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PRIZIDILOL

METABOLISM BY CYTOCHROME P-450 AND ACETYLTRANSFERASE*

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Abstract—The hepatic microsomal cytochrome P-450 enzyme system bound and metabolized the experimental drug prizidilol. Prizidilol bound to two distinct sites on cytochrome P-450. At low concentrations (less than *ca* 20 μM), prizidilol bound to the substrate binding site of the enzyme and produced a Type I difference spectrum. At higher concentrations (25–190 μM), prizidilol bound to the oxygen binding site of the enzyme and produced a type II difference spectrum. Prizidilol stimulated hepatic microsomal CO-inhibitable NADPH oxidation. Prizidilol metabolism by hepatic microsomes assessed by prizidilol disappearance was inhibited by $CO:O_2$ (80:20; v/v), SKF 525-A and metyrapone. Prizidilol disappearance was monitored using a newly developed TLC assay for prizidilol following derivatization with quinolin-3-al. The apparent binding constants (K_s), maximum extents of binding (ΔA_{max}), Michaelis constants (K_m) and maximum velocities (V_{max}) for the interaction of prizidilol with hepatic microsomal cytochrome P-450 were assessed in rats pretreated or not with the inducing agents phenobarbital, β-naphthoflavone and pregnenolone-16α-carbonitrile. For the differently pretreated rats the apparent K_s values for the type I site and the type II site and the apparent K_m were *ca* 3 μM, 150 μM and 2 μM, respectively. Apparent V_{max} values varied from 20 to 70 pmol per min per mg microsomal protein. The observed effects of induction on the apparent equilibrium constants and maximum extents of binding and metabolism of prizidilol indicate that the forms of cytochrome P-450 induced by phenobarbital, pregnenolone-16α-carbonitrile or β-naphthoflavone do not play a major role in the metabolism of prizidilol. Prizidilol was also metabolized by hepatic cytosolic *N*-acetyltransferase. The apparent K_m values for prizidilol and acetyl CoA were 0.8 and 22 μM. Apparent V_{max} values were 50 and *ca* 2 pmol per min per mg protein for partially purified transferase and cytosol, respectively. It is concluded that the rates of oxidation and acetylation of this drug would be expected to be relatively low, being limited by low apparent V_{max} values for both oxidation and acetylation.

Prizidilol (I) is an experimental drug which combines the structural features of the β-adrenoceptor antagonist propranolol (II) and the long acting vasodepressor hydralazine (III) (Fig. 1). The aim in the design of this drug molecule was to provide a vasodilator effect while attempting at the same time to avoid undesirable reflex effects such as tachycardia and increased cardiac output. At the time of the initiation of this study, prizidilol was a candidate for clinical trials (e.g see ref. 1).

Propranolol is metabolized primarily via oxidative pathways, including initial oxidative deamination, hydroxylation and *O*-dealkylation (e.g. see refs 2 and 3). Hydralazine metabolism is via acetylation, condensation, dehydrazinolysis, hydroxylation and conjugation [4–7]. The majority of the oxidative reactions for both compounds are thought to be catalyzed by hepatic microsomal cytochrome P-450 (EC 1.14.14.1) while acetylation of the latter is via cytosolic *N*-acetyltransferase (EC 2.3.1.5) [3, 7–14]. Studies of the metabolism of prizidilol have been limited to partial characterization of major urinary metabolites in the human and two other species by TLC, HPLC and mass spectroscopy. The hydrazino group was extensively conjugated with α-keto carboxylic acids and acetylated to a lesser degree. Little cytochrome P-450 dependent metabolism was observed (Dr Ann Bell, Smith Kline & French: personal communication).

Cytochrome P-450 is a group of isoenzymes involved in the oxidative and reductive metabolism of xenobiotics in the liver and other tissues. The first step in the metabolism of diverse drugs and xenobiotics is catalyzed by this enzyme system [15]. Different forms of cytochrome P-450 can be induced by pretreatment of animals with xenobiotics such as phenobarbital, pregnenolone-16α-carbonitrile and β-naphthoflavone [16].

Acetylation is a major route for the metabolism of organic amines in many different species. The liver cytosolic *N*-acetyltransferases catalyze the acetylation of a number of commonly used drugs such as isoniazid, sulfamethazine and hydralazine in

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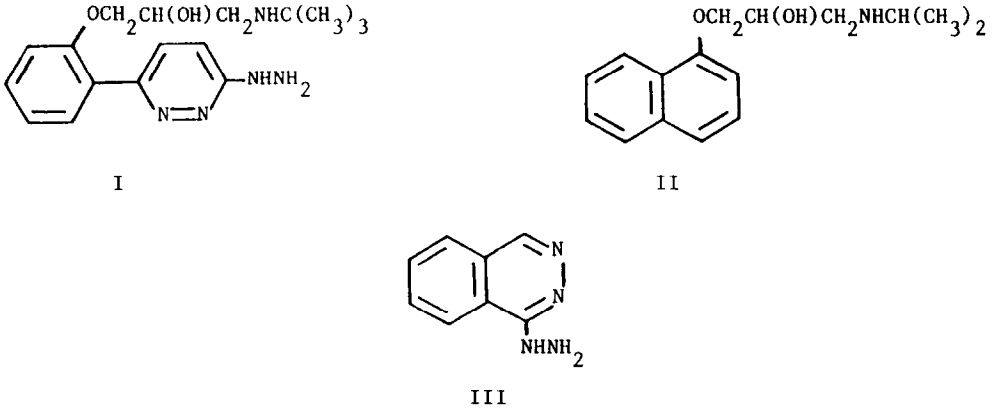

