complexes in a Varian Cary 210 spectrophotometer in 1-ml quartz cuvettes as follows. One-milliliter samples of microsomes from rats or patients (1 mg of protein per ml) were placed in reference and sample cuvettes maintained at 37°C, and the difference absorbance spectrum was scanned from 500 to 360 nm. Troleandomycin, dirithromycin, erythromycin, erythromycylamine, or dirithromycin in solution in dimethyl sulfoxide (3 μl) was added to the sample cuvette to a final concentration of 5 μM, an equivalent amount of dimethyl sulfoxide was added to the reference cuvette, and the difference absorbance spectrum was again scanned from 500 to 360 nm to monitor the type I binding spectrum (maximum, 390 nm; minimum, 418 nm). NADPH in phosphate buffer (40 mM, pH 7.4) was then added to both cuvettes to a final concentration of 0.6 mM. The ensuing cytochrome P-450 MI complex (ΔA_{456-490}) formed at 37°C was monitored by scanning the 500- to 420-nm spectrum at 0, 5, 15, and 25 min to confirm that maximal MI complex formation had occurred. An extinction coefficient of 64 mM−1 cm−1 was used to quantitate the cytochrome P-450 MI complex (10). All compounds were soluble in the microsomal suspensions. The assay of cytochrome P-450 3A in human hepatic microsomal samples was accomplished by immunooquantitation as previously reported (17).

**RESULTS**

The possibility of hepatic cytochrome P-450 MI complex formation by dirithromycin in microsomes from untreated, phenobarbital-pretreated, and dexamethasone-pretreated rats was investigated on the basis of the observation that some macroclide antibiotics cause cytochrome P-450 MI complex formation as evidenced by a 456-nm absorption peak in hepatic microsomes (9, 11). Troleandomycin readily formed a cytochrome P-450 type I binding spectrum with hepatic microsomes isolated from dexamethasone-pretreated rats and to a lesser extent with microsomes from phenobarbital-pretreated rats (Table 1). Troleandomycin did not form a detectable type I binding spectrum in microsomes from untreated rats. When NADPH was added to both cuvettes containing microsomes from dexamethasone-pretreated rats, the troleandomycin type I binding spectrum was abolished and a cytochrome P-450 MI complex with an ΔA_{456} peak at 456 nm formed. The ΔA_{456} peak increased with time of incubation at 37°C such that by 25 min the ΔA_{456-490} was 0.053 (Table 1), which corresponds to 0.83 nmol of complexed cytochrome P-450 per mg of protein. These results are similar to those reported by Wrighton et al. (16). In contrast to the results with troleandomycin, dirithromycin did not produce a cytochrome P-450 type I binding spectrum with microsomes from untreated, phenobarbital-pretreated, or dexamethasone-pretreated rats, and only a very small cytochrome P-450 MI complex was formed in microsomes from dexamethasone-pretreated rats upon addition of NADPH to the cuvettes (6% of that observed with troleandomycin). No cytochrome P-450 MI complex was detected after the addition of dirithromycin and NADPH to microsomes from untreated or phenobarbital-pretreated rats (Table 1).

Troleandomycin formed a cytochrome P-450 type I binding spectrum with hepatic microsomes isolated from patients who had received barbiturate and/or phenytoin therapy (subjects E and I), but only a very small type I binding spectrum was observed in subjects not exposed to barbiturate or phenytoin therapy (subjects A and B) (Table 2). When NADPH was added to microsomes containing troleandomycin, the type I binding spectrum was abolished and a cytochrome P-450 MI complex ΔA_{456} peak developed with time. MI complex formation was greatest in the microsomes from subjects E and I, i.e., the subjects who received barbiturate and/or phenytoin therapy; little or no cytochrome P-450 MI complex formation was observed in the two subjects with no recent barbiturate exposure. The percentages of enzyme bound as the cytochrome P-450 MI complex in microsomes from subjects A (17%), E (18%), and I (31%) after incubation with troleandomycin and NADPH were very similar to the total percentages of cytochrome P-450 3A isozyme determined by immunoblot analyses to be present in microsomes from the same subjects (20, 25, and 32%, respectively). Microsomes obtained from subject B contained 28% of the total cytochrome P-450 in the 3A form; however, no binding or MI complex was observed.

Although erythromycin formed a type I binding spectrum and a cytochrome P-450 MI complex in human hepatic microsomes, they were generally smaller than those observed with troleandomycin (Table 2). The largest type I binding spectrum and cytochrome P-450 MI complex formed by erythromycin were associated with microsomes from subject E, who had been recently exposed to the cytochrome

