The Influence of Quinolines on Coumarin 7-Hydroxylation in Bovine Liver Microsomes and Human CYP2A6

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Quinoline is a chemical with potential pharmaceutical components, such as antimalaria, antiulcer, and antibiotic agents. Quinoline is metabolized by CYP2A6, whose activity is generally shown by coumarin 7-hydroxylation, and the principal product is the 5,6-epoxide of quinoline. We found coumarin 7-hydroxylase activity in bovine liver microsomes and studied the interaction of quinoline and some quinoline derivatives with coumarin 7-hydroxylase activity by fluorometry. Quinoline inhibited coumarin metabolism, and the apparent V_{max} value decreased to 0.39 nmol/min/nmol cytochrome P-450 (CYP) in the presence of quinoline from the value (V_{max} = 0.63 nmol/min/nmol CYP) in the absence of quinoline. 5-fluoroquinoline (5FQ), 6-fluoroquinoline (6FQ) and 8-fluoroquinoline (8FQ) showed stronger inhibition than quinoline, whereas 3-fluoroquinoline (3FQ) showed weaker inhibition (apparent V_{max} was 0.59 nmol/min/nmol CYP). Almost the same inhibition pattern of fluoroquinolines were found in assays of cDNA-expressed human CYP2A6. The results suggest that bovine CYP2A enzymes (s) as well as human CYP2A6 can interact strongly with monofluoroquinolines such as 5-, 6-, and 8-FQ, but weakly with 3-FQ.

Key words —— CYP2A6, bovine microsomes, quinoline, coumarin 7-hydroxylase, fluoroquinoline, cytochrome P-450

INTRODUCTION

Quinoline is the backbone of some drugs, including quinine, chloroquine and mefloquine as antimalaria agents, quinidine as an antiarrhythmic agent, rebamipide as an antiulcer agent, and norfloxacin as an antibiotic agent. Thus, quinoline is a promising chemical for use as a drug. Meanwhile, 4-nitroquinoline-*N*-oxide, a derivative of quinoline, is a toxic and typical potent carcinogen.[1] Quinoline itself shows weak mutagenicity after oxidation by liver microsomal enzymes, as shown in Fig. 1.[2–5] The oxidized enamine epoxide, as shown in the right side of Fig. 1, reacts with DNA, and quinoline adducts on DNA bases may induce carcinogenesis.

Cytochrome P-450 (CYP) participates in the oxidative metabolism of phase I biotransformation. A contribution of CYP subfamilies to the reaction of quinoline has been reported.[6] Meanwhile, we have studied the drug metabolism of quinoline and have manifested that CYP2A6 in human liver microsomes is the principal cytochrome P-450 involved in the formation of 5,6-dihydroquinoline 5,6-epoxide in the left side of Fig. 1, and quinoline *N*-oxide in the middle. CYP2E1 plays a major role in quinoline 3-hydroxylation, a pathway that may be related to the genotoxicity of quinoline *in vitro*[2] and *in vivo*.[7,8] On the other hand, CYP subfamilies in rat liver microsomes show slightly different patterns from those in human liver microsomes. CYP1A2 or 1A1 is the principal cytochrome P-450 involved in the formation of quinoline 5,6-epoxide, and CYP2E1 also plays a major role in quinoline 3-hydroxylation in rat liver microsomes.[6] The % ratios of quinoline 5,6-epoxide, quinoline *N*-oxide and 3-hydroxyquinoline in the quinoline metabolites are 82%, 5% and 3%, respectively.[2–5]

The amount of CYP2A6 in human liver is 4% of all CYP subfamilies, which makes it the fourth most abundant next to CYP3A, CYP2C, CYP1A and 2E.[9–11] CYP2A6 metabolizes ordinary chemicals, such as coumarin, methoxyfluorane, fadrozole

