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Short Communication

ENOXACIN IS AN INDUCER OF CYP1A2 IN RAT LIVER

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Abstract—The induction of cytochrome P450 by enoxacin, ciprofloxacin, and ofloxacin was investigated in female Wistar rats. Animals were treated orally with daily doses ranging from 10 to 400 mg enoxacin per kg body wt, 400 mg ciprofloxacin, or 400 mg ofloxacin per kg body wt for up to 7 days. Activities of methoxyresorufin O-demethylase (MROD) and ethoxyresorufin O-deethylase (EROD) were determined fluorimetrically in hepatic microsomes. MROD activity was increased 2.6-fold after treatment with 100 mg enoxacin per kg body wt for 7 days. Lower doses of enoxacin did not induce MROD activity significantly. Antipeptide antibodies directed specifically against different rat cytochrome P450 enzymes demonstrated that CYP1A2, but not CYP1A1, was induced in rats treated with enoxacin. After ciprofloxacin or ofloxacin treatment, no induction of MROD or EROD activity was observed. Neither ciprofloxacin nor ofloxacin caused any change in CYP1A1 or CYP1A2 apoprotein levels. Further investigations with antipeptide antibodies showed that there was no induction of CYP2B1, CYP2B2, CYP2E1, CYP3A1, CYP3A2, CYP4A1, or CYP4A2 following treatment with enoxacin, ciprofloxacin, or ofloxacin. It is concluded that enoxacin, but not ciprofloxacin or ofloxacin, is an inducer of CYP1A2 in rat liver.

Key words: quinolones; induction; CYP1A2; ethoxyresorufin; methoxyresorufin

Fluoroquinolones are important in antimicrobial drug therapy due to their broad antibacterial spectrum and favourable pharmacokinetics [1]. Experience from the therapeutic use of these antimicrobial agents shows that some (e.g. enoxacin and ciprofloxacin) interfere with the metabolism of concomitantly administered drugs such as theophylline. Under such conditions, adverse effects are due to increased theophylline levels [2–5]. Studies with human liver microsomes [6, 7], rat liver microsomes [8], and with cell lines expressing specific cytochrome P450 enzymes have shown that enoxacin specifically inhibits CYP1A2 activity [9].

Many inhibitors of monooxygenases have a biphasic effect on enzyme activity. After an initial period of lowered activity follows a phase of increase in activity commensurate with enzyme induction [10, 11]. However, it has been reported previously that hepatic microsomal ethoxycoumarin O-deethylase, benzphetamine N-demethylase, and aniline hydroxylase activities were not elevated following treatment of rats with enoxacin, norfloxacin, or ofloxacin [12]. Thus, it was concluded that fluoroquinolones are not inducers of cytochromes P450. However, none of the enzyme activities measured are catalyzed predominantly by CYP1A2, the enzyme which is inhibited. Therefore, the possible inductive capacity of fluoroquinolones was reassessed using enzyme reactions relatively specific for CYP1A enzymes, in particular, MROD‡ and EROD [13–15]. In addition, the effect of fluoroquinolones on the levels of several cytochrome P450 enzymes, including CYP1A1 and CYP1A2, were determined using antipeptide antibodies directed specifically against each enzyme.

Materials and methods

Chemicals. Ethoxyresorufin was synthesized from resorufin as described previously [16]. Methoxyresorufin was obtained from Molecular probes (Eugene, OR). Resorufin was purchased from Aldrich-Chemie (Steinheim, Germany). The suppliers of electrophoresis and immunoblotting reagents are stated elsewhere [17, 18].

Treatment of rats. Female Wistar rats (Bor: Wisw/spf, TNO) weighing 200–220 g were purchased from Winkelmann (Borchen, Germany). The fluoroquinolones were suspended in a 2% starch solution (w/v) and administered by gavage. Five animals per group received daily doses of 10, 30, 100, 200, or 400 mg enoxacin/kg body wt, 400 mg ciprofloxacin/kg body wt, or 400 mg ofloxacin/kg body wt for 7 days, or 200 mg enoxacin/kg body wt daily for 1, 3, 5, or 7 days. Control animals were treated with a 2% starch solution only. After receiving the final dose, the animals were starved overnight and killed the next day. Rats treated with 100 ng TCDD/kg body wt were used as a positive control for the induction of CYP1A1 and CYP1A2 [19]. In the immunoblotting experiments microsomes from rats treated with phenobarbital (PB), pregnenolone-16α-carbonitrile (PCN), isoniazid (INH), or clofibric acid (CFA) were used as reference. The treatment protocols have been described elsewhere [20]. The preparation of microsomal fraction has been described previously [19]. Measurement of EROD and MROD activities was performed directly after the microsomal fraction had been prepared.