Fig. 1. Structures of prizidilol (I), propranolol (II) and hydralazine (III).

man and other mammalian species [12, 17]. The activity and cellular distribution of the *N*-acetyltransferases varies widely between species [12]. The *N*-acetylation of xenobiotics in man exhibits a hereditary polymorphism, which is readily observable in the rate of drug acetylation [12, 17]. *N*-acetyltransferase activity is inhibited by various metals such as Cu^{+}, Zn^{2+}, Mn^{2+}, and Ni^{2+} [18].

In view of the limited information concerning the metabolism of prizidilol, a study of its metabolism by hepatic microsomal cytochrome P-450 and hepatic cytosolic *N*-acetyltransferase was undertaken. A role for both enzymes in prizidilol metabolism is demonstrated. These studies utilize a newly developed TLC assay for prizidilol following derivatization with quinolin-3-al.

MATERIALS AND METHODS

Materials. Materials were obtained as follows: sodium phenobarbital: Maybaker Ltd., Port Elizabeth, R.S.A.; β-naphthoflavone: Aldrich Chemical Company, Milwaukee, WI; acetyl CoA: Sigma Chemicals, St Louis, MO; NADPH and the components of the NADPH-generating system: Miles Laboratories, Cape Town, R.S.A.; cuprous chloride: May & Baker Ltd, Dagenham, U.K.; manganese sulfate: BDH Ltd, Poole, U.K. and cylinders of O_2 and CO: Afrox Ltd., Cape Town, R.S.A.; quinolin-3-al (3-quinoline carbaldehyde): Merck, Darmstadt, F.D.R. Metyrapone [2-methyl-1,2-bis(3-pyridyl)-1-propane] was a gift from Ciba-Geigy Ltd, Basle, Switzerland. SKF 525-A (β-di-ethylaminoethyl-2,2-diphenyl valerate), prizidilol, prizidilol pyruvate hydrazone, cyclized acetylated prizidilol, and SKF 93238 trihydrochloride were gifts from Smith Kline & French Ltd., Isando, Transvaal, R.S.A. Pregnenolone-16α-carbonitrile was a gift from G. D. Searle & Co., Chicago, IL. Water was glass distilled and deionized.

Experimental animals. Male Long Evans rats (200 ± 10 g) were used for all experiments. Housing and pretreatment of animals was as described earlier [19].

Hepatic microsomes—Preparation and assays. Microsomes and post-microsomal supernatant were prepared from fresh rat liver homogenate at 4° by differential ultracentrifugation essentially by the method of Holtzman and Carr [20]. The microsomes were suspended at a concentration of 2 mg protein per ml 0.05 M Tris–HCl, pH 7.4, and were used within 5 hr of preparation. The protein concentration of the microsomal suspension was determined by the method of Lowry *et al.* [21] as modified by Chaykin [22] using bovine serum albumin as a standard.

The concentration of cytochrome P-450 in hepatic microsomes was determined from measurement of the difference spectrum of CO-ferrocytochrome P-450 versus ferrocytochrome P-450, as described by Omura and Sato [23]. The extinction coefficient used for the difference in absorbance at 450 nm and 490 nm was 91 mM^{-1} cm^{-1} [23].

Hepatic microsomal difference spectra. Difference spectra were recorded as follows. Two Teflon-stoppered 4 ml cuvettes each containing 2.5 ml of microsomal suspension were equilibrated to 25°. An aqueous solution of prizidilol (5–100 μl) was added to the sample cuvette below the surface of the microsomal suspension. An equal volume of water was added to the reference cuvette. The cuvettes were stoppered and mixed. The magnitude of the difference spectrum was measured as the difference in absorbance between the peak at 431 nm and the trough at 396 nm at high concentrations of prizidilol (25–190 μM) or the absorbance peak at 380 nm and the trough at 416 nm at low concentrations of prizidilol (1–20 μM). Correction was made for the intrinsic differences in absorbance at these wavelengths for cuvettes containing microsomal suspension only.

Prizidilol metabolism in hepatic microsomes. In some experiments, the metabolism of prizidilol was measured by monitoring the rate of hepatic microsomal NADPH consumption in the presence of prizidilol with and without $CO:O_2$ (80:20; v/v) [24]. Two Teflon-stoppered 4 ml cuvettes each containing 2.5 ml of microsomal suspension were equilibrated to 25°. Prizidilol (14 μM or 142 μM) was added to the sample cuvette, and the reaction was initiated with the addition of NADPH (0.12 mM) to the sample cuvette. Background rates of NADPH oxidation were measured in the presence of prizidilol and $CO:O_2$ (80:20; v/v). The decrease in absorbance

Table 1. Recovery and reproducibility of prizidilol analysis by the reported and newly developed TLC methods