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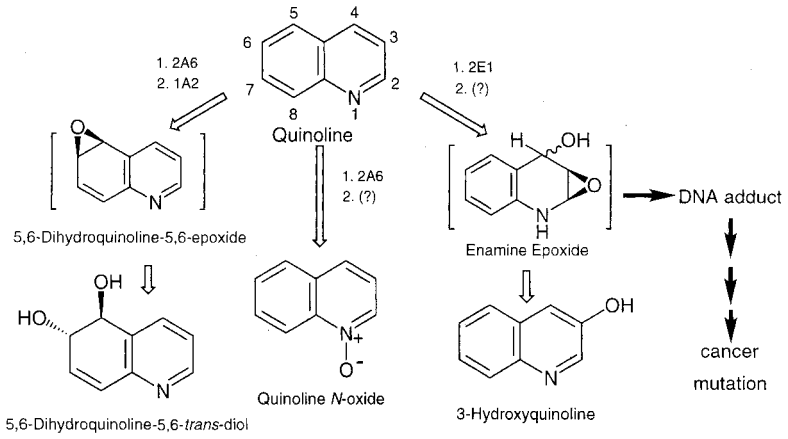

Fig. 1. Quinoline Metabolism in Human Microsomes
This is summarized from schemes in the reported literature.[2,3]

losigamone and tegafur. It is also involved in the metabolic activation of several xenobiotics including aflatoxin B1,[12] carcinogenic nitrosoamines,[13] nicotine,[14] and cotinine.[15] Recently SM-12502 was also reported to be a substrate of CYP2A6.[16]

The human cytochrome P-450 2A subfamily contains three enzymes: CYP2A6, CYP2A7 and CYP2A13. CYP2A6 is responsible for coumarin 7-hydroxylase activity. Rat has CYP2A1 and CYP2A2, and mouse has CYP2A4 and CYP2A5. In rat liver microsomes, coumarin was metabolized to 3-hydroxycoumarin by CYP1A1[17,18] and 2B subfamilies.[19] In the case of mouse, coumarin is 7-hydroxylated by CYP2A5.[20]

During the course of investigation of the mechanism of selenocysteine synthesis with cytosol from bovine liver,[21] we reserved a large amount of bovine liver microsomes and found that bovine liver microsomes had the ability to catalyze coumarin to 7-hydroxycoumarin, which is generally dependent on CYP2A6 in human. We have also studied quinoline metabolism by the phase I reaction.[22] In this study, we examined the influence of quinoline and fluoroquinolines on the reaction of coumarin 7-hydroxylation in bovine liver microsomes and human CYP2A6.

MATERIALA AND METHODS

Chemicals —— Quinoline was obtained from Wako Pure Chemical Industries, Ltd., (Osaka, Japan) coumarin and 7-hydroxycoumarin (umbelliferone) from Sigma-Aldrich Co., Japan, and human CYP2A6 from Gentest Corporation, Woburn, (MA, U.S.A.). Monofluoroquinolines shown in Fig. 2 were produced as previously reported.[7,22]

Preparation of Enzymes —— Bovine liver microsomes were prepared as previously reported.[23] Briefly, fresh bovine liver was minced and mixed in 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 105000 × *g* for 1 hr. The precipitate at 105000 × *g* (microsomes) was suspended in 0.1 M Tris–HCl–10% glycerol and centrifuged once more at 105000 × *g*. The precipitate was resuspended in 0.1 M Tris–HCl (pH 7.5)–10% glycerol and stored at –80°C.

Coumarin 7-Hydroxylase Activity —— Coumarin 7-hyroxylation activity was measured as previously reported.[3] Briefly, the incubation mixture (0.3 ml) contained 1.3 mM $NADP^+$, 3.3 mM glucose 6-phosphate, 4 U/ml glucose 6-phosphate dehydrogenase, 0.1–10 μM coumarin and 10 μM 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 preincubation at 37°C. The mixtures were incubated for 30 min at 37°C. The reactions were terminated by the addition of 100 μl 20% trichloroacetic acid and the mixtures were centrifuged at 14000 × *g* for 10 min at 4°C. The supernatant (40 μl) was added to 760 μl 100 mM Tris–HCl (pH 9.0). Each of the three 200 μl as triplicates 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 ARVO1420 Multilabel Counter.[24] The mean ± S.D. values of each point was calculated from 5 determinations. The significance between two values is shown in the legend of each of the figures. Cytochrome P-450 con-

