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Study of inhibition of CYP2A6 by some drugs derived from quinoline

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Abstract

CYP2A6 metabolizes coumarin to 7-hydroxycoumarin showing fluorescence, as measured by fluorometry. Firstly, we measured the inhibition of coumarin 7-hydroxylase of cDNA-expressed human CYP2A6 and in bovine liver microsomes, by quinoline and fluoroquinolines (FQ). Quinoline, 5-FQ, 6-FQ and 8-FQ inhibited activity but 3-FQ showed little inhibition. This suggests that the position 3 of quinoline is a recognition site for CYP2A6. We found similar patterns of coumarin 7-hydroxylase activity with human pooled liver microsomes. The level of CYP2A6 in human and bovine microsomes is the same as that detected by immunological titration with monoclonal antibody against CYP2A6. Secondly, we studied the inhibition of CYP2A6 with clinically used drugs of quinoline compounds, such as norfloxacin as an antibacterial agent, quinidine as an antiarrhythmic agent, quinine and chloroquine as antimalaria agents and rebamipide as an anti-ulcer agent. IC50 values (concentration producing 50% inhibition in activity) of norfloxacin, rebamipide and chloroquine at mM concentrations showed them to possess almost no inhibitory activity or influence on drug interaction. Meanwhile, the IC50 value of quinidine was 1.12 mM. The IC50 value of quinine was 160 μM with weak inhibition, suggesting that quinine, at a high dose, influences the metabolism of substrates for CYP2A6 by drug–drug interaction. These results also show that CYP2A6 discriminates the structure difference between the diastereoisomers quinidine and quinine.

Introduction

We studied coumarin 7-hydroxylation (Figure 1) of CYP2A6, which metabolizes ordinary drugs, such as methoxyflurane, fadrozole, zidovudine tegafur, paracetamol (acetaminophen) and phenacetin. CYP2A6 is also involved in the metabolic activation of several xenobiotics including aflatoxin B1, nicotine and cotinine. In man, CYP2A6 accounts for approximately 4% of all cytochrome P450s (CYPs) (Lewis & Pratt 1998). Human oesophagus expresses CYP2A6/2A7 and CYP2E, which can also activate the nitrosamine of a carcinogenic compound (Crespi et al 1991; Godoy et al 2002). There was a positive relationship between CYP2A6 activity and colorectal cancer risk (Nowell et al 2002), and deficient CYP2A6 activity coming from genetic polymorphism may reduce the risk of lung cancer (Miyamoto et al 1999). Expression of murine CYP2A5 is induced by inflammation (Montero et al 1999).

The history of the naming of human CYP2A6 (coumarin 7-hydroxylase) is as follows. The activity in man had initially been called cytochrome P450 II A3, and the gene encoding a polypeptide made of 448 amino acids was 85% identical with rat CYP2A3 (Lazard et al 1990). The human CYP II A3 gene product was named CYP2A6, as the name CYP2A3 was already occupied by rat CYP. CYP2A13 in human nasal mucosa has coumarin 7-hydroxylation activity (Su et al 1996), but its level in liver is much lower than that of CYP2A6 (Su et al 2000). Distribution and some properties of the CYP2A subfamily are summarized in Table 1 (Lindberg et al 1989; Lai & Chiang 1990; Peng et al 1993; Kurose et al 1998; Zhuo et al 1999; Su et al 2000). Coumarin 7-hydroxylase activity was not observed in rat and mouse livers, but found in olfactory mucosa (Zhuo et al 1999). For this reason, coumarin is used as ratsbane, because coumarin accumulates in the rat body and shows total toxicity.

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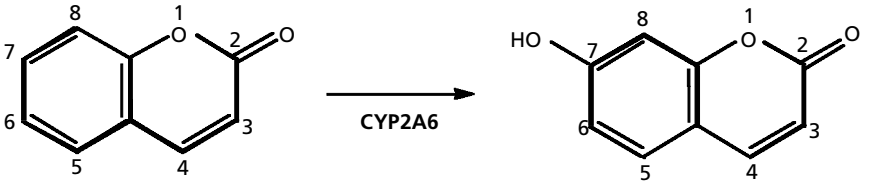


Figure 1 Coumarin 7-hydroxylation (CYP2A6).