Reported assay [1, 26]						Developed TLC assay					
Spiked concentration of prizidilol (μM)	Peak height ratio	Mean	Recovered concentration (μM)	Mean	Coefficient of variation (%)	Spiked concentration of prizidilol (μM)	Peak height ratio	Mean	Recovered concentration (μM)	Mean (SD)	Coefficient of variation (%)
0.05	0.28	0.237	0.20	0.17	34	0.15	0.16	0.157	0.14	0.14	11
	0.25		0.21				0.15		0.12	(0.02)	
	0.18		0.11				0.16		0.14		
							0.16		0.14		
0.30	0.41	0.367	0.32	0.28	14		0.16		0.14		
	0.35		0.26								
	0.34		0.25			0.30	0.26	0.269	0.30	0.30	3
							0.27		0.31	(0.01)	
0.60	0.69	0.723	0.55	0.58	8		0.26		0.30		
	0.70		0.56				0.28		0.32		
	0.80		0.64				0.27		0.31		
	0.72		0.57								
						0.75	0.55	0.544	0.80	0.79	6
1.20	1.66	1.453	1.36	1.19	12		0.53		0.77	(0.05)	
	1.26		1.03				0.53		0.75		
	1.45		1.19				0.55		0.80		
	1.44		1.18				0.55		0.80		
							0.56		0.81		
1.80	2.21	2.274	1.82	1.88	9						
	2.38		1.97			1.50	0.85	0.829	1.54	1.47	5
	2.14		1.76				0.79		1.36	(0.07)	
	2.16		1.78				0.84		1.49		
	2.48		2.06				0.83		1.47		
							0.84		1.49		
2.40	2.79	2.818	2.31	2.33	5		0.83		1.47		
	2.92		2.42								
	2.68		2.22			2.25	1.04	1.038	2.18	2.19	5
	2.88		2.39				1.01		2.09	(0.11)	
							1.05		2.26		
3.00	3.55	3.618	2.95	3.00	1		1.05		2.26		
	3.63		3.02				1.01		2.09		
	3.66		3.04				1.06		2.31		
	3.63		3.02								
						3.00	1.22	1.204	3.16	3.07	4
							1.19		2.96	(0.11)	
							1.19		2.99		
							1.21		3.09		
							1.21		3.09		
							1.21		3.13		

at 340 nm was recorded. The reported rates for the oxidation of NADPH were calculated using $\varepsilon_{340\,nm}$ of 6.2 mM^{-1} cm^{-1} [24]. Spectral measurements were performed using a Beckman 5230 recording spectrophotometer. In all cases, cuvettes with a pathlength of 1 cm were used in the compartment adjacent to the photomultiplier.

Hepatic microsomal metabolism of prizidilol was also assessed by prizidilol disappearance in incubation mixtures (2.5 ml, total volume) containing prizidilol (0–2.85 μM), NADPH-generating system [25] and hepatic microsomes in 0.05 M Tris–HCl, pH 7.4. Incubations were at 37° with shaking at 60 oscillations per min. One ml aliquots of reaction mixtures were treated as follows: The reaction was stopped by adjustment of the pH of the reaction mixture to 3 by the addition of 100 μl of orthophosphoric acid solution (20% v/v initial concentration). One hundred μl of pepsin solution (2% w/v initial concentration), 20–100 μl of SKF 93238 trihydrochloride internal standard (2% w/v initial concentration) and 50 μl of quinolin-3-al (2% w/v in propan-2-ol initial concentration) were added. The mixture was heated at 45° for 45 min and cooled. The pH was adjusted to 13 with 250–300 μl of 3.5 M NaOH, and the aqueous phase was extracted with 6 ml of dichloromethane. The filtered organic layer was evaporated to dryness at 25° under nitrogen, redissolved in 10 μl of acetic acid:butan-2-one (1:1), and 5 μl thereof was applied to Merck HPTLC precoated silica gel plates. The plates were developed with acetone:acetic acid (100:1), air dried and then developed with acetone:butan-2-one:ammonium hydroxide (15:5:1). The plates were dried and scanned at 366 nm using a Zeiss KM3 chromatogram scanning spectrophotometer.

The sensitivity and reproducibility of the assay and of the reported method for prizidilol quantification in biological samples [1] are presented in Table 1. Calibration curves for prizidilol were prepared daily. A typical example thereof is shown in Fig. 2. Where necessary, reaction mixtures were diluted before analysis to ensure that the concentrations of prizidilol analyzed always fell between 0.15 and 3.0 μM. The efficiency of extraction for prizidilol was *ca* 97%.

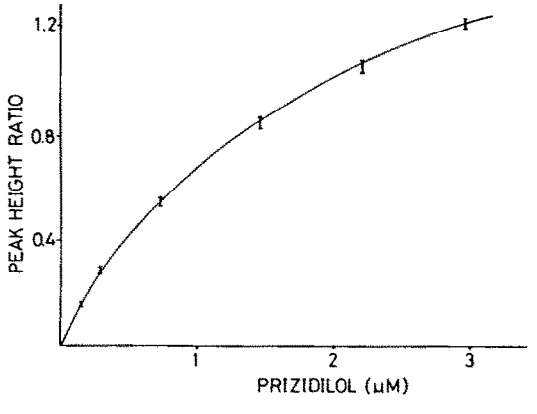

Fig. 2. Calibration curve for the determination of prizidilol. Peak height ratio: Peak height of prizidilol to peak height of fixed concentration of internal standard. $y = 1.43\ (1\text{-}e^{-1.58x})$, $r = 0.9979$.

