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Selective In-vitro Inhibition of Hepatic Oxidative Metabolism by Quinolones: 7-Ethoxyresorufin and Caffeine as Model Substrates

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Abstract—The in-vitro inhibition of several metabolic pathways has been studied in 3-methylcholanthrene-treated rats. The specificity of the 7-ethoxyresorufin *O*-de-ethylase reaction has been determined in the presence and absence of ciprofloxacin, enoxacin, norfloxacin, ofloxacin, nalidixic acid, oxolinic acid and pipemidic acid. For the caffeine N^3-demethylation reaction, enoxacin and pipemidic acid were used. Enoxacin (IC50 = 105 μM, K_i = 65 μM) and pipemidic acid (IC50 = 115 μM, K_i = 160 μM) significantly inhibited 7-ethoxyresorufin *O*-de-ethylase reaction and caffeine N^3-demethylation (IC50 = 60 μM for enoxacin and IC50 = 185 μM for pipemidic acid) by a competitive mechanism. Other quinolones had lower or no (ofloxacin) inhibitory capacity. The order of inhibitory activity observed is in agreement with results obtained previously from in-vivo studies in man. No activity was detected towards ethylmorphine *N*-demethylation.

Some antibiotics of the quinolone family can alter the clearance of methylxanthines like caffeine (Staib et al 1987; Carbó et al 1989) at the metabolic level. Caffeine has been used in previous metabolic drug interaction studies (Desmond et al 1980; Segura et al 1986, 1989), and we have used it here as a model compound to study the quinolones-methylxanthines interactions because of its widespread daily consumption, which may have toxicological relevance. The primary pathway of its metabolism in rat and man is *N*-demethylation (Wietholtz et al 1981; Grant et al 1983), which takes place almost exclusively in the liver by the hepatic microsomal oxidative system (cytochrome P450), mediated by isozymes related to the biotransformation of polycyclic aromatic hydrocarbons (Kotake et al 1982; Campbell et al 1987; Grant et al 1987). The present study investigates the in-vitro inhibitory potency of several quinolones (Fig. 1) on the primary metabolic pathway of caffeine in the rat (N^3-demethylation to paraxanthine). The enzymatic reaction of 7-ethoxyresorufin *O*-de-ethylase (EROD) (Khanna et al 1972; Campbell et al 1987) has been used previously to study the mechanism involved. The specificity of quinolones as inhibitors of microsomal hepatic activity has also been studied with model substrates.

Material and Methods

Chemicals

[8-^{3}H]Caffeine (spec. act. 22.2 Ci mmol^{-1}, 98% purity) was obtained from Amersham, UK. 3-Methylcholanthrene (3-MC), NADH, NADP, isocitric dehydrogenase, 7-ethoxyresorufin (7-ER), benzo[a]pyrene (BP), aniline, 4-aminophenol, cytochrome c, caffeine, theophylline, theobromine, paraxanthine and 1,3,7-trimethyluric acid (137-TMU) were from Sigma Chemical Co. (St. Louis, MO, USA). Isocitric acid and $MgSO_4 \cdot 7H_2O$ were from Merck (Barcelona, Spain) and resorufin from Aldrich Chemie (Steinheim, Germany). Bio-Rad Protein Assay was from Bio-Rad Laboratories (München, Germany). Sodium phenobarbitone was from Laboratorios Miquel (Barcelona, Spain) and the quinolones (nalidixic acid, oxolinic acid, pipemidic acid, enoxacin, ciprofloxacin, norfloxacin and ofloxacin, were provided by Dr C. Roy (IMIM, Barcelona, Spain).

Animals

Male Wistar rats, 225–250 g, (Panlab S.L., Barcelona, Spain), were housed in plastic cages and allowed free access to food and water. Four groups of rats (n = 6) received either sodium phenobarbitone 80 mg kg^{-1} in 0·9% NaCl (saline) (i.p.), or 3-MC, 25 mg kg^{-1} in corn oil (i.p.), once daily for three days. Control animals for each group received saline or corn oil alone, respectively.

Preparation of microsomes

After the last injection the animals were starved for 10 h and then decapitated. Livers were perfused in-situ with ice-cold 50 mM Tris-HCl buffer, pH 7·4 (containing 0·154 M KCl and 3 mM EDTA), rapidily excised, blotted dry, weighed and minced. All liver fractions from the same treatment group were pooled and homogenized with the same buffer (1 : 4 w/v). Microsomal fractions were separated by differential ultracentrifugation (Lake 1987) and stored at −80°C until use.

