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DIFFERENT INHIBITION AND INDUCTION PROFILES OF HEPATIC DRUG METABOLISM IN RATS AND DOGS BY TWO STRUCTURALLY RELATED PYRIDYL DIAZINONE CARDIOTONIC AGENTS

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Abstract—ICI 153,110 and ICI 170,777, two pyridyl diazinone cardiotonic agents, produced a different profile of effects on hepatic microsomal mixed function oxidase enzymes following multiple oral dosing to rats and dogs; these differences may be related to the molecular dimensions of the two molecules. ICI 153,110 significantly increased levels of total P450, ethoxycoumarin *O*-deethylase and ethoxyresorufin *O*-deethylase in rat microsomes, indicating an induction profile (P448) similar to that of β-naphthoflavone. This was supported by gel electrophoresis (SDS–PAGE) of microsomal proteins; a similar type of induction was observed in dog microsomes. In contrast, ICI 170,777 produced no changes indicating enzyme induction in either rat or dog. Instead, ICI 170,777 appeared to inhibit specifically the activity of aldrin epoxidase in the rat. Inhibitory activity was also indicated in the rat by prolongation of pentobarbitone sleeping time following single oral doses of either ICI 153,110 or ICI 170,777. The time-course of this effect appeared to correlate more closely with the profile of circulating metabolites, although both parent compounds were found to produce type II spectral changes on interaction with control rat microsomes. The molecular dimensions (area/depth^2) of the compounds supported the finding that only ICI 153,110 should interact with or induce P448 isozymes.

ICI 153,110 [4,5-dihydro-6[(*E*)-2-(pyrid-4-yl)vinyl]-2H-pyridazin-3-one] and ICI 170,777 [(6*RS*)-6-methyl-5-(pyrid-4-yl)-3H,6H-1,3,4-thiadiazin-2-one] (Fig. 1) are structurally related cardiotonic agents which possess both inotropic and vasodilator activity [1]. This type of compound has been shown to be clinically effective in the chronic treatment of heart failure [2, 3, 4]. Several compounds with some common structural features and similar pharmacological activities have been found to have effects on the hepatic drug metabolizing enzyme system. Inhibition of the hepatic microsomal mixed function oxidase enzymes was observed *in vitro* with amrinone and milrinone, two other pyridine-containing inotropic agents [5]; these compounds, however, showed no enzyme inducing potential following *in vivo* administration to rats. Sulmazole and related imidazopyridine-containing compounds were reported to be inducers of the drug metabolizing enzyme system in the rat, producing profiles of induction similar to those of 3-methylcholanthrene and β-naphthoflavone; in addition, these compounds had the ability to interact with microsomal P450 and inhibit drug metabolizing activity [6, 7].

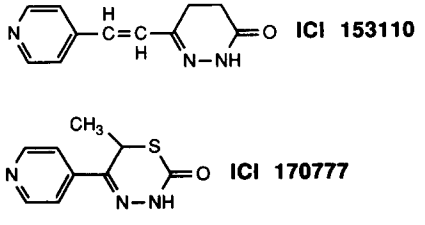

Fig. 1. The chemical structures of ICI 153,110 and ICI 170,777.

As part of the safety evaluation of ICI 153,110 and ICI 170,777, studies were conducted in rat and dog to assess the effects of these compounds on the hepatic microsomal mixed function oxidase enzymes. Since no attempt was made to characterize the P450 isozymes involved, the recommended nomenclature of Nebert *et al.* [8] will not be followed; instead, the descriptive term, P448, will be used to refer to the isozymes induced by such compounds as 3-methylcholanthrene and β-naphthoflavone, and specifically monitored by ethoxyresorufin *O*-deethylase. This paper reports the findings of these studies and discusses the possible reasons for the markedly different effects of these compounds on the mixed function oxidase enzymes.