Purification of rat liver N-*acetyltransferase.* Hepatic cytosolic *N*-acetyltransferase was purified *ca* 25-fold essentially according to the method of Weber and Cohen [18]. The fraction of rat liver cytosol between 50% and 70% saturation with respect to ammonium sulfate was chromatographed on a Sephadex G-100 column (2.5 cm × 50 cm) equilibrated with 5 mM phosphate buffer, pH 7.2.

Acetylation of prizidilol. The acetylation of prizidilol was assayed by the rate of disappearance of prizidilol from incubation mixtures normally containing prizidilol (0–4 μM), acetyl CoA (0.02–1.0 mM) and cytosol or partially purified acetyltransferase (0.8 mg protein/ml) in 0.01 M phosphate buffer, pH 7.0 (2.5 ml total volume). Incubations were at 37° with shaking at 60 cycles per min, with the order of additions indicated above. One hundred μl of aqueous orthophosphoric acid (20% v/v, initial concentration) was added to 1-ml aliquots of incubation mixtures to terminate the reaction. Thereafter, analysis of prizidilol was performed exactly as described above.

Calculations and statistical analysis. All of the results are presented as means ± SD for determinations in triplicate or greater in each of two or more separate experiments. In the case of studies on microsomes, each experiment utilized a separate microsomal preparation from a group of three animals. Student's *t*-test for unpaired data was utilized to assess the significance of differences between means. Apparent constants for the binding and metabolism of prizidilol were calculated from Hanes plots.

RESULTS

Binding of prizidilol to cytochrome P-450

Prizidilol exhibited two distinct types of difference spectra with hepatic microsomal cytochrome P-450. With low concentrations (1–20 μM) of prizidilol, a type I difference spectrum (λ_{max} = 380 nm; λ_{min} =

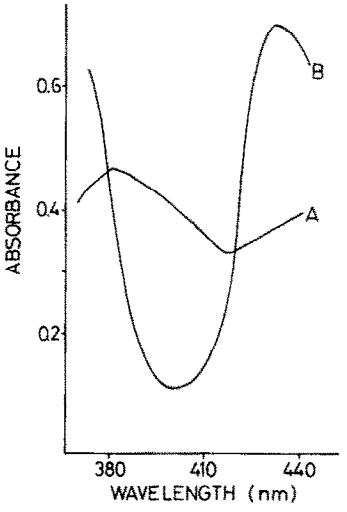

Fig. 3. Difference spectra of hepatic microsomes from phenobarbital treated rats. Addition to sample cuvette: A, 20 μM prizidilol; B, 190 μM prizidilol.

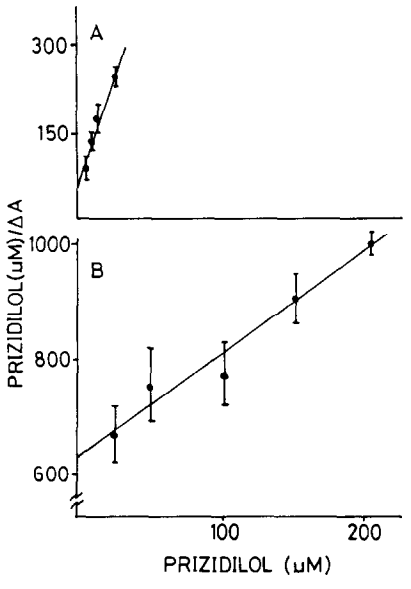

Fig. 4. Hanes plot for the binding of prizidilol to cytochrome P-450 in hepatic microsomes from untreated rats. (A) For type I difference spectrum $\Delta A = A_{380}$ nm $- A_{416}$ nm. (B) For type II difference spectrum $\Delta A = A_{431}$ nm $- A_{396}$ nm.

416 nm) was obtained. In contrast, high concentrations (25–190 μM) of prizidilol resulted in the production of a type II difference spectrum (λ_{max} = 431 nm; λ_{min} = 396 nm) (Fig. 3). The effects of induction of different forms of cytochrome P-450 on the binding of prizidilol to hepatic cytochrome P-450 was assessed.

Hanes plots for the binding of prizidilol to the type I and type II sites on cytochrome P-450 in hepatic microsomes from uninduced, phenobarbital, pregnenolone-16α-carbonitrile and β-naphthoflavone induced rats were monophasic, with a single apparent K_s value for each type of binding being calculable for each type of induction (See e.g. Fig. 4, Table 2). The pretreatment of rats with inducing agents did not result in significant alterations in the values of K_s, ΔA_{max} or ΔA_{max} per nmole cytochrome P-450 for the binding of prizidilol to the type I site (Table 2).

For the binding of prizidilol to the type II site, the value of the apparent K_s was decreased following β-naphthoflavone or phenobarbital pretreatment, but not pregnenolone-16α-carbonitrile treatment. The value of the apparent ΔA_{max} was increased following pregnenolone-16α-carbonitrile or phenobarbital treatment but not following β-naphthoflavone treatment. For the binding of prizidilol to the type II site, there were no significant changes in the apparent ΔA_{max} per nmole cytochrome P-450, following any type of pretreatment (Table 2).