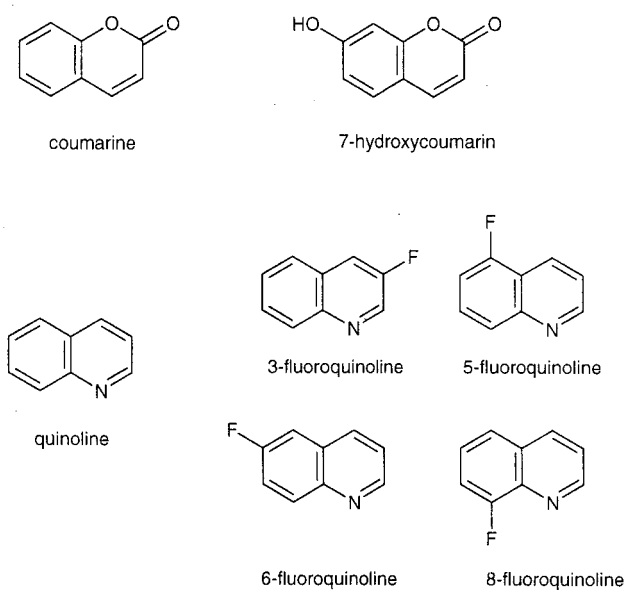

Fig. 2. Structure of Some Chemicals Used

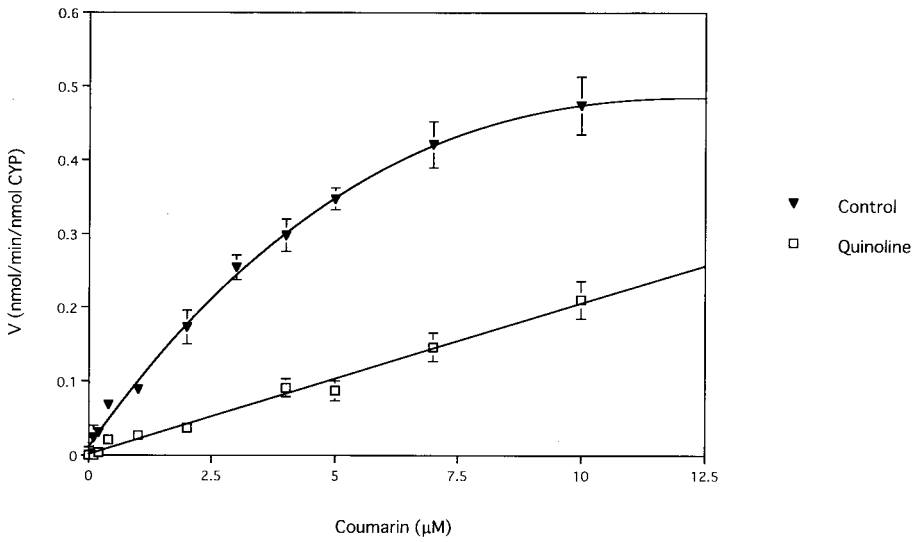

Fig. 3. The Relationships of Velocity and Substrate Concentration to Bovine Liver Microsomes

Closed triangles indicate in the absence of quinoline and open squares are in the presence of 10 μM quinoline. The mean ± S.D. values of each point were calculated from 5 determinations.

tent in each microsomal preparation was determined by the standard method of spectrophotometry with CO gas at 450 nm.[25]

RESULTS

The Assay Conditions of Coumarin Metabolism with Bovine Liver Microsomes

The formation of 7-hydroxycoumarin at 0.4 mM coumarin was detected in bovine liver microsomes, and it increased linearly for 60 min of incubation. The incubation time for 30 min was used to establish the initial rate conditions for the formation of 7-hydroxycoumarin. The linearity was found up to a protein concentration of 0.5 mg/ml, and the concentration of 0.3 mg/ml was used. Figure 3 shows a typical pattern of plots of a velocity-substrate concentration relationship at a concentration of 0.1–10 μM coumarin on coumarin 7-hydroxylation with bovine liver microsomes (V_{max}: 0.63 nmol/min/nmol CYP, K_M: 4.6 μM, by Lineweaver-Burk plots).