MATERIALS AND METHODS

Chemicals. ICI 153,110 and ICI 170,777 and their respective pyridine *N*-oxides were synthesized by ICI Pharmaceuticals; their chemical purities were greater than 99%. Aldrin and dieldrin were obtained from Applied Sciences (Pennsylvania, U.S.A.). 7-Ethoxyresorufin was purchased from Pierce and Warriner (Chester, Cheshire) and resorufin from Pfaltz and Bauer Inc. (Connecticut, U.S.A.). Sodium pentobarbitone (Euthatal and Sagatal) was obtained from May and Baker (Dagenham, Essex). The

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materials for gel electrophoresis were purchased from Bio-Rad Laboratories (Watford, Herts), while all other reagents were obtained from BDH (Poole, Dorset), Aldrich Chemical Co. (Gillingham, Dorset) or Sigma Chemical Co. (Poole, Dorset).

Animals. Male Alpk:APfSD albino rats (Animal Breeding Unit, Alderley Park) with body weights 160–252 g and male beagle dogs (Dog Breeding Unit, Alderley Park) weighing between 10 and 16 kg were used in the various studies. Rats had access to food (NDD irradiated diet from Special Diets Services, Witham, Essex) and water at all times, while dogs had free access to water and were fed (Laboratory diet A from Special Diets Services) each morning before being dosed.

Enzyme induction studies. For the rat studies, both compounds were formulated as suspensions in 0.5% aqueous Tween 80. ICI 153,110 was administered as ten daily oral doses, by gavage, at 0 (vehicle only), 50, 100 and 200 mg/kg. In one study, ICI 170,777 was administered as ten daily oral doses at 0, 1, 2, 4, and 12.5 mg/kg and, in another study, was given daily at 0, 5, 20 and 80 mg/kg for fourteen days. Twenty-four hours after the final dose the animals were killed by CO_2 inhalation and the livers quickly removed into ice-cold buffer prior to microsome preparation. In the dog studies, both compounds were administered as powder in a gelatin capsule. Both compounds were given as seven daily oral doses at 0 (lactose control), 8 and 40 mg/kg. Twenty-four hours after the final dose the dogs were killed by overdosing with Euthatal (sodium pentobarbitone) and an aliquot (10–12 g) from the edge of the central lobe of the liver was taken for preparing microsomes. Microsomes were prepared and assayed for protein, P450, aldrin epoxidase, ethoxycoumarin *O*-deethylase and ethoxyresorufin *O*-deethylase using standard methods as described previously [9]. Gel electrophoresis (SDS–PAGE) of the microsomes was carried out by the method of Laemmli [10].

Sleeping time studies. Pentobarbitone sleeping time was measured in rats following administration of single oral doses of ICI 153,110 (5–250 mg/kg), ICI 170,777 (1–80 mg/kg) or metyrapone (100 mg/kg; Sigma Chemical Co.). After either 1 hr (ICI 153,110) or 1.5 hr (ICI 170,777), the rats received a single intraperitoneal dose (30 mg/kg) of pentobarbitone (Sagatal). The sleeping time was recorded as the period between the loss and return of the righting reflex.

Ligand binding spectra. Spectral changes arising from substrate interaction with P450 were determined using a Pye-Unicam SP8-200 UV-VIS spectrophotometer. Methanol solutions (1–20 μL: 10 mM) of ICI 153,110 and ICI 170,777 were added to sample cuvettes containing control rat microsomes (2 mL; 1 nmol P450/mL); equivalent volumes of methanol were added to the reference cuvette, and the samples scanned between 350 and 500 nm. The spectral dissociation constant (K_s) and maximum spectral change were estimated from Lineweaver–Burk plots of the data.