Quinoline is the backbone of some drugs, including quinine, chloroquine and mefloquine as antimalaria agents, quinidine as an antiarrhythmic agent, rebamipide as an anti-ulcer agent and norfloxacin as an antibiotic. Contribution of CYP subfamilies to the reaction of quinoline has been reported and CYP2A6 in human liver microsomes is the principal cytochrome P450 involved in the formation of 5,6-dihydroquinoline 5,6-epoxide and quinoline *N*-oxide (Reigh et al 1996).

Coumarin 7-hydroxylase activity was not found in rat liver microsomes but found in bovine liver microsomes by us (Hirano et al 2002) and Sivapathasundaram et al (2001). Bovine liver microsomes can be obtained in large quantities at reasonable costs. If bovine liver had an enzyme like CYP2A6, it would be very useful for preclinical biochemical tests. We have already shown preliminarily the inhibition of coumarin 7-hydroxylase by quinolines but the results are complicated and not clear (Hirano et al 2002). In this paper, we show the clear results of the inhibition and the comparison between the levels of coumarin 7-hydroxylase in human liver microsomes and bovine liver microsomes. We also report the IC50 values (concentration producing 50% inhibition in activity) of several drugs used clinically and discuss the drug–drug interactions for CYP2A6.

Table 1 Summary of origin and function of the CYP2A subfamily.

CYP	Species	Organ	Function
CYP2A1	Rat	Liver	Steroid 7α-hydroxylation
CYP2A2	Rat	Liver	Steroid 15α-hydroxylation
CYP2A3	Rat	Olfactory	Coumarin 7-hydroxylation
Cyp2A4	Mouse	Liver, kidney	Steroid 15α-hydroxylation
Cyp2A5	Mouse	Olfactory, respiratory	Coumarin 7-hydroxylation
CYP2A6	Man	Liver	Coumarin 7-hydroxylation
CYP2A7	Man	Liver	No function
CYP2A8	Hamster	Liver	Steroid 15α-hydroxylation
CYP2A9	Hamster	Liver	Steroid 7α-hydroxylation
CYP2A10	Rabbit	Olfactory, respiratory	Steroid 7α-hydroxylation Coumarin 7-hydroxylation
CYP2A11	Rabbit	Olfactory, respiratory	Steroid 15α-hydroxylation
Cyp2A12	Mouse	Liver	Steroid 7α-hydroxylation
CYP2A13	Man	Olfactory, respiratory	Coumarin 7-hydroxylation

Materials and Methods

Materials

Quinoline was obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan), coumarin, chloroquine, quinidine and norfloxacin from Sigma-Aldrich Co. (Japan), quinine from Fluka, and rebamipide was kindly supplied by Otsuka Pharmaceutical Co. (Japan). Recombinant human CYP2A6 expressed in B-lymphoblastoid cells, pooled human liver microsomes and IH-MAB-CYP2A6 were products of Gentest Corporation (Woburn, MA). We purchased IH-MAB-CYP2A6 as a monoclonal antibody against CYP2A6. Some monofluoroquinolines (FQ) were generously synthesized by Dr Kato according to a previous report (Kato et al 2000).

Preparation of enzymes

Bovine liver microsomes were prepared as previously reported (Mizutani et al 1984; Kanou et al 2002). Briefly, fresh bovine liver was minced and mixed 4-fold with 0.25 M sucrose–10 mM Tris-HCl (pH 7.4). The extract was centrifuged at 8000 *g* and the supernatant was centrifuged at 105 000 *g* for 1 h. The precipitate at 105 000 *g* (microsomes) was suspended in 0.1 M Tris-HCl–10% glycerol and centrifuged once more at 105 000 *g*. The precipitate was suspended in 0.1 M Tris-HCl (pH 7.5)–10% glycerol and stored at −80 °C.