Prizidilol stimulation of CO-inhibitable NADPH oxidation

Prizidilol stimulated CO-inhibitable NADPH oxidation in hepatic microsomes from uninduced and pretreated rats. The effect of pretreatment of rats with inducing agents for cytochrome P-450 on the initial rates of CO-inhibitable hepatic microsomal oxidation of NADPH in the presence of prizidilol is shown in Table 3. With a low concentration of prizidilol (14 μM), pretreatment of rats with β-naphthoflavone or pregnenolone-16α-carbonitrile did not significantly alter the rate of CO-inhibitable NADPH oxidation per mg microsomal protein. Microsomes from rats pretreated with phenobarbital exhibited significantly increased rates of NADPH oxidation per mg microsomal protein. None of the pretreatments altered the rate of CO-inhibitable NADPH oxidation per nmole cytochrome P-450 at 14 μM prizidilol (Table 3). With a high concentration of prizidilol (142 μM), the rate of CO-inhibitable NADPH oxidation per mg protein was not affected by pretreatment of the rats with phenobarbital, but was diminished following pretreatment with pregnenolene-16α-carbonitrile or β-naphthoflavone. CO-inhibitable NADPH oxidation per nmole cytochrome P-450 was diminished by the three pretreatments (Table 3).

For microsomes from untreated rats, the rates of CO-inhibitable NADPH oxidation were identical for the low and high concentrations of drug. However, the rates of CO-inhibitable NADPH oxidation per mg protein and per nmol cytochrome P-450 were significantly lower with 142 μM prizidilol than with 14 μM prizidilol, for each type of induction (Table 3) ($P < 0.01$).

Table 2. Effect of induction on the apparent constants for the binding of prizidilol to hepatic microsomal cytochrome P-450

Inducing agent	Binding to type I site*			Binding to type II site†		
	K_s (μM)	ΔA_{max}	ΔA_{max}/nmol cytochrome P-450	K_s (μM)	ΔA_{max}	ΔA_{max}/nmol cytochrome P-450
None	5.2 ± 1.6	0.15 ± 0.09	0.13 ± 0.07	300 ± 81	0.48 ± 0.08	0.37 ± 0.04
BNF	3.5 ± 3.0	0.06 ± 0.02	0.05 ± 0.02	136 ± 23§	0.46 ± 0.12	0.30 ± 0.04
PCN	7.3 ± 1.4	0.10 ± 0.01	0.06 ± 0.01	224 ± 10	0.85 ± 0.11§	0.50 ± 0.11
PB	5.7 ± 1.3	0.20 ± 0.07	0.07 ± 0.02	76 ± 5‡	0.91 ± 0.07‡	0.37 ± 0.01

The abbreviations used are: BNF, β-naphthoflavone; PCN, pregnenolone-16α-carbonitrile; PB, phenobarbital.
* $\Delta A = A_{380}$ nm $- A_{416}$ nm.
† $\Delta A = A_{431}$ nm $- A_{396}$ nm.
‡ Differs from corresponding value for microsomes from uninduced rats, $P < 0.01$.
§ Probably differs from corresponding value for microsomes from uninduced rats, $P < 0.05$.

Table 3. Effect of induction on the prizidilol stimulation of hepatic microsomal CO-inhibitable NADPH oxidation

	CO-inhibitable NADPH oxidation (nmol/min)			
	Prizidilol (14 μM)		Prizidilol (142 μM)	
Inducing agent	per mg microsomal protein	per nmol cytochrome P-450	per mg microsomal protein	per nmol cytochrome P-450
None	1.32 ± 0.07	1.04 ± 0.09	1.51 ± 0.04	1.25 ± 0.14
BNF	1.37 ± 0.11	0.92 ± 0.09	0.85 ± 0.05*	0.56 ± 0.03*
PCN	1.60 ± 0.18	1.07 ± 0.05	0.97 ± 0.13†	0.67 ± 0.15†
PB	2.60 ± 0.19*	1.11 ± 0.12	1.54 ± 0.16	0.63 ± 0.02*

Abbreviations used are: BNF, β-naphthoflavone; PCN, pregnenolone-16α-carbonitrile; PB, phenobarbital.
* Differs from corresponding value for microsomes from uninduced rats, $P < 0.01$.
† Probably differs from corresponding value for microsomes from uninduced rats, $P < 0.05$.

Validation of the prizidilol assay

Prizidilol metabolism was monitored by a recently developed TLC assay for prizidilol in biological samples. The sensitivity and reproducibility of the assay were comparable to the only reported method for prizidilol determination in complex samples (Table 1). However, the specificity of the two assays was somewhat different with regard to the determination of certain prizidilol metabolites. In the existing method, acidic conditions were utilized which partially reconverted prizidilol hydrazones to prizidilol (J. Pearce, Smith Kline & French Ltd., personal communication) [26]. Strongly acid conditions for protein precipitation and derivatization were replaced in the developed method by pepsin digestion under mildly acidic conditions. Under these conditions, no measurable reconversion (<3%) of authentic samples of prizidilol pyruvate hydrazone or cyclized acetylated prizidilol to the parent drug was observed. Hydrazones and other metabolites of prizidilol in which the hydrazine moiety was altered, would be unable to form a hydrazone with the quinolin-3-al, and thus be undetectable by the developed TLC method. Prizidilol metabolites arising from the cytochrome P-450 enzyme system would differ in polarity from the parent drug and would be expected to be separable from prizidilol quinolin-3-al hydrazone during the two TLC stages of the assay.

