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Competitive inhibition of coumarin 7-hydroxylation by pilocarpine and its interaction with mouse CYP 2A5 and human CYP 2A6

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1 We have shown earlier that pilocarpine strongly inhibits mouse and human liver coumarin 7-hydroxylase activity of CYP 2A and pentoxyresorufin O-deethylase activity of CYP 2B *in vitro*. Since pilocarpine, like coumarin, contains a lactone structure we have studied in more detail its inhibitory potency on mouse and human liver coumarin 7-hydroxylation.

2 Pilocarpine was a competitive inhibitor of coumarin 7-hydroxlase *in vitro* both in mouse and human liver microsomes although it was not a substrate for CYP 2A5. K_i values were similar, 0.52 ± 0.22 μM in mice and 1.21±0.51 μM in human liver microsomes.

3 Pilocarpine induced a type II difference spectrum in mouse, human and recombinant CYP 2A5 yeast cell microsomes, with K_s values of 3.7±1.6, 1.6±1.1 and 1.5±0.1 μM, respectively.

4 Increase in pH of the incubation medium from pH 6 to 7.5 increased the potency of inhibition of coumarin 7-hydroxylation by pilocarpine.

5 Superimposition of pilocarpine and coumarin in such a way that their carbonyls, ring oxygens and the H-7' of coumarin and N-3 of pilocarpine overlap yielded a common molecular volume of 82%.

6 The results indicate that pilocarpine is a competitive inhibitor and has a high affinity for mouse CYP 2A5 and human CYP 2A6. In addition the immunotype nitrogen of pilocarpine is coordinated towards the haem iron in these P450s.

Keywords: CYP 2A5; pilocarpine; coumarin; enzyme inhibition

Introduction

Coumarin (1,2-benzopyrone), a plant alkaloid, possesses immunomodulatory and antitumour activity (Egan *et al.*, 1990). Its metabolism varies in different species (Cohen, 1979). Coumarin is mainly 7-hydroxylated by CYP 2A5 in mice and CYP 2A6 in man and excreted as the glucuronide in urine (Cohen, 1979). In rat, the 3-hydroxylated metabolite is the principal product of phase I enzymes (Cohen, 1979). Both CYP 2A5 and 2A6 enzymes have been purified and cloned and they show 83% amino acid homology (Juvonen *et al.*, 1988; Lindberg *et al.*, 1989; Negishi *et al.*, 1989; Miles *et al.*, 1990; Yamano *et al.*, 1990; Yun *et al.*, 1991). The furanocumarins, methoxsalen and sphondin, menadione and the imidazole antimycotics, miconazole and clotrimatzole, are efficient inhibitors of these enzymes (Mäenpää *et al.*, 1993). However, some chemicals such as metyrapone and psoralen inhibit coumarin 7-hydroxylation as catalyzed by CYP 2A5 but not the coumarin 7-hydroxylation via CYP 2A6 (Wood & Conney, 1974; Mäenpää *et al.*, 1993). The lactone structure and the carbonyl oxygen in the inhibitor are important for the inhibition of mouse CYP 2A5 (Juvonen *et al.*, 1991).

Pilocarpine is a direct acting cholinomimetic alkaloid for the control of elevated intraocular pressure associated with glaucoma. When applied to the eye, pilocarpine is absorbed into the systemic circulation (Tsujimoto *et al.*, 1972). Recently we have found that *in vitro*, pilocarpine inhibits CYP 2A and 2B activities more efficiently than other CYP activities (Kimonen *et al.*, 1995). Pilocarpine (mol.wt. 208) and coumarin (mol.wt. 146) are low molecular weight compounds, both of which contain a lactone structure (Figure. 1). On this chemical basis we studied the inhibition of coumarin 7-hydroxylation by pilocarpine *in vitro* in order to obtain more detailed data about substrate binding CYP 2A enzymes for elucidating potential drug-drug interactions. We determined the type of the inhibition of coumarin 7-hydroxylation and the pilocarpine-induced difference spectrum. The structures of pilocarpine and coumarin were compared by using SYBYL molecular modelling software to elucidate essential structure characteristics of these compounds for the binding to CYP 2A5 and 2A6.