Assays for microsomal characterization

The following three measurements were taken; microsomal protein (Bradford 1976) using a commercial kit (Bio Rad Protein Assay), total cytochrome P450 content (Omura & Sato 1964a,b), and NADPH cytochrome c reductase (Lake 1987). Four determinations of mixed function oxidase enzyme activities were carried out; benzo[a]pyrene hydroxylase (AHH) using the method of Nebert & Gelboin (1968);

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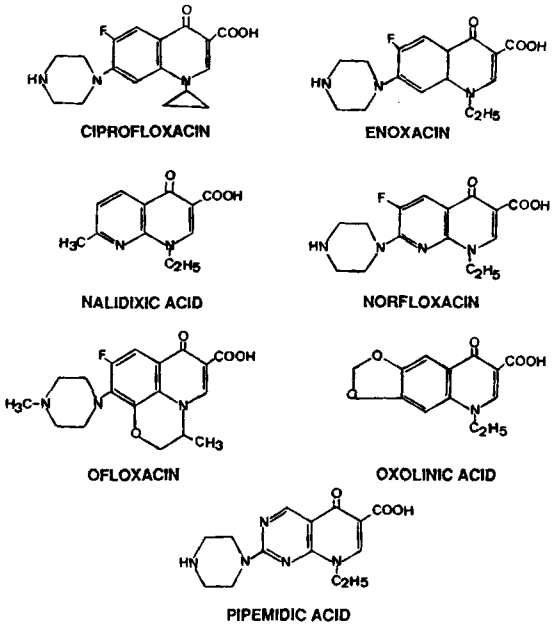

FIG. 1. Seven quinolones used.

and aniline hydroxylase, ethylmorphine *N*-demethylase and 7-ethoxyresorufin *O*-de-ethylase (EROD) according to Lake (1987), except for the NADPH generating system which consisted of NADP (0·5 mM), (±)-isocitric acid (5 mM), isocitric dehydrogenase (1 unit) and $MgSO_4 \cdot 7H_2O$ (5 mM) in a 2 mL final incubation volume.

In-vitro caffeine assay

In-vitro metabolism of caffeine was measured by a modification of the method of Bonati et al (1980) and results were calculated on the basis of the major metabolite, paraxanthine. Two μCi (90 nmol) [8-^{3}H]caffeine plus unlabelled caffeine (0 to 1000 μM) (100 μL), microsomal fraction induced with 3-MC (1 mg mL^{-1}) and 0·3 M phosphate buffer, pH 7·4, in a final volume of 1 mL, were incubated at 37°C for 30 min with the NADPH-generating system using NADP (1 mM). Reactions were stopped by cooling on ice and adding 1 g $(NH_4)_2SO_4$. Ten μL of authentic standards of 1-MX, theophylline, theobromine, paraxanthine, 137-TMU and caffeine were added to visualize absorbance peaks during chromatography. The aqueous reaction mixture was extracted with 10 mL of ethyl acetate–chloroform–isopropanol (45:45:10 v/v/v), organic extracts evaporated under nitrogen at 40°C, and the residue redissolved in 50 μL of mobile phase for chromatographic analysis. Reconstituted samples (20 μL) were injected onto a reversed-phase C_{18} column (Ultrasphere ODS 5 μm; 0·46 × 25 cm, Beckman). The mobile phase composition was 84% (v/v) (0·05%) acetic acid, 14% (v/v) methanol and 2% (v/v) acetonitrile. Elution and separation of caffeine and metabolites was monitored together with added standards by recording UV absorption at 280 nm. The flow rate was 1·2 mL min^{-1}. Retention times for 1-MX, theobromine, paraxanthine, theophylline and caffeine were 4·81, 5·6, 8·88, 9·83 and 18·29 min, respectively. 137-TMU (retention time 11·87 min) was not detected.

Column effluent was collected and counted on a liquid scintillation counter (LKB-Wallac Model 1214). Chromatography was performed using a Beckman liquid chromatographic modular system, consisting of a 112 solvent delivery module, a 340 organiser and a 165 variable wavelength detector, with an integrator LCI-100, (Perkin-Elmer).

Kinetics assays

The kinetics of EROD was evaluated using different concentrations of the substrate 7-ER [55 to 800 nM] and microsomal fraction induced with 3-MC (10 μg). K_m and V_{max} were calculated using Augustinsson-Hofstee plots (Segel 1976). The kinetics of caffeine *N*-demethylase was also evaluated using different concentrations of the unlabelled substrate caffeine (0–1000 μM) and the microsomal fraction induced with 3-MC (1 mg) and the K_m and V_{max} values were calculated as above.