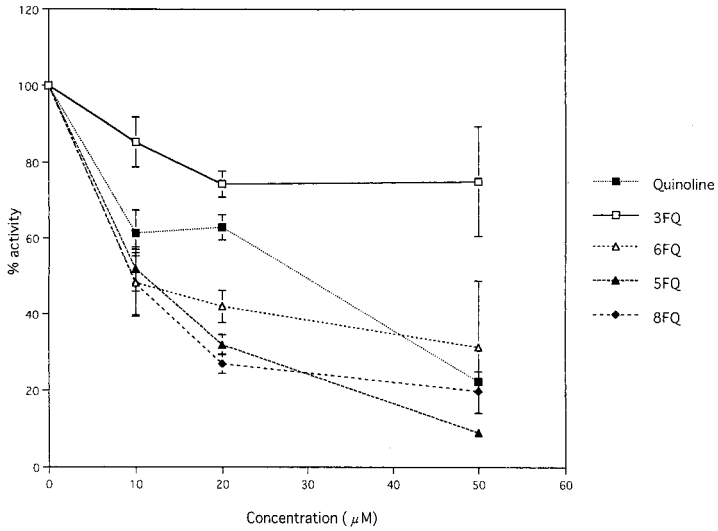

Fig. 4. The Relative Inhibition of Coumarin 7-hydroxylation by Quinolines

At a concentration of 10 μM, the values are significantly different between 3FQ and quinoline ($p < 0.05$), and between 3FQ and other FQs, such as 5FQ, 6FQ and 8 FQ ($p < 0.01$). At a concentration of 50 μM, the values are significantly different between 3FQ and other quinolines, such as quinoline, 5FQ, 6FQ and 8FQ ($p < 0.01$). The mean ± S.D. values of each point were calculated from 5 determinations.

Inhibition Analysis

Quinoline inhibited coumarin 7-hydroxylation when 10 μM quinoline was added to the reaction mixture, as shown in Fig. 3 (appearent V_{max}: 0.39 nmol/min/nmol CYP). Next, one kind of 10 μM monofluoroquinoline was also added, and 5-fluoroquinoline (5FQ), 6-fluoroquinoline (6FQ) and 8-fluoroquinoline (8FQ) showed stronger inhibition than quinoline (apparent V_{max}: 0.28, 0.18, 0.12 nmol/min/nmol CYP, respectively). However, 3-fluoroquinoline (3FQ) showed less (apparent V_{max}: 0.59 nmol/min/nmol CYP). The lowered V_{max} values with the addition of quinoline and FQs, except 3FQ, indicated the inhibition of coumarin metabolism by these compounds. We observed some kinetic parameters indicating a mixed type of competitive and non-competitive inhibition and could not show clear Ki values for these inhibitions.

Instead, we compared the coumarin 7-hydroxylase activity at 10 μM quinoline or one of the quinoline derivatives. A weak inhibition by 3FQ was found, as shown in Fig. 4, in which the activities were normalized relative to the activity of 7-hydroxycoumarin formed at 10 μM coumarin. Quinoline, 5FQ, 6FQ, and 8FQ showed significant inhibition of 39%, 41%, 47% and 52%, respectively, as shown in Fig. 4. On the other hand, inhibition by 3FQ at 10 μM was 17.5%, and this value was significantly different from those of control, quinoline and quinoline derivatives, by Student's *t*-test.

To clarify the specificity of 3FQ, the relative activities at higher concentrations of each quinoline and monofluoroquinoline were also compared, as shown in Fig. 4. The activities were normalized relative to the activity of 7-hydroxycoumarin formed at a coumarin concentration of 10 μM. The level of inhibition of 3FQ was 15–20% at a concentration range of up to 50 μM, at which concentration, quinoline and the other monofluoroquinolines inhibited coumarin 7-hydroxylase by more than 60%. Thus, quinoline, 5FQ, 6FQ and 8FQ were inhibitors of coumarin 7-hydroxylation in bovine liver microsomes, whereas the inhibition by 3FQ was weak. The conversion of coumarin to 7-hydroxycoumarin is generally performed by CYP2A6 in human liver microsomes. In this study, we showed that quinolines inhibited coumarin 7-hydroxylation by the enzyme in bovine liver microsomes. Taken together, the strong inhibition of coumarin 7-hydroxylation by quinoline, 5FQ and 6FQ, and the weak inhibition by 3FQ, suggest that the position-3 of quinoline interacts with the active site of CYP2A6.