Methods

Biological material

Male and female (7-12 weeks old) DBA/2N//Kuo mice were given pyrazole in saline (200 mg kg^{-1}, i.p.) on three consecutive mornings and killed 24h after the last injection. Livers were removed into ice cold saline and microsomes prepared as earlier described by Lang & Nebert (1981). Human liver

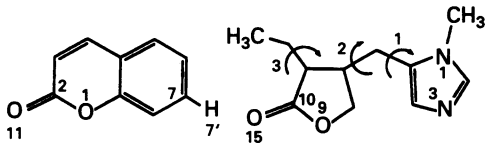

Figure 1 Chemical structures of coumarin and pilocarpine.

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samples were obtained from patients undergoing surgery in connection with laparoscopy under the approval of the ethics committee of the University of Kuopio. Liver samples were frozen in liquid nitrogen and stored at − 80 °C. Only tissue free of tumour was used for the preparation of microsomes that were isolated within one week of biopsy according to Lang & Nebert (1981). The patients have been described by Kimonen *et al.* (1995).

Biochemical assays

Microsomal protein content was determined by the method of Smith *et al.* (1985) and P450 content by the method of Omura & Sato (1964). The 7-hydroxycoumarin formed was measured as described by Aitio (1978). An incubation time of 5 min with 6 μg mouse and 30 μg human liver microsomal protein was used for all kinetic measurements. These conditions resulted in a linear response at all substrate concentrations. The incubation mixture contained 50 mM phosphate buffer pH 7.4, 5 mM $MgCl_2$, 0–1000 μM coumarin, 0, 0.5, 1, 2 or 4 μM pilocarpine and 75 μM NADPH. For studying the pilocarpine-induced difference spectrum, the microsomes at 3 mg ml^{-1} protein concentration were first solubilized for 30 min with a 0.6% cholate solution containing 100 mM phosphate buffer pH 6.4, 20 % glycerol, 0.1 mM EDTA and 0.1 mM dithiothreitol an then centrifuged for 1 h at 105 000 *g*. The supernatant was divided into two spectrophotometer cells, pilocarpine (0.2 μM–40 μM) was added from 10 μM stock solution in 100 μM phosphate buffer pH 7.4 to the sample cell and buffer to the reference cell and the spectrum records from 460 nm to 360 nm (Jefcoate, 1978). Analysis and possible metabolism of pilocarpine were checked in 50 mM potassium phosphate buffer pH 7.4 containing 5 mM $MgCl_2$, 0.1 mM NADPH, 0.150 mM pilocarpine and 1 mg mouse liver microsomal protein or 40 pmol CYP 2A5 purified from recombinant yeast cells (Juvonen *et al.*, 1991), 120 pmol NADPH cytochrome P450 reductase and 30 nmol dilauroylphosphatidylcholine. After 1 h incubation at 37°C, the samples were immediately analyzed by use of a deactivated Supelcosil LC8-DB reversed phase column (15m × 4.6 mm i.d., 5 μM) with an isocratic mobile phase consisting of 5% potassium dihydrogenphosphate pH 2.5 and methanol (97:3, v:v) (Järvinen *et al.*, 1991). K_m and V_{max} were analyzed by Lineweaver-Burk equation and K_i was estimated using their secondary plots.

Chemicals

These were obtained from the following sources: bicinconic acid, bovine serum albumin, coumarin, dilauroylphosphatidylcholine, 7-hydroxycoumarin, pyrazole (Sigma Chemical Co., St Louis, MO, U.S.A.), NADPH (Boehringer Mannheim Gmbh, Germany), pilocarpine hydrochloride (Leiras, Finland).

Computer analysis

Molecular modelling was carried out by using SYBYL (6.0) molecular modelling software (Tripos Ass. Inc. St Louis, MO, U.S.A.). The molecular structure of pilocarpine and coumarin were created using the sketch option of SYBYL. Structures were minimized using standard Tripos forcefield included in SYBYL (Clark *et al.*, 1989). Conformational analysis for pilocarpine was carried out by using the systematic search method in SYBYL. The increment for torsion angles (angles 1, 2 and 3 in Figure 1) in the search was 10°. Rings were kept rigid during the search and energy was calculated with Tripos forcefield; 500 lowest energy conformations were used to calculate distances between functional groups. Molecular volumes were also calculated using the lowest energy confirmation.