Inhibition studies

Ethylmorphine N-*demethylase.* Inhibition of ethylmorphine *N*-demethylase activity, using the microsomal fraction induced by sodium phenobarbitone, was determined by calculation of the IC50 for the reaction using enoxacin (0 to 500 μM) as inhibitor. The inhibitor, dissolved in methanol, was added (100 μL) at the start of the reaction and IC50 values were obtained by plotting reciprocal of % activity of reaction versus concentration of inhibitor for the substrate concentration of 50 mM.

Ethoxyresorufin O-*de-ethylase.* Inhibition of enzymatic activity of EROD, using the microsomal fraction induced by 3-MC, was carried out in two phases:

Calculation of the IC50. Different concentrations of the quinolones as inhibitors were: nalidixic acid (0 to 500 μM), oxolinic acid (0 to 250 μM), pipemidic acid (0 to 500 μM), enoxacin (0 to 250 μM), ciprofloxacin (0 to 500 μM), ofloxacin (0 to 1000 μM) and norfloxacin (0 to 1000 μM). The inhibitors (dissolved in methanol) were added (100 μL) at the start of the reaction, and the substrate concentration was maintained at 5 mmol. The IC50 values were obtained by plotting reciprocal of % activity of reaction versus concentration of inhibitor.

Calculation of the K_i. The K_i values of the quinolones were calculated using three different concentrations of substrate ([S]) according to Dixon plots (these concentrations were K_m, $K_m/2$ and $2K_m$ of the EROD reaction values).

Activity of EROD was measured in the presence of the following quinolones (100 μL, methanolic solutions): enoxacin [0 to 400 μM], pipemidic acid [0 to 200 μM] and norfloxacin [0 to 200 μM]. The inhibition type was estimated by the replotting of Dixon plots (slope versus 1/[S]) (Segel 1976).

Caffeine N-*demethylase.* The inhibition study of the enzymatic activity of caffeine *N*-demethylase, using microsomal fraction induced by 3-MC, was carried out by calculation of the IC50 for the reaction using enoxacin (0 to 150 μM) and pipemidic acid (0 to 300 μM) as inhibitors. The inhibitors (dissolved in methanol) were added (100 μL) at the start of the reaction. The unlabelled substrate concentration was 4 μM and the IC50 values were again obtained by plotting the

Table 1. Rat hepatic microsomal characterization.

Group*	Protein (1)	Cytochrome P450 (2)	NADPH-cytochrome c reductase (3)	Ethyl-morphine *N*-demethylase (4)	Aniline hydroxylase (5)	AHH (6)	EROD (7)
Control	17·80	0·85 (449·8)	217·80	1200·20	36·80	1·40	0·15
Phenobarbitone	23·40	2·40 (450·0)	250·40	2443·30	119·90	2·20	0·05
Control	15·50	0·95 (449·3)	117·70	588·00	22·10	1·60	0·05
3-MC	23·80	1·40 (448·2)	120·00	582·60	71·50	5·80	5·30

* Mean data (n = 6).
Data are expressed in the following units: (1) mg (g liver)$^{-1}$; (2) nmol (mg prot.)$^{-1}$ (λ); (3) nmol cytochrome c (reduced form) min^{-1} (mg prot.)$^{-1}$; (4) nmol formaldyhide h^{-1} (mg prot.)$^{-1}$; (5) nmol *p*-aminophenol h^{-1} (mg prot.)$^{-1}$, (6) nmol 3-OH benzo[a]pyrene min^{-1} (mg prot.)$^{-1}$ (7) nmol resorufin min^{-1} (mg prot.)$^{-1}$.

reciprocal of % activity of reaction versus inhibitor concentrations.

In each assay, control activity was measured in the presence of pure diluent for the appropriate inhibitor. All assays were performed in duplicate.