RESULTS

Enzyme induction studies

Dog. The concentration of total P450 was increased by 100% and ethoxyresorufin *O*-deethylase was increased by 200% following administration of ICI 153,110 to dogs at 40 mg/kg for seven days (Table 1). At the lower dose level (8 mg/kg), the concentration of P450 was unaffected while ethoxyresorufin *O*-deethylase was increased by 100%. Aldrin epoxidase activity remained constant at both dose levels. This pattern of enzyme changes was similar to that produced by β-naphthoflavone [9] indicating that ICI 153,110 was a P448 inducer, but of lower potency than β-naphthoflavone. In addition, the wavelength of the reduced P450-CO peak was found to have shifted from 449.8 nm in microsomes from control dogs to 448.5 nm in the higher dose group animals.

In contrast, ICI 170,777 produced no obvious induction of the dog mixed function oxidase enzymes (Table 1), when it was administered in the same dosing regimen and achieved blood concentrations similar to those of ICI 153,110 (unpublished data).

Rat. When ICI 153,110 (50, 100 and 200 mg/kg) and ICI 170,777 (5, 20 and 80 mg/kg) were administered daily to rats for 10 or 14 days, a decrease in daily body weight gain was observed in some of the higher dose groups during the first few days of treatment. However, only the highest dose level of ICI 153,110 continued to produce an obvious inhibition of growth throughout the dosing period (Fig. 2). Neither compound produced a significant effect on liver weight relative to body weight.

ICI 153,110 produced dose-related increases in both ethoxycoumarin *O*-deethylase and ethoxyresorufin *O*-deethylase (Table 2). The magnitude of the ethoxyresorufin *O*-deethylase increase (29 times greater than control) together with concomitant elevation of ethoxycoumarin *O*-deethylase is indicative of P448 induction. The concentration of total P450 also showed a small but significant increase in the microsomes from the two higher dose groups, while the reduced P450-CO peak shifted from 450.2 nm in controls to 449.1 nm in the highest dose group; microsomes from β-naphthoflavone-treated rats showed a wavelength maximum at 448.8 nm, when measured as a positive control.

Aldrin epoxidase activity was markedly decreased by ICI 153,110 administration. This compound also inhibited body weight gain indicating some toxicity which might be expected to cause a decrease in enzyme activity; however, it is well known that inhibition of specific P450 markers such as aldrin epoxidase and ethylmorphine *N*-demethylase occurs as a consequence of P448 induction [7, 9, 11].

The protein patterns of a microsome sample from each of the dose groups together with microsomes from phenobarbitone and β-naphthoflavone induced rats were examined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Microsomes from animals in the two higher dose groups showed that ICI 153,110 produced a protein pattern similar to that induced by β-naphthoflavone. In effect, ICI 153,110 slightly increased the intensity of the protein band at 48,500 daltons, made the band at 51,000 daltons more diffuse and induced a new protein at 54,500 daltons.

The effect of ICI 170,777 on the mixed function oxidase enzymes was quite different from that of

Table 1. The effects of 7 daily oral doses of ICI 153,110 and ICI 170,777 on the hepatic microsomal mixed function oxidase enzymes of the male dog

Compound	Dose level (mg/kg)	Cytochrome P450 (nmol/mg)	Aldrin epoxidase (nmol/mg/min)	Ethoxyresorufin *O*-deethylase (pmol/mg/min)
ICI 153,110	0	0.67 ± 0.06	1.04 ± 0.04	369 ± 50
	8	0.59 ± 0.06	1.16 ± 0.04	600 ± 20†
	40	1.35 ± 0.14†	1.21 ± 0.04*	1081 ± 116†
ICI 170,777	0	0.48 ± 0.02	0.98 ± 0.19	530 ± 79
	8	0.42 ± 0.04	1.05 ± 0.10	425 ± 54
	40‡	0.37 , 0.56	0.36 , 1.00	519 , 588

Values show the mean ± SE obtained from 4 animals in each group, except where noted‡, when only two dogs were considered sufficiently healthy to complete the dosing period.
Significantly different (* $P < 5\%$; † $P < 1\%$) from control by Student's *t*-test.