Results and discussion

Inhibition of coumarin 7-hydroxylation

In vitro, pilocarpine appeared to be a potent competitive inhibitor of this activity both in mouse and human liver microsomes as the V_{max} values did not change and K_m values of coumarin 7-hydroxylase activity increased with increasing pilocarpine concentrations in both sets of microsomes (Table 1). K_m values of coumarin in the coumarin 7-hydroxylation reaction were the same in both mouse and human liver microsomes (Table 1) and agree well with the earlier data for mouse and human liver microsomes (Pelkonen *et al.*, 1985; Juvonen *et al.*, 1988; Honkakoski *et al.*, 1993). The K_i values of pilocarpine for coumarin 7-hydroxylation were similar in mice and human enzymes to the K_m values for coumarin (Table 2) indicating whether equivalent affinity of pilocarpic and coumarin for CYP 2A5 and CYP 2A6 (Tables 1 and 2). Although pilocarpine possessed a high affinity for CYP 2A5, we could not detect either a decrease in pilocarpine concentration or the appearance of any new h.p.l.c. peak after a 2 h incubation with CYP 2A5 (Figure 2b). However, pilocarpine acid, which is one

Table 1 Effect of pilocarpine on kinetic parameters of coumarin 7-hydroxylase

Pilocarpine (μM)	K_m (μM)	V_{max} (nmol min^{-1} $nmol^{-1}$ P450)
In mouse		
0	0.74 ± 0.07	2.9 ± 0.3
2	5.9 ± 2.4	3.0 ± 0.4
4	10.4 ± 4.1	3.4 ± 0.4
8	15.3 ± 1.0	3.3 ± 0.5
16	38.4 ± 10.3	3.1 ± 0.4
In man		
0	0.64 ± 0.06	0.72 ± 0.12
0.5	0.73 ± 0.06	0.70 ± 0.10
1.0	1.27 ± 0.02	0.68 ± 0.06
2.0	1.97 ± 0.07	0.64 ± 0.07
4.0	3.13 ± 0.10	0.61 ± 0.06

Coumarin 7-hydroxylase activity was measured at various pilocarpine concentrations: 0, 2, 4, 8 and 16 μM pilocarpine were used with mouse liver microsomes and 0, 0.5, 1, 2 and 4 μM pilocarpine with human liver microsomes. With the use of each inhibitor concentration the activity was obtained with at least six different coumarin concentrations (0.5–1000 μM). The results are the average ± s.d. of three separate experiments with one set of pooled mouse liver microsomes and three different human liver microsomes.

Table 2 Affinity of pilocarpine for CYP 2A5 and CYP 2A6

Source of microsomes	K_i *of pilocarpine* (μM)	K_a *of pilocarpine* (μM)
Mouse liver	0.52 ± 0.22	3.7 ± 1.6
Human liver	1.21 ± 0.51	1.6 ± 1.1
CYP 2A5 recombinant yeast cell	ND	1.4,1.5

K_i values of pilocarpine were calculated from the secondary plots of each separate experiment done in Table 1. The pilocarpine-induced difference spectrum was measured with 0.2, 0.4, 0.8, 1.8, 3.8, 8.8, 18.8 and 39 μM concentrations. From these results K_a was calculated by double reciprocal analysis. Two results with yeast cells are shown and all the others are the average ± s.d. of three separate experiments. ND denotes not determined.

of the major *in vivo* metabolites of pilocarpine was formed during incubations with mouse liver microsomes (Figure 2c). These results indicate that the affinity of pilocarpine is high and equal in mouse and human liver microsomes and that *in vitro* pilocarpine is a strong competitive inhibitor for CYP 2A5 and 2A6 but that CYP 2A5 is unable to metabolize it.