No endogenous substances in serum, hepatic microsomes or hepatic cytosol measurably interfered with the analysis of prizidilol. Possible interference by other drugs was not examined, with the exception of metyrapone and SKF 525-A which did not interfere with prizidilol determination.

Prizidilol metabolism

Metyrapone (1 mM), SKF 525-A (200 μM) and $CO:O_2$ (80:20; v/v) inhibited the metabolism of prizidilol by hepatic microsomes. As inhibitors of prizidilol disappearance, metyrapone and SKF 525-A were equivalent (77 ± 15% inhibition), while CO was less effective (54 ± 2% inhibition).

The disappearance of prizidilol in incubations containing NADPH-generating system and hepatic microsomes from uninduced and pretreated rats is shown in Fig. 5. No loss of prizidilol was seen in the absence of NADPH (data not shown). The disappearance of prizidilol with microsomes from phenobarbital pretreated rats was linear for 10 min: this time period was utilized for kinetic studies for this type of pretreatment. With microsomes from pregnenolone-16α-carbonitrile or β-naphthoflavone treated and untreated rats, prizidilol was lost rapidly for a short period of time (<3 min); subsequently the loss of prizidilol with time was linear for 20 min. An incubation period of 20 min was of necessity used in kinetic studies for microsomes from rats subjected to these pretreatments. In these cases, sufficient prizidilol was not lost during the initial rapid phase of the reaction for accurate measurement. The apparent constants calculated under these conditions may represent composite constants.

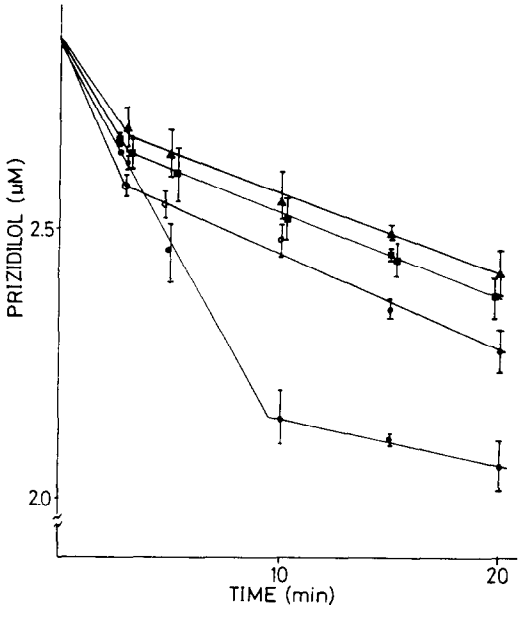

Fig. 5. Effect of incubation of prizidilol with NADPH-generating system plus hepatic microsomes on prizidilol concentration as a function of time. Microsomes from (▲) untreated rats; (■), β-naphthoflavone; (○), pregnenolone-16α-carbonitrile; and (●), phenobarbital treated rats.

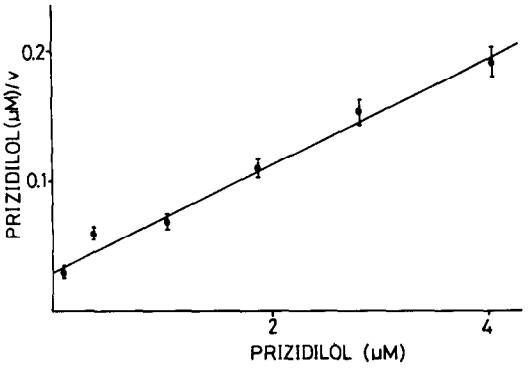

Fig. 6. Hanes plot for prizidilol disappearance with hepatic microsomes from β-naphthoflavone treated rats plus NADPH-generating system. v, rate of prizidilol disappearance in pmol/min/mg microsomal protein.

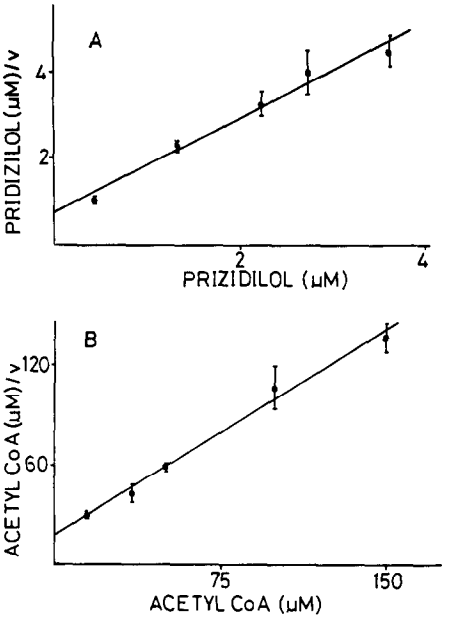

Fig. 7. Hanes plot of prizidilol metabolism by partially purified *N*-acetyltransferase. v, rate of prizidilol disappearance in μmol/30 min/2 mg protein: (A) As a function of prizidilol concentration, at 1 mM acetyl CoA; (B) as a function of acetyl CoA concentration, at 3.7 μM prizidilol.

Hanes plots for the disappearance of prizidilol from incubations of NADPH-generating system plus hepatic microsomes from rats pretreated or not with phenobarbital, pregnenolone-16α-carbonitrile or β-naphthoflavone were linear (see e.g. Fig. 6).