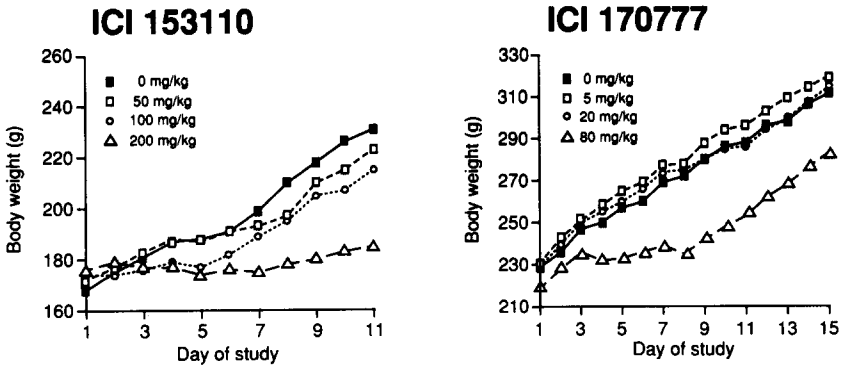

Fig. 2. The effects of ICI 153,110 and ICI 170,777 on rat body weight gain. The compounds were administered once daily, by gavage. Each profile shows the mean value obtained from either five (ICI 153,110) or four (ICI 170,777) animals.

Table 2. The effects of ICI 153,110 and ICI 170,777 on the hepatic microsomal mixed function oxidase enzymes of the male rat

Compound	Dose level (mg/kg)	Cytochrome P450 (nmol/mg)	Aldrin epoxidase (nmol/mg/min)	Ethoxycoumarin *O*-deethylase (nmol/mg/min)	Ethoxyresorufin *O*-deethylase (pmol/mg/min)
(a)					
153,110	0	0.94 ± 0.04	3.5 ± 0.2	0.29 ± 0.01	16 ± 2
	50	0.96 ± 0.05	2.4 ± 0.3*	0.49 ± 0.02‡	130 ± 9‡
	100	1.11 ± 0.04*	2.6 ± 0.3*	0.65 ± 0.01‡	216 ± 12‡
	200	1.23 ± 0.04‡	1.8 ± 0.3‡	1.01 ± 0.04‡	458 ± 29‡
(b)					
170,777	0	0.68 ± 0.06	8.4 ± 0.6	0.26 ± 0.03	11 ± 1
	5	0.53 ± 0.03	5.9 ± 0.6*	0.23 ± 0.01	12 ± 1
	20	0.51 ± 0.03	3.1 ± 0.2‡	0.26 ± 0.01	13 ± 1
	80	0.60 ± 0.02	1.7 ± 0.2‡	0.17 ± 0.01*	24 ± 2†

(a) Values represent the mean ± SE obtained from 5 animals per group following daily oral dosing for 10 days.
(b) Values represent the mean ± SE obtained from 4 animals per group following daily oral dosing for 14 days.
Significantly different (* $P < 5\%$; † $P < 1\%$; ‡ $P < 0.1\%$) from control by Student's *t*-test.

Table 3. The effect of ICI 170,777 treatment on rat hepatic microsomal aldrin epoxidase

Dose level (mg/kg)	Aldrin epoxidase (nmol/mg/min)
0	3.4 ± 0.1
1	3.5 ± 0.2
2	3.4 ± 0.1
4	2.7 ± 0.1*
12.5	2.4 ± 0.2*

Values represent the mean ± SE obtained from 4 animals per group following administration of ten daily oral doses. Significantly different (* $P < 1\%$) from control by Student's *t*-test.

ICI 153,110 (Table 2). The only marked effect produced by ICI 170,777 was to inhibit aldrin epoxidase in a dose-related manner. A further study conducted to determine a nil effect dose level showed that the inhibition of aldrin epoxidase still occurred following 10 oral doses of ICI 170,777 at 4 mg/kg but not at dose levels of 1 or 2 mg/kg (Table 3). When the microsomes from these studies were examined by SDS–PAGE no obvious changes in the P450 isozyme profile were observed. This suggested that the decreases in aldrin epoxidase were not related to effects on protein synthesis, but were more likely caused by a direct inhibition of the specific P450 isozyme by ICI 170,777 or its metabolite(s).