Analytical procedures. Enzymatic measurements were performed fluorimetrically as described elsewhere [19]. The substrate concentrations used were 0.5 μM for ethoxyresorufin and 1 μM for methoxyresorufin. The protein content was measured by a Biuret-based method, using an automatic bichromatic analyzer (ABA 100, Abbott Diag. Products, Wiesbaden, Germany).

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‡ Abbreviations: CFA, clofibric acid; EROD, ethoxyresorufin O-deethylase; INH, isoniazid; MROD, methoxyresorufin O-demethylase; PB, phenobarbital; PCN, pregnenolone-16α-carbonitrile; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Immunoblotting was performed essentially as described previously [17], with modifications described elsewhere [18], using polyclonal antipeptide antibodies directed against rat CYP1A1 [17], CYP1A2 [21], CYP2E1 [22], CYP2B1/2, CYP3A1, CYP3A2, or CYP4A1/2. Antibodies directed against CYP2B1/2, CYP3A1, CYP3A2, and CYP4A1/2 were produced by immunising rabbits with the peptides Ile-Asp-Thr-Tyr-Leu-Leu-Arg-Met-Glu-Lys-Glu-Lys, Ile-Ile-Thr-Gly-Ser, Val-Ile-Asn-Gly-Ala, and Leu-Lys-Lys-Leu-His, respectively, by methods described previously [22, 23]). Quantification of CYP1A2 was performed as described elsewhere [18] using appropriate dilutions of microsomal fractions.

Results are presented as the mean ± SEM. Statistical significance was evaluated by Student's *t*-test using the Minitab software (Pennsylvania State College, University Park, PA, 1987).

Results and Discussion

Hepatic microsomal MROD and EROD activities. After 7 days' treatment of groups of rats with a range of doses of enoxacin, hepatic microsomal MROD and EROD activities were determined. A slight but significant increase in EROD activity (1.6-fold) was observed in rats receiving 30 mg enoxacin/kg body wt; however, this dose had no influence on the MROD activity (Fig. 1). Higher doses of enoxacin had no further effect on EROD activity, but caused a significant elevation of MROD activity (Fig. 1). MROD activity reached a maximum following treatment with 200 mg enoxacin/kg body wt, which corresponds to a 3.6-fold induction compared to the control group (Fig. 1).

The dose exhibiting maximum induction of MROD activity was selected for a time course study. A single dose of 200 mg enoxacin/kg body wt induced hepatic microsomal MROD activity by 2.6-fold; further treatment for 3, 5, and 7 days increased the activity 3.3-, 3.5-, and 3.6-fold, respectively (data not shown). EROD activity was induced after a single dose (1.9-fold), but treatment for longer periods did not further increase this activity (data not shown).

The effects of ciprofloxacin and ofloxacin were compared to the effect of enoxacin following administration for 7 days at a dose of 400 mg/kg body wt. Whereas the group of rats treated with enoxacin had a 3.0-fold elevation of hepatic microsomal MROD activity, neither treatment with ciprofloxacin nor ofloxacin caused any increase in MROD activity (Table 1). EROD activity in rats treated with enoxacin was significantly increased compared to the control group. In contrast, both ciprofloxacin and ofloxacin caused a slight but significant reduction in EROD activity (Table 1). In the group of rats treated with 100 ng TCDD/kg body wt, EROD activity was induced more potently (24-fold) than MROD activity (3.7-fold). MROD activity in this group was similar to that measured in the enoxacin-treated group (Table 1).

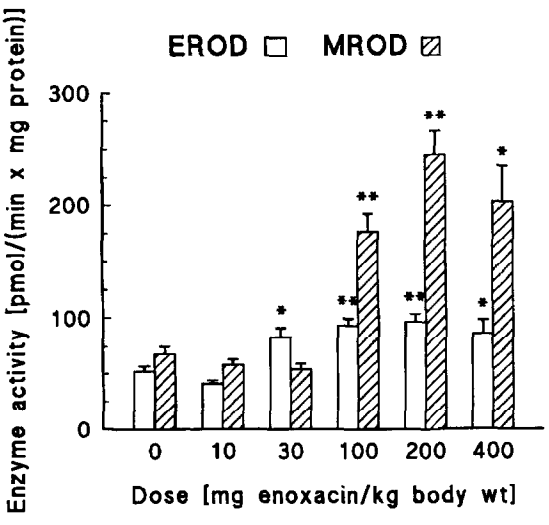

Fig. 1. The effect of the dose of enoxacin on hepatic microsomal EROD (□) and MROD (▨) activities in female Wistar rats. Rats were treated daily for 7 days with the doses of enoxacin indicated. The results shown are means ± SEM for 5 animals. *Significantly different from control animals at $P < 0.05$. **Significantly different from control animals at $P < 0.01$.