Coumarin 7-hydroxylase activity

Coumarin 7-hydroxylation activity was measured as previously reported (Hirano et al 2002). Briefly, the incubation mixture (0.3 mL) contained 1.3 mM $NADP^+$, 3.3 mM glucose 6-phosphate, 4 U mL^{-1} glucose-6-phosphate dehydrogenase, 0.1–10 μM coumarin and 10 μM (for bovine liver microsomes) or 20 μM (for cDNA-expressed CYP2A6) quinoline or quinoline derivatives prepared in 0.1 M Tris-HCl buffer (pH 7.4). The reactions were started

by the addition of 0.15 mg protein after 5 min pre-incubation at 37 °C. The mixture was incubated for 30 min at 37 °C. The reactions were terminated by the addition of 100 μL 20% trichloroacetic acid and the mixture was centrifuged at 14 000 *g* for 10 min at 4 °C. The supernatant (40 μL) was added to 700 μL of 100 mM Tris-HCl (pH 9.0). Each of the three 200-μL triplicate samples was added to wells in a 96-well plate and the fluorescence (Ex 390 nm, Em 460 nm) of these mixtures was measured with an ARVO 1420 Multilabel Counter. The mean ± s.d. value of each point was calculated from 5 determinations. The significance of the difference between two values is shown in the legend of each figure. The cytochrome P450 content in each microsomal preparation was determined by the standard method of spectrophotometry with CO gas at 450 nm (Omura & Sato 1962).

The IC50 values of drugs were estimated from the values obtained by addition of those drugs at various concentrations to the above reaction mixture, according to the method described in the brochure of Gentest.

Inhibition of CYP2A6 by monoclonal antibody

For estimation of the level of CYP2A6, the assay described above was carried out with the enzyme preparations pre-incubated with IH-MAB-CYP2A6 at various concentrations for 10 min at 4 °C. Then the assay mixture containing substrates was added to the antibody–enzyme solution and incubated for 30 min. Further steps are as described above (see Coumarin 7-hydroxylase activity).

Results

CYP species metabolizing quinoline are described by Reigh 1996, and quinoline 5,6-hydroxylation proceeds by CYP2A6. Therefore, we investigated the inhibition of CYP2A6 coumarin 7-hydroxylation by quinoline and its fluorinated derivatives. The results with bovine microsomes and cDNA-expressed human CYP2A6 as enzyme sources are shown in Figure 2 (A and B, respectively). In Figure 2, the coumarin concentration is 10 μM and the inhibitor concentration is 10 μM for bovine enzymes and 20 μM for human CYP2A6. Quinoline, 5-FQ, 6-FQ and 8-FQ significantly inhibited 39%, 41%, 47% and 52% of the coumarin 7-hydroxylation, respectively (Figure 2A). On the other hand, the inhibition of 3FQ was low. These results also show that bovine liver microsomes have coumarin 7-hydroxylation activity and are valuable as an enzyme source of CYP2A6. The inhibition pattern with bovine microsomes was clearer than with human CYP2A6 (Figure 2B). This result suggests that the position 3 of quinoline is important in the recognition by human CYP2A6 and bovine enzymes.

The similarity of the bovine enzyme to human CYP2A6 was further confirmed by titration of an inhibi-