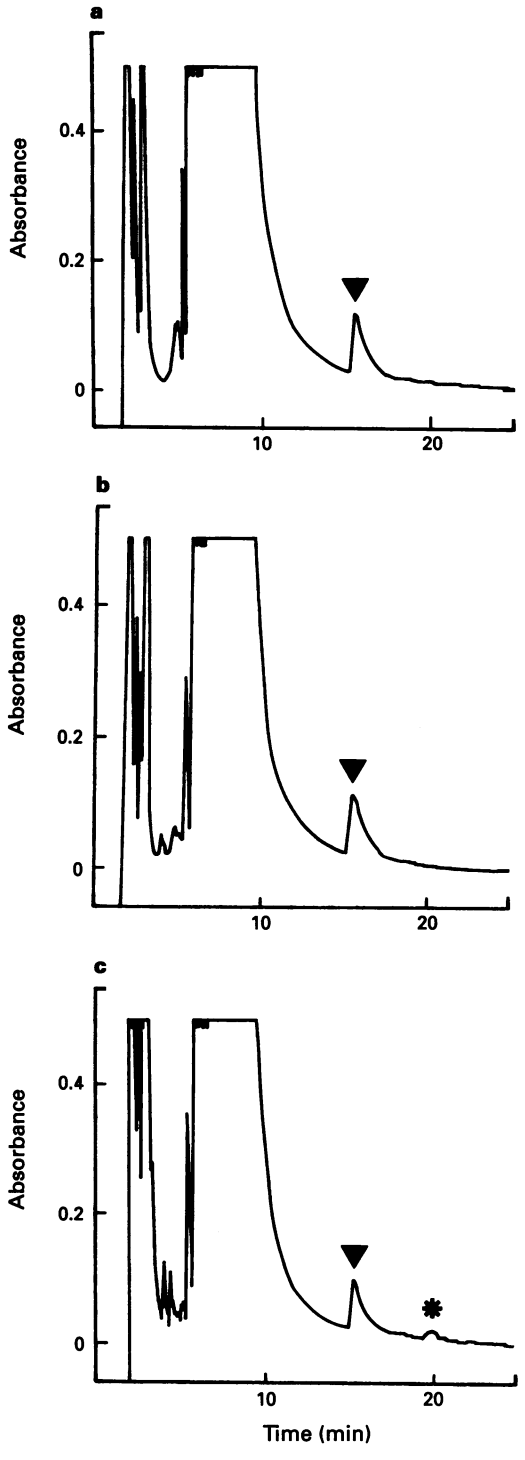

Figure 2 Metabolism of pilocarpine *in vitro*. Pilocarpine (0.150 mM) was incubated with 40 pmol reconstituted CYP 2A5 or 1 mg mouse liver microsomes for 1 h in 50 mM phosphate buffer pH 7.4 containing 5 mM $MgCl_2$ and 0.1 mM NAPDH and then analyzed with h.p.l.c. reversed phase column. (a) Represents incubation with microsomes without NAPDH, (b) with reconstituted CYP 2A5 and (c) with untreated mouse liver microsomes. (▼) Shows the elution peak of pilocarpine and the asterisk the elution peak of pilocarpic acid.

Binding of pilocarpine to P450

To measure more directly the binding of pilocarpine to P450 we measured the substrate-induced difference spectrum in mouse and human liver microsomes. In a recent study, pilocarpine gave a type II substrate-induced difference spectrum in microsomes of both species (Figure 3). Its peak was at 428 nm and it had a broad trough around 400 nm. Tsujimoto *et al.* (1972) have reported that pilocarpine induces a type II spectrum in rabbit liver microsomes. The association constant K_a in mouse liver microsomes was two times higher than in human microsomes which means that the affinity of pilocarpine is weaker in mice microsomes than in the human samples. The response may be caused by several P450 enzymes but would be largely by CYP 2A and 2B enzymes since their marker monooxygenase activities were inhibited by the lowest pilocarpine concentrations (Kimonen *et al.*, 1995). The difference in K_a between mouse and human might be due to either different P450 enzyme composition in these species or different affinity of the P450 enzymes. However, the K_a values were in the same range as the K_i values for inhibition of coumarin 7-hydroxylation which support the conclusion that pilocarpine has a high affinity for CYP 2A5 and 2A6.

Since pilocarpine-induced difference spectra in mice or human liver microsome do not indicate direct binding only to the active centre of CYP2A5 or 2A6, the spectrum was also measured in recombinant yeast cell microsomes expressing only CYP 2A5. The spectrum was type II with a K_a value of 1.5 μM which is two times smaller than in mice liver microsomes and the same as in human liver microsomes. This gives clear evidence that pilocarpine binds with a high affinity to CYP 2A5, and since CYP 2A6 is the human counterpart of CYP 2A5 and the inhibition of coumarin 7-hydroxylation is similar in the human and mice liver microsomes, it seems likely that pilocarpine binds in the same way also to CYP 2A6.