cDNA-Expressed CYP2A6

cDNA-expressed CYP2A6 purchased from GENTEST was used instead of bovine liver microsomes to confirm its effect on quinoline metabolism. Reaction mixtures were likewise incubated for 30 min. A protein concentration of 0.1 mg/ml was used, because at that concentration we could detect similar intensities to fluorometry for comparison to bovine liver microsomes. At that concentration, we

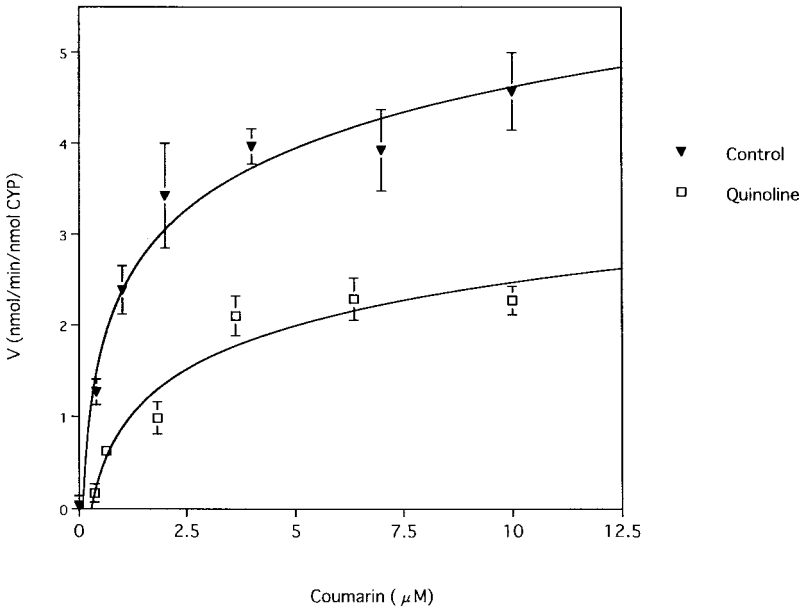

Fig. 5. The Relationships of Velocity and Substrate Concentration to Human CYP2A6

Closed triangles are in the absence of quinoline and open squares are in the presence of 20 μM quinoline. The mean ± S.D. values of each point were calculated from 5 determinations.

confirmed that cDNA-expressed CYP2A6 catalyzed coumarin 7-hydroxylation (V_{max}: 6.9 nmol/min/nmol CYP, K_M: 2.7 μM). This preparation has about ten-times higher activity than that of bovine liver microsomes. To assess the correlation of the effects of quinoline and monofluoroquinolines, we followed the same protocol as in Figs. 3 and 4 with human CYP2A6 in place of bovine liver microsomes. The velocity-substrate concentration relationship is shown in Fig. 5. The inhibition by quinoline at 20 μM is shown in Fig. 6. 3FQ did not show any inhibition, but quinoline, 5FQ, 6FQ and 8FQ showed inhibition of 20–30%. The same property of quinoline derivatives was found at higher concentrations, as shown in Fig. 6. The 50% inhibition by 3FQ was not reached in the concentration range up to 100 μM, at which concentration quinoline and the other monofluoroquinolines inhibited coumarin 7-hydroxylase at rates greater than 50%. Thus, we found the same reaction profile between bovine liver microsomes and human CYP2A6. However, the inhibition pattern with human CYP2A6 was not clear compared with the pattern with bovine microsomes. To explain this difference, we considered that the human CYP2A6 used was not a product from human liver, but it was over-expressed due to CYP2A6 cDNA. It is also possible that the difference in inhibition patterns between bovine liver microsomes and human CYP2A6 is dependent upon the species-specificity. Finally, these findings suggest that the coumarin 7-hydroxylation in bovine liver microsomes is due to the enzyme-like human CYP2A6.

DISCUSSION

Isolated bovine liver microsomes that contained 0.1 nmol CYP/mg protein converted coumarin to 7-hydroxycoumarin. This fact indicates that bovine liver microsomes contain a coumarin metabolizing enzyme, like human liver microsomes. We studied the inhibitory properties with quinoline of the enzyme, which was assessed in the metabolic pathways and was involved in cytochrome P-450 subfamilies.[6] In human liver microsomes, CYP2A6 was involved in the pathways of 5,6-epoxidation and *N*-oxidation, and CYP2E1 is involved in 3-hydroxylation. In this report, quinoline and four kinds of monofluoroquinolines were used as inhibitors of coumarin 7-hydroxylation. It was reported that human CYP2A6 principally metabolized quinoline to 5,6-dihydroquinoline-5,6-epoxide.[6] Thus, we expected that 5FQ and 6FQ would not interact with CYP2A6, and thus be weaker inhibitors than quinoline, if bovine liver microsomes have a similar enzyme to CYP2A6. However, the findings in Fig. 3 showed that 3FQ appeared to show the weakest inhibition of the reaction with CYP2A6 in bovine liver microsomes.