### Table 1. Type I binding spectrum and MI complex formation by troleandomycin and dirithromycin in hepatic microsomes from control and phenobarbital- and dexamethasone-induced rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Troleandomycin</th>
<th>Dirithromycin</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Type I binding</td>
<td>MI complex</td>
</tr>
<tr>
<td>Control</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Phenobarbital-pretreated</td>
<td>0.006</td>
<td>0.010</td>
</tr>
<tr>
<td>Dexamethasone-pretreated</td>
<td>0.038</td>
<td>0.053</td>
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</table>

a Hepatic microsomes were prepared from untreated, phenobarbital-pretreated, or dexamethasone-pretreated male Sprague-Dawley rats.
b For type I binding spectrum, ΔA_{456-490} was measured; for MI complex, ΔA_{456-490} was measured.
TABLE 2. Cytochrome P-450 type 1 binding spectrum and MI complex formation in human hepatic microsomes incubated with troleandomycin, erythromycin, erythromyclamine, and dirithromycin

<table>
<thead>
<tr>
<th>Subjecta</th>
<th>Total P-450 (nmol/mg of protein)b</th>
<th>P-450 3A isoyme (% of total P-450)c</th>
<th>Drugd</th>
<th>Formation of:</th>
<th>Type I binding spectrum ((\Delta A))f</th>
<th>Cytochrome P-450 MI complex ((\Delta A))g (nmol/mg of protein) (% of total P-450)h</th>
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<tbody>
<tr>
<td>A</td>
<td>0.29</td>
<td>20</td>
<td>TAO</td>
<td>0.002</td>
<td>0.003</td>
<td>0.05</td>
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<td></td>
<td></td>
<td></td>
<td>ERY</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DIR</td>
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</tr>
<tr>
<td>B</td>
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<td>28</td>
<td>TAO</td>
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<tr>
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<td></td>
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<td></td>
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<td></td>
<td>DIR</td>
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</tr>
<tr>
<td>E</td>
<td>0.61</td>
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<td>TAO</td>
<td>0.006</td>
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<tr>
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<td></td>
<td>DIR</td>
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<td>0.00</td>
</tr>
<tr>
<td>I</td>
<td>0.57</td>
<td>32</td>
<td>TAO</td>
<td>0.010</td>
<td>0.011</td>
<td>0.18</td>
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<td>DIR</td>
<td>0.000</td>
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a Patient drug history: A, 25-year-old male smoker with no recent drug history; B, 50-year-old male smoker with no recent drug history; E, 14-year-old male nonsmoker with a recent drug history of pentobarbital (coma induced 1 week before death), pancuronium bromide, dopamine, furosemide, mannitol, heparin, and cefazolin; I, 43-year-old male nonsmoker with a recent drug history of phenobarbital, phenytoin, and propranolol.
b Total microsomal cytochrome P-450 measured as the reduced carbon monoxide (450 to 490 nm) binding spectrum.
c Cytochrome P-450 3A isoyme content determined by immunoquantitation and expressed as percentage of total cytochrome P-450.
d TAO, troleandomycin; ERY, erythromycin; EMA, erythromyclamine; DIR, dirithromycin.
e Results are expressed as \(\Delta A_{450-490}\) per milligram of microsomal protein.
f Results are expressed as \(\Delta A_{450-490}\) per milligram of microsomal protein.

P-450-inducing agent pentobarbital; 10% of the total cytochrome P-450 and 40% of the cytochrome P-450 3A content were bound as the erythromycin–cytochrome P-450 MI complex. Dirithromycin did not form either a type I binding spectrum or a cytochrome P-450 MI complex in microsomes from any subject regardless of recent drug history. Similarly, erythromyclamine, a metabolite of dirithromycin, did not form a type I binding spectrum in microsomes from any of the four subjects and formed only a very small cytochrome P-450 MI complex in subject I; only 1% of the total cytochrome P-450 was bound as a cytochrome P-450 MI complex.

**DISCUSSION**

Cytochromes P-450 are a family of isozymes which are important in the metabolic clearance of numerous therapeutic agents, and some of these isozymes are inducible by a wide variety of drugs. The concentration and activity of the isozyme cytochrome P-450 3A are strongly induced by glucocorticoids such as pregnenolone-16a-carbonitile and dexamethasone (16). Additionally, macrolide antibiotics such as troleandomycin induce the hepatic concentration of cytochrome P-450 3A in rats (16), rabbits (1), and humans (14). This induction occurs primarily through stabilization of the enzyme as a drug metabolite-enzyme complex (i.e., MI complex) (15). Nevertheless, while the cytochrome P-450 3A concentration is induced by troleandomycin administration, formation of a cytochrome P-450 MI complex with a troleandomycin metabolite effectively inactivates the isozyme, leading to the pharmacokinetic drug interactions observed with this and other macrolide antibiotics. In the present study with microsomes isolated from rats pretreated with dexamethasone to induce hepatic cytochrome P-450 3A concentration, troleandomycin readily bound to the enzyme to form a cytochrome P-450 type I binding spectrum and when metabolized, subsequent to the addition of NADPH, formed a substantial and time-dependent cytochrome P-450 MI complex (Table 1 and Fig. 1). Phenobarbital is a less-potent inducer of cytochrome P-450 3A, and therefore incubation of troleandomycin with microsomes from phenobarbital-pretreated rats results in a smaller type I binding spectrum and a smaller cytochrome P-450 MI complex relative to those observed with dexamethasone-induced microsomes. Cytochrome P-450 3A is present in a very low concentration in microsomes from untreated rats, and therefore, a troleandomycin type I binding spectrum and a cytochrome P-450 MI complex were not observed in mi-