Pilocarpine contains only two nitrogen atoms, i.e. N(1) and N(3) atoms (Figure 1). Type II substrate-induced spectra are typical of chemicals containing nitrogen which is coordinated towards the P450 haem iron (Jefcoate, 1978; Gibson & Tamburini, 1984). Therefore the pilocarpine-induced type II spectrum indicates that one nitrogen of pilocarpine is coordinated towards the haem iron of the P450. Nitrogen N(3) in pilo-

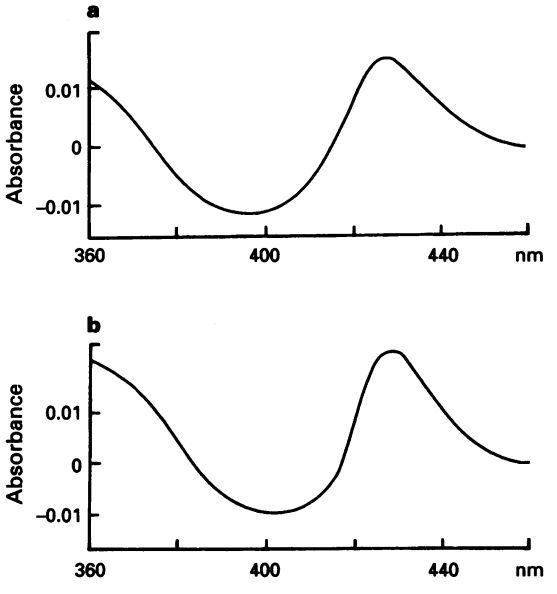

Figure 3 Pilocarpine-induced difference spectrum. Pyrazole-treated D2 mouse liver microsomes (a) or human liver microsomes (b) were solubilized, centrifuged and divided into two cells as described in Methods. Pilocarpine (40 μM in cells) was added into the sample cell and the spectrum recorded against solvent cell from 460 to 360 nm.

carpine is basic being approximately half protonated and charged at physiological pH while nitrogen N(1) is not (Figure 1, Table 3). A protonated nitrogen shows reduced coordination to the positively charged haem iron. If nitrogen N(1) was coordinated towards the haem iron then increasing the pH in the range of 6.0–7.5 should not have any effect on the inhibition of coumarin 7-hydroxylation by pilocarine since the change in pH will not affect the protonation of the nitrogen N(1). In addition, as the nitrogen N(1) is methylated, the coordination between haem iron and the nitrogen N(1) is very difficult if not impossible. In contrast, if nitrogen N(3) was coordinated towards haem iron, the inhibition should be reduced by lowering pH since this would move the equilibrium between unprotonated and protonated nitrogen N(3) towards the protonated fraction.

This hypothesis was tested by inhibiting coumarin 7-hydroxylation by pilocarpine at pH 6.0, 6.5, 7.0 and 7.5 in phosphate buffer. The inhibition was reduced by lowering the pH of the incubation mixture. IC_{50} values of pilocarpine for the reaction were 50, 16, 7 and 6 μM in mice and 50, 30, 13 and 9.5 μM in human liver microsomes at 10 μM coumarin in phosphate buffers of pH 6.0, 6.5, 7.0 and 7.5, respectively (Figure 4). To be sure that the effect was due to the protonation equilibrium of pilocarpine and not the effect of pH on the enzymes, we tested coumarin 7-hydroxylase inhibition by 7-methylcoumarin, a substrate for CYP 2A5 and a compound not affected by pH. Its IC_{50} values were 2, 2, 3.5 and 4.7 μM in pH 6.0, 6.5, 7.0 and 7.5, respectively, in mouse liver microsomes. The effect of pH on inhibition was weak and the opposite of that found with compared pilocarpine, which strengthens the proposal that N(3) protonation of pilocarpine influenced its binding to the enzyme. In human liver microsomes pH changed the inhibition by 7-methylcoumarin weakly as the IC_{50} values were 96, 92, 92 and 91 μM in pH 6.0, 6.5, 7.0 and 7.5 respectively. The results of these pH experiments support the hypothesis that the nitrogen N(3) in pilocarpine is coordinated towards the haem iron and that the protonated pilocarpine cannot bind to CYP 2A5 or 2A6 or inhibit the reaction.