Pretreatment of rats with β-naphthoflavone or pregnenolone-16α-carbonitrile did not significantly alter the apparent values of K_m or V_{max} per mg microsomal protein. Pretreatment of rats with phenobarbital increased the apparent values of K_m and V_{max} per mg microsomal protein relative to microsomes from untreated rats. The apparent values of V_{max} per nmole cytochrome P-450 were not affected by the pretreatment of rats with any of the inducing agents (Table 4).

Acetylation of prizidilol

The disappearance of prizidilol from reaction mixtures containing acetyl CoA (1 mM), rat liver cytosol and prizidilol (3.7 μM) was linear for 30 min (data not shown). This time period was used in all further studies of the acetylation of prizidilol. The loss of prizidilol per 30 min was proportional to the amount of cytosol added, up to a concentration of 2 mg protein per ml. For partially purified enzyme, the reaction was linear up to *ca* 1.2 mg protein per ml and to 30 min.

There was no significant loss of prizidilol when acetyl CoA or enzyme preparation was omitted from incubation mixtures. There was also no disappearance of substrate in reaction mixtures containing prizidilol and a preparation of *N*-acetyltransferase that had been heated for 10 min at 90° prior to incubation.

The inhibitors of *N*-acetyltransferase, cuprous chloride (0.14 mM) and manganese sulfate (0.14 mM), diminished the metabolism of prizidilol by partially purified hepatic *N*-acetyltransferase by 51% and 69% respectively ($P < 0.01$).

Hanes plots of the effect of acetyl CoA and prizidilol concentrations on the acetylation of prizidilol by partially purified rat liver cytosol *N*-acetyltransferase are shown in Fig. 7. Apparent K_m and V_{max} values calculated therefrom were as follows. At saturating concentrations of acetyl CoA (1 mM), the apparent K_m for prizidilol was 0.78 μM and the apparent V_{max} was 41 pmol per min per mg protein. In the presence of 4 μM prizidilol, the apparent K_m

Table 4. Effect of induction on the apparent constants for metabolism of prizidilol by microsomal cytochrome P-450

		V_{max} (pmol/min)	
Induction	K_m (μM)	per mg microsomal protein	per nmol cytochrome P-450
None	1.3 ± 0.5	22 ± 5	21 ± 4
BNF	1.0 ± 0.2	24 ± 4	18 ± 2
PCN	1.6 ± 0.5	26 ± 1	18 ± 8
PB	3.8 ± 0.5*	71 ± 18†	29 ± 8

Abbreviations used are: BNF, β-naphthoflavone; PCN, pregnenolone-16α-carbonitrile; PB, phenobarbital.

* Differs from corresponding value for microsomes from uninduced rats, $P < 0.01$.

† Probably differs from corresponding value for microsomes from uninduced rats, $P < 0.05$.

for acetyl CoA was 22 μM, and the apparent V_{max} was 50 pmol per mg protein per min. The apparent V_{max} for rat liver cytosol was *ca* 2 pmol per mg protein per min.

Accompanying a decrease in the levels of prizidilol following the incubation of prizidilol with partially purified *N*-acetyltransferase and acetyl CoA, was an increase in the levels of cyclized acetylated prizidilol, assessed by the TLC method for the determination of quinolin-3-al derivatized prizidilol and cyclized acetylated prizidilol. The yield of cyclized acetylated prizidilol was very low and this material could not be quantified accurately.

DISCUSSION

A role for rat hepatic microsomal cytochrome P-450 in the binding and metabolism of prizidilol is demonstrated. Prizidilol bound to cytochrome P-450 as a type I compound and stimulated microsomal CO-inhibitable NADPH oxidation (Fig. 3 and Table 3), indicating that the drug interacted with the substrate binding site of cytochrome P-450. The metabolism of prizidilol by hepatic microsomes required NADPH and was inhibited by three known inhibitors of cytochrome P-450, viz. SKF 525-A, metyrapone and CO. Finally, phenobarbital pretreatment increased NADPH oxidation per mg microsomal protein in the presence of 14 μM prizidilol and increased the apparent V_{max} for prizidilol disappearance (Tables 3 and 4).

The effects of three inducing agents for cytochrome P-450 on NADPH oxidation and prizidilol metabolism provides information on the forms of cytochrome P-450 capable of metabolizing this drug. The forms of the enzyme induced by β-naphthoflavone and pregnenolone-16α-carbonitrile do not appear to metabolize prizidilol. These inducing agents did not affect the apparent K_s for the binding of prizidilol to the type I site or the apparent K_m for prizidilol metabolism and did not increase the apparent ΔA_{max} or V_{max} (Tables 2 and 4). Since phenobarbital induction altered the apparent K_m and increased the apparent V_{max} for prizidilol metabolism per mg microsomal protein but not per nmole cytochrome P-450 (Table 4), it would appear that the phenobarbital inducible forms of cytochrome P-450 may at most play a minor role in prizidilol metabolism. Thus, one or more forms of cytochrome P-450 present in hepatic microsomes from untreated rats, but not induced by the agents utilized in this study, may be of primary importance in prizidilol metabolism. Different forms of cytochrome P-450 appear to be involved in the metabolism of prizidilol versus propranolol or hydralazine. In contrast to prizidilol, the cytochrome P-450 dependent metabolism of propranolol and hydralazine was stimulated by both phenobarbital and 3-methylcholanthrene [6, 10, 27]. Furthermore, SKF 525-A but not metyrapone inhibited propranolol metabolism [28], while both compounds inhibited the metabolism of prizidilol (see Results).