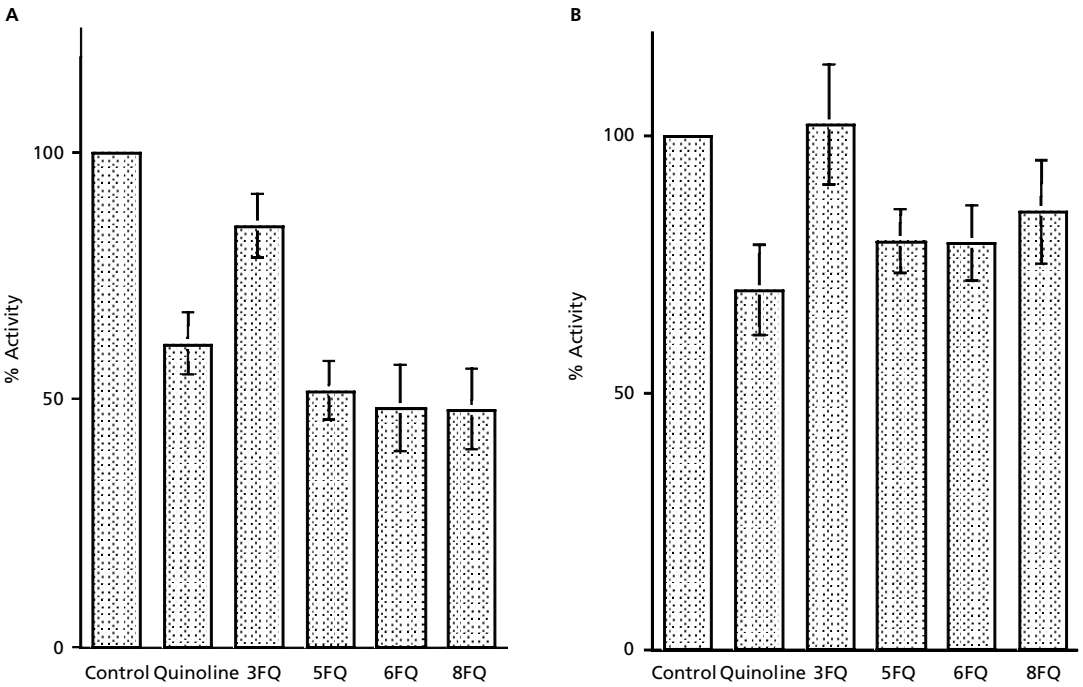


Figure 2 The relative inhibition of coumarin 7-hydroxylation by quinoline and fluoroquinolines (FQ) using bovine liver microsomes (A) or cDNA-expressed human CYP2A6 from B-lymphoblastoid cells (B). Coumarin was used at 10 μM; the concentration of inhibitors (quinolines) was 10 μM (A) or 20 μM (B). In A, $P < 0.05$, 3FQ vs quinoline; $P < 0.01$, 3FQ vs other FQs, such as 5FQ, 6FQ and 8FQ. In B, $P < 0.05$, 3FQ vs other quinolines.

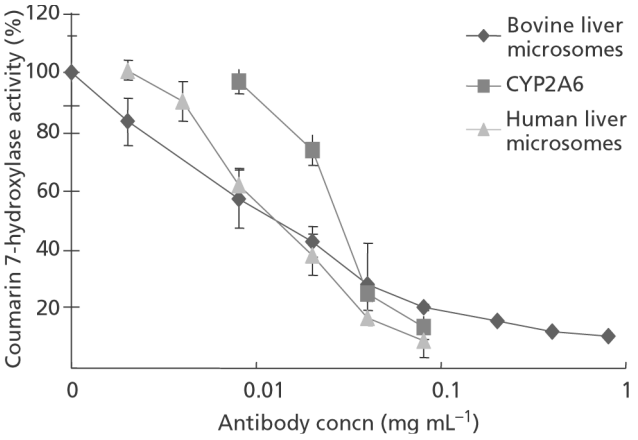


Figure 3 The level of CYP2A6 by estimation from inhibition experiment with monoclonal antibody against CYP2A6. Diamonds, bovine liver microsomes; triangles, pooled human liver microsomes; squares, cDNA-expressed human CYP2A6. The antibody used is IH-MAB-CYP2A6.

tion experiment with monoclonal antibody against human CYP2A6 (IH-MAB-CYP2A6). Figure 3 shows that CYP2A6 presented at a similar level in pooled human liver microsomes (Gentest) and bovine liver microsomes, with a concentration of 0.01 mg mL^{-1} giving 50% inhibition. The CYP2A6 level was slightly higher in the recombinant human CYP2A6 expressed in B-lymphoblastoid cells (Figure 3). These results confirmed that human and bovine microsomes contain a similar level of CYP2A6.

Due to inhibition of CYP2A6 by quinolines, we were interested in the inhibition exhibited by some drugs derived from quinoline (norfloxacin, rebamipide, chloroquine, quinine and quinidine). We carried out inhibition experiments with cDNA-expressed human CYP2A6 at a concentration of 10 μM coumarin. The structure and IC50 values of the quinoline derivatives are shown in Table 2. The IC50 value of norfloxacin was more than 10 mM, and rebamipide and chloroquine inhibited 50% of the coumarin 7-hydroxylation at 6.8 and 7.8 mM, respectively. These concentrations are about a thousand-fold that of coumarin. Therefore, these three drugs are almost non-inhibitors and did not influence CYP2A6 reaction at the clinical blood level of those drugs, such as 3.5 μM for norfloxacin, 0.6 μM for rebamipide and 10 μM for chloroquine.