![FIG. 1. Formation of a cytochrome P-450 MI complex upon addition of troleandomycin and NADPH to rat hepatic microsomes.](http://aac.asm.org/)
cromes from control rats as has been noted previously (16). Contrary to the results with troleandomycin, dirithromycin did not form a type I binding spectrum with microsomes from dexamethasone-pretreated rats, and the cytochrome P-450 MI complex formed was negligible. Furthermore, dirithromycin did not form either a type I binding spectrum or a cytochrome P-450 MI complex in microsomes from untreated or phenobarbital-induced rats. Such a lack of interaction with cytochrome P-450 was suggested by previous observations in rats administered five 30-mg/kg/day doses of dirithromycin lactobionate. The isolation of hepatic microsomes and assay for a cytochrome P-450 MI complex resulted in no evidence of the formation of such a complex by dirithromycin in vivo (data not shown). Both troleandomycin and erythromycin have been shown to form cytochrome P-450 MI complexes in vivo in animals and humans (6, 12, 13).

Human subjects administered troleandomycin for 7 days have increased levels of hepatic cytochrome P-450, appreciable concentrations of which are present as a cytochrome P-450 MI complex (12). In the present in vitro study, troleandomycin formed a small type I binding spectrum and a small cytochrome P-450 MI complex in microsomes from one of the subjects without a recent history of exposure to known inducers of cytochrome P-450 3A (subject A). In contrast, troleandomycin formed an appreciable type I binding spectrum and an appreciable cytochrome P-450 MI complex in microsomes from both subjects who had had recent exposure to barbiturates and/or phenytoin, known inducers of cytochrome P-450 3A. Interestingly, the sizes of the troleandomycin-induced type I binding spectrum and cytochrome P-450 MI complex in microsomes from these barbiturate- and/or phenytoin-exposed subjects were very similar to those observed in microsomes from phenobarbital-dosed rats (Table 1).

The cytochrome P-450 3A content of the human hepatic microsomes used in this study was 20 to 32% of the total cytochrome P-450 content of the sample (Table 2). Wrighton et al. have shown that cytochrome P-450 3A is the specific isozyme induced by troleandomycin and bound to a troleandomycin metabolite which gives rise to the cytochrome P-450 MI complex (16). In this study, incubation of troleandomycin and NADPH with three of four human microsomal samples resulted in the formation of a cytochrome P-450 MI complex which was approximately the same concentration as that of the total cytochrome P-450 3A in each of the respective microsomal samples. These data suggest near-complete complexation of the cytochrome P-450 3A present in the human microsomal sample. Similar results have been reported with troleandomycin in rat hepatic microsomes (15).

Erythromycin also induces hepatic cytochrome P-450 concentration, leading to a cytochrome P-450 MI complex, and, as a result, exhibits pharmacokinetic drug interactions with theophylline and cyclosporin in humans (2, 5, 6). In the present studies, erythromycin resulted in a much lower degree of cytochrome P-450 MI complex formation than troleandomycin, which is consistent with the fewer pharmacokinetic drug interactions observed with erythromycin than with troleandomycin (6, 12).

Unlike troleandomycin and erythromycin, dirithromycin does not induce a type I binding spectrum or a cytochrome P-450 MI complex in hepatic microsomes from subjects with or without recent barbiturate and/or phenytoin exposure (Table 2). Similarly, erythromycinylamine, a metabolite of dirithromycin, does not produce a type I binding spectrum in microsomes from any of the subjects examined. Erythromycinylamine produced only a very small cytochrome P-450 MI complex in microsomes from only one of the four subjects tested, and that subject had recently been exposed to the cytochrome P-450-inducing agents phenobarbital and phenytoin.

The results of these in vitro studies with rat and human hepatic microsomes may have significant clinical relevance. The basis of the pharmacokinetic interaction of several macrolide antibiotics with other drugs is considered to be the binding of a macrolide metabolite to, and inactivation of, the cytochrome P-450 3A isozyme involved in the metabolism of drugs such as ergotamine, glucocorticoids, theophylline, oral contraceptives, and cyclosporin (9, 13). Human cytochrome P-450 3A is also responsible for the metabolism of nifedipine and other dihydropryridines, lidocaine, quinidine, midazolam, triazolam, and lovastatin (18). Troleandomycin and erythromycin bind to cytochrome P-450 3A and form an inactive cytochrome P-450 MI complex, which results in pharmacokinetic interactions with various drugs which are metabolized by cytochrome P-450 3A. The data presented herein demonstrate that dirithromycin does not bind to cytochrome P-450 or induce a cytochrome P-450 MI complex with human liver samples. It is, therefore, expected that there is little or no probability of dirithromycin causing the clinical pharmacokinetic drug interactions characteristic of other macrolide antibiotics, which produce a cytochrome P-450 MI complex and alter metabolic clearance of coadministered therapeutic agents.

REFERENCES

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