Immunoblotting with antipeptide antibodies. Immunoblotting using a specific antibody against rat CYP1A2 confirmed that this enzyme was present in hepatic microsomal fractions of untreated rats (Fig. 2). Higher levels of CYP1A2 apoprotein were observed following treatment with 400 mg enoxacin/kg body wt or 100 ng TCDD/kg body wt (Table 1, Fig. 2). However, the level in rats treated with ciprofloxacin or ofloxacin was similar to untreated rats (Table 1, Fig. 2).

CYP1A1 was not detected either in control rats or rats treated with fluoroquinolones. This enzyme was present after treatment with TCDD (Fig. 2). Immunoblotting was also performed using antibodies directed against rat CYP2B1/2, CYP2E1, CYP3A1, CYP3A2, CYP4A1/2. Administration of fluoroquinolones failed to produce any change in the amount of the apoproteins in hepatic microsomal fraction. The reactivity of the antibodies to their target P450 enzymes is demonstrated using hepatic microsomal fractions from rats treated with appropriate inducing compounds, PB for CYP2B1 and CYP2B2, INH for CYP2E1, PCN for CYP3A1 and CYP3A2 and CFA for CYP4A1 and CYP4A2 (Fig. 2). The pattern of induction of hepatic microsomal EROD and MROD activities in enoxacin-treated and TCDD-treated rats is similar to the respective expression of CYP1A1 and CYP1A2 apoproteins. This indicates that MROD activity is suitable for the determination of CYP1A2 activity. In the untreated rat and in rats treated with polycyclic aromatic hydrocarbons, MROD activity is catalyzed mainly by CYP1A2 [13, 15]. However, EROD activity that is catalysed by CYP1A1 in rats treated with polycyclic aromatic hydrocarbons is catalysed by CYP2C6 in untreated rats [15]. In contrast to the effect of enoxacin, neither ciprofloxacin nor ofloxacin were found to be inducers of MROD activity or CYP1A2 apoprotein. Thus, it appears that amongst the three fluoroquinolones studied here, the ability of enoxacin to inhibit enzyme activity may be related to its ability to induce CYP1A2.

General discussion. At the present time, the mechanism of induction of CYP1A2 is poorly understood [24]. Although there is a mechanism for CYP1A2 regulation via the *Ah* receptor [25], this does not always occur. Isosafrole is a model compound used in studies of CYP1A2 induction, and it potently induces CYP1A2 through a non-*Ah* receptor mechanism [26]. Interestingly, isosafrole, like enoxacin, is an inhibitor of CYP1A2 activity. Possible mechanisms could involve post-translational stabilization of specific P450 forms as well as mRNA.

More recently, musk xylene has been claimed to be a specific inducer of CYP1A2 [27]. However, it is evident from their work that this compound also induces CYP1A1. In addition, no data has been presented for the induction of P450 forms other than CYP1A1 or CYP1A2 [27]. Thus, it is difficult to conclude that musk xylene is a specific inducer of CYP1A2.

The results of the present study show the importance of using specific methods for the determination of enzyme induction. Okazaki and coworkers [12] measured relatively nonspecific monooxygenase activities, none of which is catalysed predominantly by CYP1A2, and consequently were unable to detect any induction of cytochrome P450. In contrast, here, MROD activity was measured that is catalysed principally by CYP1A2 [13, 14]. In addition, monospecific antipeptide antibodies targeted to various cytochrome P450 enzymes were used to study the levels of a number of cytochromes P450 including CYP1A2. Both approaches clearly show induction of CYP1A2 in rat liver following treatment with enoxacin, but not ciprofloxacin or ofloxacin.