Enzyme inhibition studies

Pentobarbitone sleeping time. Prior administration of single oral doses of either ICI 153,110 (1 hr) or ICI 170,777 (1.5 hr) significantly prolonged the sleeping time caused by a single intraperitoneal dose (30 mg/kg) of pentobarbitone (Table 4). The barbiturate was administered at the time that maximum blood concentrations of ICI 153,110 and ICI 170,777 had been observed in earlier pharmacokinetic studies [12]. The prolongation of sleeping time, due to the inhibition of pentobarbitone metabolism, was clearly related to the dose of each compound. Metyrapone, a known inhibitor of P450 activity, was included as a positive control and also produced a significant increase in sleeping time. Metyrapone was a more potent inhibitor than ICI 153,110 but appeared to be less potent than ICI 170,777. The inhibitory effect of ICI 170,777 on pentobarbitone metabolism occurred at dose levels down to 4 mg/kg, but was not observed following single doses of 1 and 2 mg/kg; this dose response was remarkably similar to the inhibition of microsomal aldrin epoxidase observed following multiple doses of ICI 170,777.

As the more potent compound, the effect of ICI 170,777 on pentobarbitone sleeping time was investigated further, principally to determine the time-course of the inhibition following a single oral dose (20 mg/kg) (Fig. 3). While the sleeping times of both control and test animals at 90 min were similar to those of the earlier study (cf. Table 4), the inhibitory effect of ICI 170,777 was apparently greater after 6 hr. A prolongation of sleeping time was still evident 24 hr after ICI 170,777 administration, but no effect was observed after 72 hr. A comparison of the time course of this inhibition with the blood profiles of ICI 170,777 and its metabolite(s) obtained previously [12], indicated that the inhibition appeared to correlate more closely with the concentration of circulating metabolite(s) than with unchanged ICI 170,777 (Fig. 3).

ICI 170,777 reached maximum blood concentrations at 90 min and was cleared with a half-life of about 4 hr; in contrast, blood concentrations of metabolite(s) formed a plateau between 3 and 7 hr and diminished with an apparently much longer half-life. Although the very low concentrations in the blood precluded identification of the circulating metabolite(s), the major metabolite isolated from rat urine was found to be the (pyridine) *N*-oxide of ICI 170,777 [12]; similar blood profiles and the corresponding *N*-oxide metabolite were also observed with ICI 153,110 (unpublished observations).

Table 4. The effects of ICI 153,110 and ICI 170,777 on pentobarbitone-induced sleeping time in rats

ICI 153,110 Dose level (mg/kg)	Sleeping time (min)	ICI 170,777 Dose level (mg/kg)	Sleeping time (min)
0	34 ± 1	0	29 ± 1
5	41 ± 2*	1	31 ± 3
10	48 ± 1†	2	29 ± 2
250	106 ± 6†	4	38 ± 3*
Metyrapone	177 ± 3†	5	54 ± 8*
		20	107 ± 15†
		80	198 ± 12†
		Metyrapone	167 ± 24†

ICI 153,110, ICI 170,777 and metyrapone (100 mg/kg) were administered as single oral doses either 1 hr (ICI 153,110) or 1.5 hr (ICI 170,777) prior to a single intraperitoneal dose (30 mg/kg) of pentobarbitone. Values show the mean ± SE obtained from 4 animals.

Significantly different (* $P < 5\%$, † $P < 0.1\%$) from controls by Student's *t*-test.