The IC50 value of quinidine was 1.12 mM, representing slight inhibition among drugs used in this experiment; that of quinine was 160 μM and this result may slightly influence CYP2A6 reaction by drug–drug interaction, because the clinical blood level of quinine is about 10 μM (1.5 g daily). This effective inhibition of CYP2A6 by quinine must be considered in clinical cases. It is interesting that the relation of quinine and quinidine, having the same chemical structure, is a diastereoisomer. The orientation of residues binding on C4 of quinine might cause the difference in steric interaction between drugs and CYP2A6. The inhibition of CYP2D6 by quinidine is well known.

Table 2 Summary of inhibition of CYP2A6 by some quinoline derivatives.

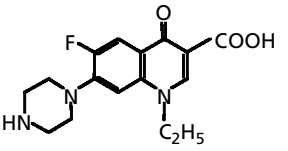
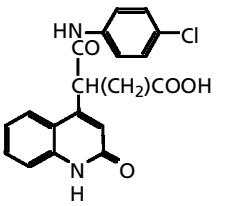
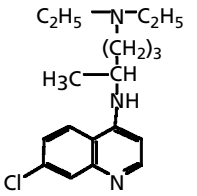
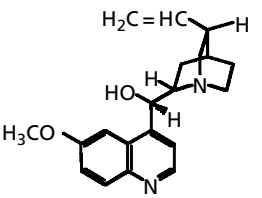
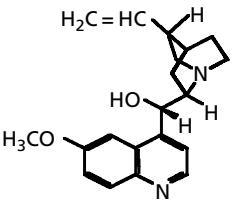
Drug	Structure	IC50
Norfloxacin		> 10 mM
Rebamipide		6.8 mM
Chloroquine		7.8 mM
Quinine		160 μM
Quinidine		1.12 mM

Figure 4 shows experimental data used to estimate the IC50 value of quinidine and quinine. There are a few reports which showed no inhibition of CYP2A6 by quinine (Draper et al 1997; Juvonen et al 2000). When quinine is used at a high dose with tegafur, paracetamol and

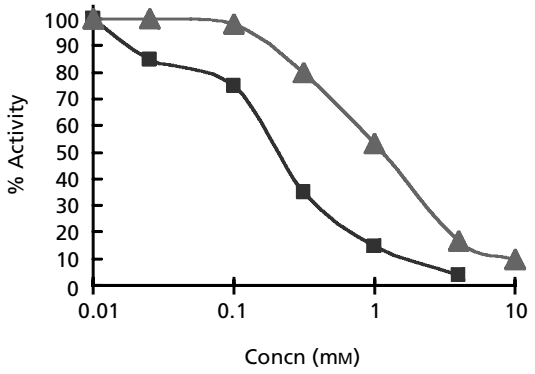


Figure 4 A typical inhibition pattern of coumarin 7-hydroxylation by quinine (squares) and quinidine (triangles). Enzyme used is cDNA-expressed human CYP2A6.

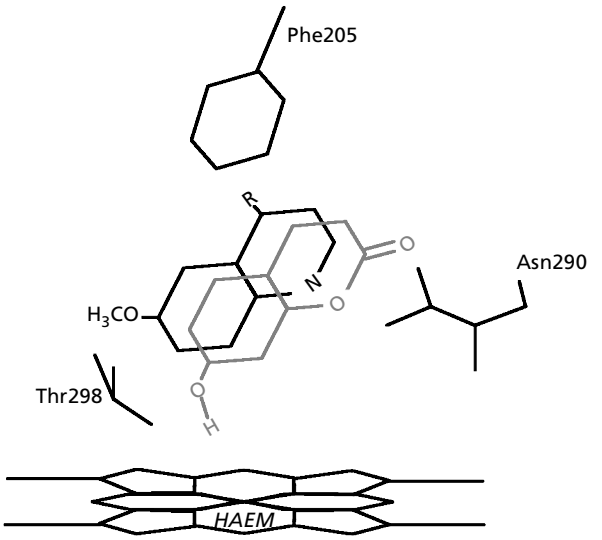


Figure 5 Molecular model of 7-hydroxycoumarin (grey) and quinine (quinidine) (black) at the putative active site of CYP2A6.

phenacetin, which are metabolized by CYP2A6, we must consider the drug–drug interaction of metabolism by CYP2A6.