To assess the relevance of these results for humans, comparisons have to be made on the basis of pharmacokinetic param-

Table 1. CYP1A2 content, MROD, and EROD activities in hepatic microsomes of rats after pretreatment with different fluoroquinolones

Substance	Dose [mg/kg body wt]	N	CYP1A2 [pmol CYP1A2/mg prot]	MROD [pmol resorufin/mg prot/min]	EROD [pmol resorufin/mg prot/min]
Control	–	10	47 ± 6	68 ± 7	52 ± 5
Ciprofloxacin	400	5	70 ± 9	46 ± 3*	27 ± 1†
Ofloxacin	400	5	67 ± 7	59 ± 5	34 ± 1†
Enoxacin	400	5	134 ± 13†	202 ± 33*	86 ± 12*
TCDD	10^{-4}	3	187 ± 27	251 ± 124	1270 ± 404*

Rats were treated with daily doses of fluoroquinolones for seven days. A single subcutaneous dose of TCDD was applied seven days before killing. Five animals of the control group were investigated for CYP1A2 content. The results shown are means ± SEM.
* $P < 0.05$ compared with the control group.
† $P < 0.01$ compared with the control group.

eters. Enoxacin is used with a therapeutic dose of 400 mg per patient, yielding plasma peak concentration of 3 mg/L [28]. In the rat, after dosing with 50 mg/kg body wt, peak plasma concentrations were found to be 1.5 mg/L [29]. Assuming a linear relationship between dose and peak plasma concentration, a dose of 100 mg/kg body wt, which is sufficient to induce MROD activity, should yield a plasma concentration comparable to the values determined in patients. Up until now, induction of CYP1A2 in humans has not been reported. However, as previous studies were designed to investigate potential interactions of enoxacin with other drugs, any inductive effect would not be found, because of the presence of enoxacin, which also inhibits enzyme activity. Induction of CYP1A2 could only be demonstrated if enoxacin is first eliminated. In our rat study the time between the last dose and the investigation was 24 hours. This corresponds to more than eight elimination half-lives [29].

As the inhibitory potency of enoxacin in the rat [8] is similar to that in human [7], it would be interesting to investigate if enoxacin is an inducer of CYP1A2 not only in rat liver, but also in human liver.

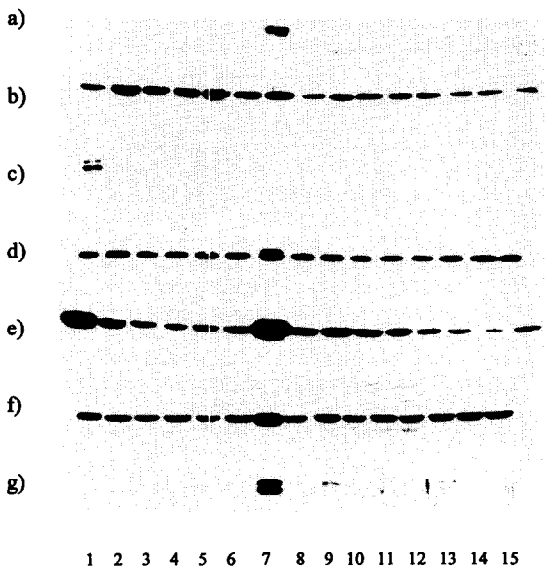

Fig. 2. Immunoblot of rat liver microsomes using antipeptide antibodies against various cytochromes P450. Hepatic microsomal proteins were resolved by SDS-PAGE, transfered to nitrocellulose filters and developed with the following antibodies: (a) anti-CYP1A1, (b) anti-CYP1A2, (c) anti-CYP2B1/2, (d) anti-CYP2E1, (e) anti-CYP3A1, (f) anti-CYP3A2, and (g) anti-CYP4A1/2. In each blot, microsomal protein was used from individual rats treated with 80 mg PB/kg body wt by intraperitoneal injection for 4 days (lane 1), 400 mg enoxacin/kg body wt by gavage for 7 days (lanes 2–6), vehicle, i.e. 2% (w/v) starch by gavage for 7 days (lanes 8 and 9), 400 mg ofloxacin/kg body wt by gavage for 7 days (lanes 10–12), and 400 mg ciprofloxacin/kg body wt by gavage for 7 days (lanes 13–15). In the different blots, lane 7 contained samples appropriate to each of the antibodies used. Thus, microsomal protein was used from rats treated with 100 ng TCDD/kg body wt by subcutaneous injection (a and b), vehicle, as described above (c), 0.1% (w/v) INH in the drinking water for 11 days followed by a single intraperitoneal injection of 50 mg INH/kg body wt (d), 100 mg PCN/kg body wt by intraperitoneal injection for 3 days (e and f), and 200 mg CFA/kg body wt by intraperitoneal injection for 3 days (g). The amount of protein loaded varied between blots and was (a) 5 µg, (b) 20 µg, (c) 25 µg except lane 1, which was 5 µg, (d), 5 µg, (e) 20 µg, (f) 25 µg except lanes 1 and 7, which contained 10 µg and (g) 10 µg.

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