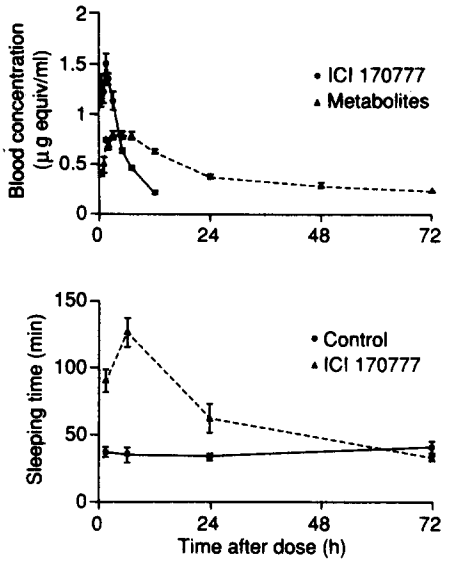

Fig. 3. A comparison of the time course of the ICI 170,777 effect on pentobarbitone-induced sleeping time with blood levels of ICI 170,777 and total metabolites observed in the rat. These profiles were obtained from separate studies. The blood profile shows the concentrations obtained following a single oral dose (5 mg/kg) of [^{14}C]ICI 170,777; total metabolite concentrations were determined from radioactivity measurements and exclude the contribution of unchanged ICI 170,777. The effect on sleeping time was determined at various times following administration of a single oral dose (20 mg/kg) of ICI 170,777. Each value shows the mean ± SE obtained from three animals.

However, with both compounds a substantial proportion of the urinary radioactivity consisted of unidentified metabolites.

Binding spectra. Spectral binding studies were performed to examine whether the inhibitory effects of ICI 153,110 and ICI 170,777 could be due to a direct interaction with P450. Using hepatic microsomes (1 nmol P450/mL) from control male rats, characteristic type II difference spectra (min 391–393; max 426–427 nm) were produced by the interaction of both compounds with P450. ICI 153,110 had a K_s of 11.8 μM and produced a maximal change of 0.0334A; the binding of ICI 170,777 was very similar exhibiting an affinity of 5.9 μM and maximal change of 0.0349A. In contrast, the pyridine *N*-oxides of both compounds failed to produce a recognizable spectral change using concentrations up to 100 μM.

DISCUSSION

On interaction with microsomes from control rats, both ICI 153,110 and ICI 170,777 produced a characteristic type II spectral change. This spectral binding is typical of many nitrogen-containing compounds, including substituted pyridines, and is thought to arise from an interaction of the accessible electrons of nitrogen with the haem iron of P450 [13, 14]. The spectral interactions of ICI 153,110 and ICI 170,777 are probably due to the nitrogen of the pyridine ring rather than the pyridazinone ring nitrogens, since the binding affinities and maximal change produced by each compound are very similar; in addition, oxidation of the pyridine nitrogen to the *N*-oxide completely abolished the type II spectrum.

Many compounds which bind to the haem iron of P450 as a sixth ligand effectively inhibit the oxidative metabolizing capability of P450 [15, 16]. Both ICI 153,110 and ICI 170,777 were shown to be inhibitors of P450-mediated reactions in the rat as indicated by the decrease in microsomal aldrin epoxidase activity *ex vivo* and by the prolongation of pentobarbitone sleeping time. However, comparison of the profile of inhibitory activity with the blood concentration–time curve for ICI 170,777 indicated that the prolongation of sleeping time correlated more closely with the concentration of metabolite(s) in the circulation than with parent compound. A role for metabolites was also suggested by the findings of the multiple dose study where microsomes were prepared 24 hr after the final dose, since it would be unusual to observe enzyme (aldrin epoxidase) inhibition due solely to a compound (ICI 170,777) with a blood half-life of about 4 hr [12]. Since there was no obvious change in the P450 population observed by gel electrophoresis following multiple dosing of ICI 170,777, the inhibition observed is probably due to a direct effect of ICI 170,777 or, more likely, a metabolite on the specific P450 isozyme responsible for aldrin epoxidase. Amrinone and milrinone, other cardiotonic agents of similar chemical structure, have also been shown to inhibit drug metabolizing activity by a mechanism which may involve a metabolite intermediate [5].