The substrate binding site of hepatic cytochrome P-450 exhibited a high affinity for prizidilol, with the apparent K_s and K_m values for this site being in the range of 1–7 μM (Tables 2 and 4). These constants compare to the reported apparent K_s values of 0.2–4.0 μM for the binding of propranolol to a high affinity site on rat hepatic microsomal cytochrome P-450 [29–31]. The apparent constants for prizidilol were far below the apparent K_m values of 17–220 μM for the metabolism of propranolol by cytochrome P-450 or of 35 μM for the cytochrome P-450 dependent activation of hydralazine [14, 31, 32]. Cytochrome P-450 exhibited a greater affinity for prizidilol than for the majority of xenobiotics and drugs such as benzene, benzo[α]pyrene, diphenylhydantoin, harmine, ethyl morphine, and volatile anaesthetic agents for which K_m values range from 10 μM to 5 mM (e.g. see refs 15 and 22).

In spite of high affinity of prizidilol binding, the cytochrome P-450 dependent metabolism of this drug proceeded extremely slowly. The apparent V_{max} values for prizidilol were in the range of 20–70 pmole per min per mg protein (Table 4). These values were below reported rates and V_{max} values for propranolol metabolism by hepatic microsomal cytochrome P-450 of 0.2–1.4 nmole per mg protein per min [9, 10, 32]. The apparent V_{max} values for cytochrome P-450 dependent metabolism of prizidilol were several orders of magnitude below the reported rate of microsomal metabolism of hydralazine of *ca* 3 nmole per mg protein per min [7] and *ca* 10^5- to 10^6-fold lower than the V_{max} value for ethyl morphine *N*-demethylase [15].

The results reported herein provide no evidence for low affinity binding of prizidilol to the substrate binding site of cytochrome P-450, with neither increased Type I binding nor increased NADPH oxidation being apparent at high concentrations (*ca* 150 μM) of prizidilol (Tables 2 and 3). In contrast, propranolol was reported to bind to both high and low affinity substrate binding sites on cytochrome P-450, the latter being characterized by a K_s of 7 mM and by a K_m of 137 μM for propranolol *N*-dealkylation [11, 30].

Low affinity binding of prizidilol was, however, observed at the Type II site of cytochrome P-450 (Table 2). The binding of prizidilol to this site suggests that prizidilol could inhibit its own metabolism and that of other drugs by cytochrome P-450 by competing with oxygen binding to the heme iron atom. Inhibition would only become apparent at concentrations of prizidilol greater than 50 μM. In support of this proposal is the diminution in the rate of prizidilol stimulated NADPH oxidation at 142 μM prizidilol, following induction of cytochrome P-450 (Table 3).

Since both phenobarbital and pregnenolone-16α-carbonitrile induction increased the apparent maximum extent of binding (ΔA_{max}) of prizidilol to the type II site, and since phenobarbital and β-naphthaflavone altered the apparent K_s for binding to this site (Table 2), it would appear that prizidilol may bind to the type II site of more forms of cytochrome P-450 than accept prizidilol as a substrate.

The observed rates of prizidilol metabolism, as assessed by substrate disappearance and NADPH oxidation (Tables 3 and 4) were not in agreement. The rate of prizidilol (14 μM) stimulated CO-inhibitable NADPH oxidation exceeded the rate of prizidilol disappearance for all types of induction

(Tables 3 and 4). For phenobarbital induction, NADPH oxidation exceeded prizidilol disappearance by a factor of 35. For all other pretreatments the factor was approximately 60-fold. For all types of pretreatment except phenobarbital, the relatively greater rate of NADPH oxidation could in part reflect the rapid burst of prizidilol metabolism seen up to 3 min. The latter could account for a factor of *ca* 3 in the ratio of the rates of NADPH oxidation and prizidilol disappearance. The remaining discrepancy between the rates of NADPH oxidation and substrate disappearance may reflect the stimulation by prizidilol of electron transfer from NADPH to oxygen via the cytochrome P-450 enzyme system.

Prizidilol was shown to be metabolized by the hepatic cytosolic *N*-acetyltransferases. The reaction required acetyl CoA and was inhibited by metal ion inhibitors of this enzyme. This reaction was not unexpected in view of the facile acetylation of hydralazine [6, 12]. The apparent K_m for prizidilol of *ca* 1 μM was below that of 6 μM reported for hydralazine [12], indicating that prizidilol may bind more tightly than its analogue to the *N*-acetyltransferases.

A comparison of the kinetic parameters for the metabolism of prizidilol by hepatic microsomal cytochrome P-450 and cytosolic *N*-acetyltransferase from untreated rats indicates that both enzymes bound prizidilol with high affinity, i.e. apparent K_m values of *ca* 1 μM. The apparent V_{max} values for prizidilol metabolism by hepatic microsomal cytochrome P-450 and hepatic cytosolic *N*-acetyltransferase were for different subcellular preparations and were not directly comparable.

It is concluded that prizidilol would be metabolized in the rat by both hepatic cytochrome P-450 and hepatic *N*-acetyltransferase. The overall rate of metabolism of this drug by these pathways would be expected to be relatively low, being restricted by the extremely low apparent V_{max} values.

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