Results

Table 1 shows the hepatic microsomal characterization for the different assays. A high specificity for the EROD reaction was observed from the hepatic microsomal fraction induced by 3-MC. The kinetics of EROD and caffeine *N*-demethylase

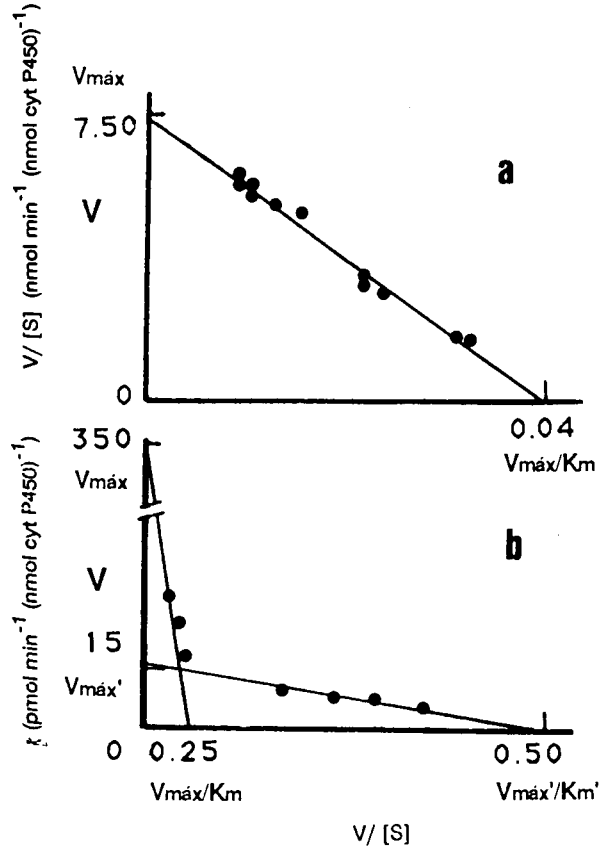

FIG. 2. (a) Kinetics of EROD reaction [V_{max} = 7·50 nmol resorufin min^{-1} (nmol cyt.P450)$^{-1}$, K_m = 190·00 nM]. (b) Kinetics of caffeine *N*-demethylase reaction [V_{max} = 350·00 pmol min^{-1} (nmol cyt.P450)$^{-1}$, K_m = 1330·00 μM, V_{max}' = 16·50 pmol min^{-1} (nmol cyt.P450)$^{-1}$, K_m' = 39·50 μM]. Microsomes of 3-MC induced fraction were used in both cases.

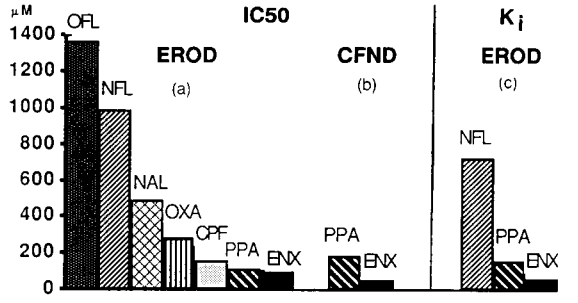

FIG. 3. Inhibition study. (a) IC50 of the EROD reaction; values: enoxacin (ENX) 105 μM; pipemidic acid (PPA) 115 μM; ciprofloxacin (CPF) 160 μM; oxolinic acid (OXO) 280 μM; nalidixic acid (NAL) 490 μM; norfloxacin (NFL) 1000 μM and ofloxacin (OFL) 1400 μM. (b) IC50 of the caffeine *N*-demethylase (CFND) fraction; values: ENX 60 μM; PPA 185 μM. (c) K_i of the EROD reaction; values: ENX 70 μM; PPA 165 μM; NFL 730 μM. Microsomes of 3-MC induced fraction were used in all cases.

are illustrated in Fig. 2 where EROD shows a linear activity while caffeine *N*-demethylase had biphasic behaviour with two sets of values for K_m and V_{max}. The IC50 assays for EROD show a gradation in the potency of quinolone inhibition (Fig. 3a). Enoxacin and pipemidic acid had a marked inhibitory effect, ciprofloxacin had a significant effect, oxolinic acid and nalidixic acid showed a lower inhibitory capacity, norfloxacin has only a slight effect and ofloxacin had the lowest. The K_i assay for EROD showed a similar gradation of inhibition to the IC50 assay (Fig. 3c). Of the three quinolones tested, enoxacin and pipemidic acid had a strong effect whilst norfloxacin had only a weak effect. The type of inhibition appears to be competitive in the three cases (Fig. 4). Inhibitory effects of enoxacin and pipemidic acid were also seen in the IC50 caffeine *N*-demethylase reaction. Enoxacin had an inhibitory capacity three times greater than pipemidic acid in the N^3-demethylation pathway (Fig. 3b). No effect was observed for enoxacin inhibition (0 to 500 μM) for ethylmorphine *N*-demethylase.