While ICI 153,110 appeared to have similar inhibitory characteristics to ICI 170,777, but of lower potency, the more obvious change produced by multiple dosing of ICI 153,110 was a pronounced induction of ethoxyresorufin *O*-deethylase and ethoxycoumarin *O*-deethylase. These effects are typical of P448 inducers such as 3-methylcholanthrene and β-naphthoflavone [9, 17]. This type of inducer also causes a marked decrease in the activity of such markers as aldrin epoxidase and ethylmorphine *N*-demethylase, presumably due to the decrease in concentration of the P450 isozymes as the hepatocytes selectively induce production of the P448 isozymes [7, 9, 11]. The decrease in aldrin epoxidase produced by ICI 153,110 may therefore arise either as a consequence of P448 induction or as a direct inhibitory effect of the compound or a metabolite.

One of the major areas of interest in this study was the markedly different spectrum of effects produced by two compounds with such structural similarities. The difference in inhibitory potency between the compounds may be a consequence of pharmacokinetic differences, since ICI 153,110 was found to have a shorter blood half-life (0.7 hr) and lower bioavailability than ICI 170,777 in the rat (unpublished observations). However, the observation of P448 induction with ICI 153,110, but not with ICI 170,777, is probably related to a difference in the physicochemical properties of the molecules. In contrast to other types of enzyme induction, inducers of the P448 isozymes are known to have characteristic

structural requirements [18, 19, 20]. The generally accepted requirements for a flat, planar aromatic structure have recently been assigned some quantitative dimensions by the molecular modelling method of Lewis *et al.* [21]. Compounds which interact primarily with P448 had a mean (±SE) area/depth^2 ratio of 6.3 ± 0.5 (N = 35); in contrast, compounds which interact with P450s other than P448 had a lower mean area/depth^2 value of 2.2 ± 0.2 (N = 25) [21]. This showed a correlation of P448 inducing potential with the area/depth^2 ratio of the molecule. Dr D. F. V. Lewis (Surrey University) kindly analysed both ICI 153,110 and ICI 170,777 using this computerized modelling method. ICI 153,110 was found to have an area/depth^2 value of 5.5, which places it firmly within the range of compounds likely to interact with or induce P448 [21]. ICI 170,777 had an area/depth^2 of 3.3 and is therefore considered unlikely to show any specificity for P448. This difference in properties is probably due to the presence of the methyl group in the *ortho* position of the thiadiazinone ring of ICI 170,777, preventing the molecule from adopting a suitably planar configuration. A similar effect has recently been reported to occur with the isomeric forms of aminobiphenyl, with only *ortho*-aminobiphenyl being non-planar [22].

It is difficult to assess whether P450 inhibition or P448 induction in laboratory animals has more importance when extrapolated to the clinical situation. Inhibitors of P450, such as cimetidine, produce drug interactions with a wide range of compounds, although only some of these interactions may have clinical relevance [23]. Induction of P448, which occurs as a result of cigarette smoking, can also produce clinically important drug interactions, such as the increased metabolic clearance of theophylline, a compound with a narrow therapeutic index [24]. However, the major area of concern is the possible toxicological implications of P448 induction in man, since most P448 inducers are mutagenic, carcinogenic, teratogenic, or produce some form of pronounced toxicity in laboratory animals [25, 26]. As a consequence, there are no known potent P448 inducers in clinical use, although enzyme induction data on a substantial proportion of current therapeutic drugs are not available [27]. Although enzyme induction in general is widely regarded as a physiological, adaptive response, the perceived association of P448 induction with some form of toxicity will probably continue until a compound, which produces pronounced P448 induction in man, has been used safely in the clinic for an extended period of time. Unfortunately, this will not be the case with ICI 153,110, which is no longer being developed as a candidate drug due to the observation of animal toxicity.

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