Discussion

The ligand binding model to CYP2A6 (Lewis 2002a, b) has been reported. In the model, Phe205, Asn290 and Thr298 are key amino acids for interaction between drug and haem in the enzyme. In Figure 5, we show the orientation of quinine (quinidine) (black line) and 7-hydroxycoumarin (grey line) constructed according to the above model. The orientation of R residues binding on C4 of quinoline is important. We consider that the R residue on quinidine interfered with Phe205, meanwhile the R residue of quinine is orientated to the vacant space between Phe205 and Thr298, and quinine fits more in the pocket for ligands of CYP2A6 than quinidine. Norfloxacin, rebamipide and chloroquine are not fitted in the ligand pocket due to steric hindrance of the structure, such as residues binding on C4 or C8 of quinoline. It has been reported that active amino acid residues on CYP2A6 are Gln74, to interact with oxygen in coumarin, His437, with ketone in coumarin, and Phe181 for π–π stacking interaction (Lewis & Gorrod 2002). From the research of site-directed mutagenesis, Val117, Arg129 and Leu160 residues on CYP2A are also important for interaction. Table 3 shows comparison of the ligand-binding domain of some CYP2A subtypes, which show two types of activity – coumarin 7-hydroxylation and steroid 7α- or 15α-hydroxylation (Table 1).

CYP2A and UDP-glucuronosyl transferase were found in bovine olfactory mucosa (Lazard et al 1990). They obtained peptide 19 from the protease digestion of CYP2A. The peptide 19 has a common sequence with the more homologous peptide 19 coming from CYP2A13 than that from CYP2A6. The peptide 19 is also similar to CYP2A3 from rat nasal mucosa. Table 3 summarizes the sequences of peptide 19 from CYP2A subfamilies that have coumarin 7-hydroxylation activity. These sequences include the substrate recognition site called SRS3, which is 6 amino acids from Phe238 to Gly243. Inside this 6-amino-acid sequence, the 239th and 240th amino acids are characteristic positions. CYP2A6 in liver has Gln239 and Leu240, and the others have Lys239 and Glu240. SRS3 is conserved in olfactory coumarin 7-hydroxylases.

Conclusion

Coumarin 7-hydroxylase of CYP2A6 was found in bovine liver microsomes at the same level as in human liver microsomes. The hydroxylation reaction was inhibited

Table 3 Comparison of sequences of peptide 19 and the homologous position in some of the CYP2A subfamily.

	227												239					243
CYP2A6	M	K	H	L	P	G	P	Q	Q	Q	A	F	Q	L	L	Q	G	L
Peptide 19	M	X	Y	L	P	G	P	Q	Q	Q	A	F	K	E	L	Q	G	L
CYP2A3	M	K	H	L	P	G	P	Q	Q	Q	A	F	K	E	L	Q	G	L
Cyp2A5	M	K	H	L	P	G	P	Q	Q	Q	A	F	K	E	L	Q	G	L
CYP2A10	M	K	H	L	P	G	P	Q	Q	Q	A	F	K	E	L	Q	G	L
CYP2A13	M	K	H	L	P	G	P	Q	Q	Q	A	F	K	E	L	E	G	L

by quinoline, 5-fluoroquinoline (FQ), 6-FQ and 8-FQ, but not by 3-FQ. We next studied the inhibition of CYP2A6 with drugs having quinoline backbones and used clinically, such as norfloxacin, quinidine, quinine, chloroquine and rebamipide. From the IC50 values, norfloxacin, rebamipide and chloroquine showed almost no inhibition. Meanwhile, the IC50 value of quinidine was 1.12 mM (slight inhibition). The IC50 value of quinine was 160 μM (weak inhibition) and this value was significant in the context of drug–drug interaction on CYP2A6 when quinine is administered at a high dose.

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