Table 3 Comparison of chemical structures of coumarin and pilocarpine

		Interatomic distances (Å)	*Molecular volumes*	*pK_a values*
Coumarin	O(1)-H(7')	5.3	112.9	–
	O(11)-H(7')	7.1		
Pilocarpine	O(9)-N(1)	3.8-5.5	184.7	N(1) 1.6 HB+
	O(15)-N(1)	5.2-6.6		
	O(9)-N(3)	5.0-6.6		
	O(15)-N(3)	6.6-7.5		N(3) 7.0 HB+

Coumarin and pilocarpine molecular modelling were carried out by SYBYL molecular modelling software as described in Methods. Numbering of atoms is indicated in Figure 1. HB+ means the acidic state of the nitrogen atom.

Comparison of coumarin and pilocarpine binding to P450 2A5 and 2A6

As pilocarpine is a competitive inhibitor for CYP 2A5 and 2A6, it occupies the same sites as coumarin in the active site of

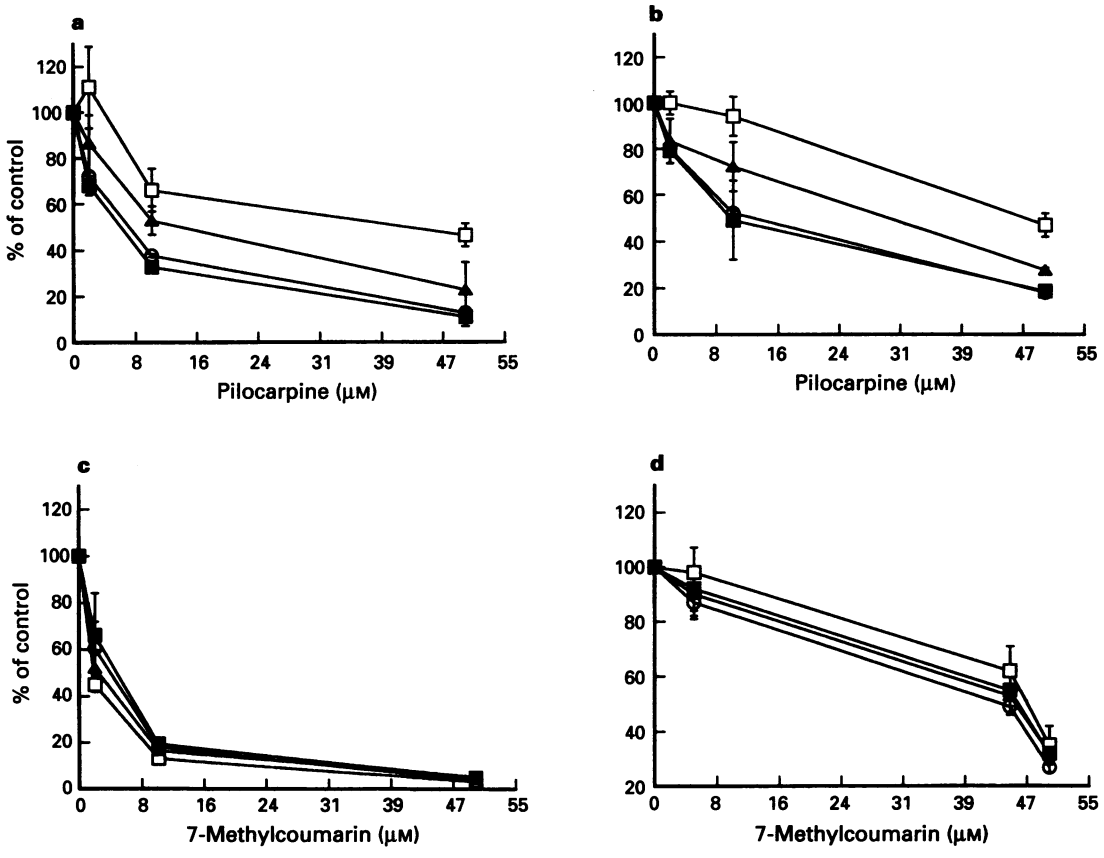

Figure 4 Effect of pH on the inhibition of coumarin 7-hydroxylation by pilocarpine and 7-methylcoumarin. Coumarin 7-hydroxylase activity at 10 μM coumarin concentration was inhibited by 2, 10 and 50 μM pilocarpine at pH 6.0 (□), 6.5 (▲), 7.0 (○) and 7.5 (■) in mouse (a,c) and human (b,d) liver microsomes. The data represent mean ± s.d. of the residual activity of three mouse and two human experiments performed individually.

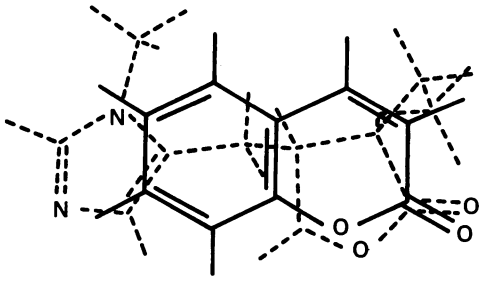

Figure 5 The superimposition of coumarin and pilocarpine. The structures were analyzed by SYBYL (6.0) molecular modelling software. The analysis gave a 82% common molecular volume. The structure of coumarin is shown with a thick line and pilocarpine with a dashed line.

the enzyme. We compared their chemical structures to elucidate important common features of the substrate and its inhibitor of CYP 2A5 and 2A6 such as molecular volumes, interatomic distances and pK_a values (Table 3).

Both compounds are small, the molecular weight of pilocarpine being 208 and coumarin 146 (Figure 1). Coumarin is mainly planar and rigid while pilocarpine is more torsional, having many possible minimum energy conformations. They both have a similar lactone ring structure although in pilocarpine the ring is composed of five atoms and in coumarin of six atoms. The lactone structure and especially its carbonyl oxygen is essential for the binding of coumarin to CYP 2A5 and probably also for CYP 2A6 (Juvonen *et al.