Discussion

The results show the selectivity in the inhibitory capacity of several quinolone antibiotics with regards to the enzymatic activity of cytochrome P450 in the rat. While quinolones are ineffective inhibitors of the cytochrome P450 reaction of ethylmorphine *N*-demethylase (isozyme P450 IIIA) (Gonzalez 1989), they are powerful inhibitors of the cytochrome

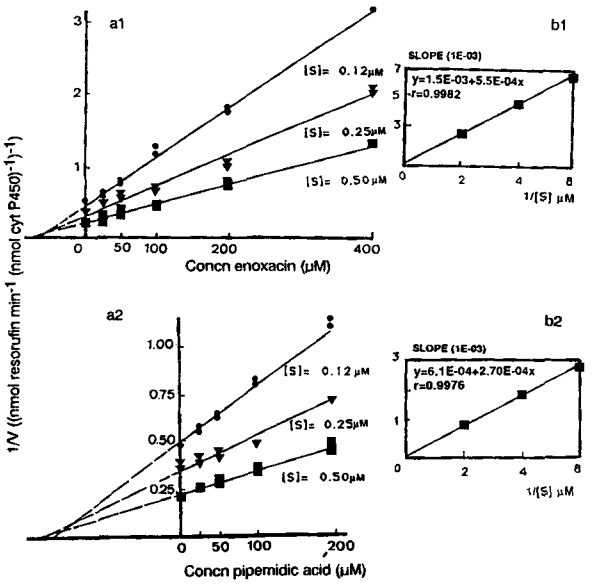

FIG. 4. Inhibition study of the EROD reaction using microsomes of 3-MC induced reaction. (a) calculation of the K_i by the Dixon method: (a1) enoxacin, (a2) pipemidic acid. The K_i for norfloxacin was calculated by the same method. (b) Replots of the slopes from the Dixon plots to study the type of inhibition: (b1) enoxacin (b2) pipemidic acid. The results for norfloxacin were: $y = 2{\cdot}20E-04+6{\cdot}20E-06x$, $r = 0{\cdot}9983$.

P450 isozyme induced by polycyclic aromatic hydrocarbons (PAH) (P450 IA).

The EROD reaction was described previously as highly selective for the isozyme induced by PAH of cytochrome P450 that also appears to be the metabolic pathway used by xanthines both in rat and man (Burke & Mayer 1974; Phillipson et al 1984; Campbell et al 1987). In view of these data, our observation that enoxacin and pipemidic acid are approximately equipotent inhibitors for both *O*-de-ethylation of 7-ER and N^3-demethylation of caffeine in rat hepatic microsomes induced by PAH, suggests that both the substrates 7-ER and caffeine share a common metabolic pathway, which appears to be inhibited by some quinolone antibiotics. Additionally, the EROD reaction seems to be a useful tool to study the metabolic pathways of caffeine.

The results obtained for the IC50 and the K_i show a strong inhibitory effect for enoxacin and pipemidic acid; the other quinolones are less potent as inhibitors, and ofloxacin shows no inhibitory effect.

The relative potencies of enoxacin and pipemidic acid obtained in the in-vitro study of caffeine inhibition are in agreement with those deduced from in-vivo studies in man (Staib et al 1987; Harder et al 1988; Barnett et al 1989; Carbó et al 1989). This correlation reveals the likely importance of *N*-demethylation (first metabolic step) in man even though the metabolic pathways for the xanthines have not been totally characterized.

Studies reported by Wijnands et al (1987) suggested that the 4-oxo-metabolite of enoxacin may be responsible for the potency of this quinolone in the inhibition of dimethylxanthine metabolism. Mulder et al (1988) disagreed with this, noting that the enoxacin molecule probably acts by directly inhibiting the enzymatic metabolic pathway. Recent studies (Harder et al 1988; Sanz et al 1988) postulate that the specific structural requirements present in enoxacin and pipemidic acid, could explain their greater potency as inhibitors when compared with other quinolones. The suggestion that the unaltered quinolone molecules may be directly responsible for inhibition agrees with the in-vitro results obtained in our study.

The mechanism of inhibition between the quinolones and 7-ER appears competitive and, on the basis of the high selectivity of EROD reaction for the metabolic pathway used by caffeine, the same mechanism may be likely for caffeine-related substances.

The high inhibitory activity towards the oxidative metabolism of cytochrome P450 IA shown by enoxacin and pipemidic acid, suggests their potential use in both in-vivo and in-vitro experimental studies, as tools to study the activation mechanisms in man of xenobiotics to compounds related with chemical toxicity and carcinogenesis, processes in which the cytochrome P450 IA is involved (Ioannides & Parke 1987; Van Wauve & Janssen 1989).

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