*, 1991). We have assumed that the lactone ring structure of pilocarpine is bound in the same site as that in the corresponding coumarin structure. Although it has not been proven experimentally, it is likely that the carbonyl oxygen O(11) of coumarin forms a hydrogen bond with a hydrogen donor amino acid residue at the enzyme. This is analogous with camphor in which the carbonyl oxygen forms a hydrogen bond with tyrosine residue 96 and stabilizes the camphor CYP 101 complex (Poulos *et al.*, 1987; Raag & Poulos, 1991).

Since CYP 2A5 and 2A6 hydroxylate coumarin to 7-hydroxycoumarin, the hydrogen H(7') in coumarin must be pointing towards the oxygen coordinated to the haem iron in these enzymes. The interatomic distances in coumarin between hydrogen H(7') to carbonyl oxygen O(15) is 5.3 and from hydrogen H(7') and to oxygen O(1) is 7.1 (Table 2). In pilocarpine the interatomic distances of both oxygens to nitrogen N(3) match well to the above interatomic distances of coumarin; but the distance of oxygen O(15) to nitrogen N(1) does not although the distance of oxygen (O9) to nitrogen N(1) corresponds with these values (Table 3). This supports the conclusion that the nitrogen N(3) is coordinated towards the haem iron, thus confirming results from the type II pilocarpine-induced difference spectra and from the effect of pH on the potency of the inhibition. Pilocarpine and coumarin could be superimposed in such a way that their carbonyls, ring oxygens and the hydrogen H(7') of coumarin and nitrogen N(3) of pilocarpine overlapped (Figure 5). This yielded 82% common molecular volume. The major groups outside this common volume in this superimposition were the methyl and ethyl groups of pilocarpine, suggesting that in these binding orientations there is free space in the active centre of the CYP 2A5 and 2A6 enzymes. This high common molecular volume supports the possibility of the identical binding sites for coumarin and pilocarpine. Therefore we suggest that chemical structure analysis of unstudied substrates of CYP enzymes with specific and well known substrates are informative, useful and have a high predictive value for their metabolic patterns.

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