We next compared the inhibition of coumarin 7-

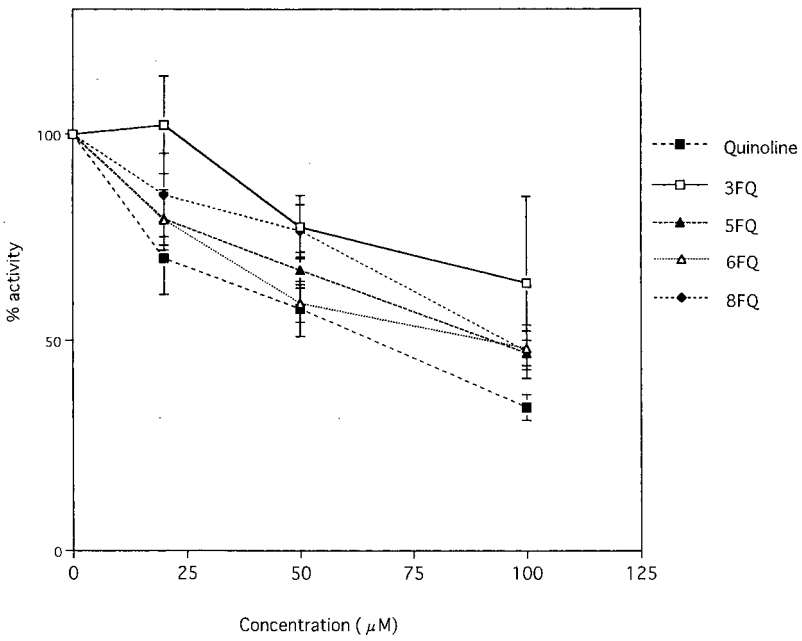

Fig. 6. The Relative inhibition of Human CYP2A6 by Quinolines

At concentrations of 20 or 100 μM, the values are significantly different between 3FQ and other quinolines ($p < 0.05$). The mean ± S.D. values of each point were calculated from 5 determinations.

hydroxylation at 10 μM coumarin with quinoline, 3FQ, 5FQ, 6FQ and 8FQ at concentrations of 10, 20, and 50 μM, as shown in Fig. 4. The intensity of inhibition by 3FQ was about half that of quinoline, and less than half that of 5FQ, 6FQ and 8FQ (Fig. 4). Moreover, Fig. 4 also showed that 3FQ might be the least inhibitor for CYP2A enzyme (s) in bovine liver microsomes. Similar results were obtained for human CYP2A6 (Figs. 5 and 6). This indicates that coumarin 7-hydroxylase in bovine liver microsomes is similar to human CYP2A6. It also indicates that position-3 might be important for the inhibition of coumarin 7-hydroxylation by CYP2A6. Meanwhile, quinidine, a quinoline derivative, is metabolized by CYP3A4, and the major product is 3-hydroxyquinidine.[26)]

In this report, it was suggeted that CYP2A6 recognizes position-3 of quinoline, because the inhibition profile with 3FQ was different from that with quinoline. It was reported that quinoline was metabolized by human CYP2E1 to 3-hydroxyquinoline in a major metabolic pathway.[6)] It is possible that CYP2A6 participates in the production of 3-hydroxyquinoline in a minor metabolic pathway. This study did not show that quinoline was mainly metabolized by CYP2A6, but rather that quinolines inhibited coumarin 7-hydroxylation by CYP2A6. Quinoline may only have an affinity to CYP2A6 and inhibit coumarin 7-hydroxylation. Fluorine in position-3 may inhibit the interaction of 3FQ with CYP2A6. Another possibility is inhibition by the chelating activity of quinoline, not by the direct interaction with the active site of CYP2A6, like 8-hydroxyquinoline (5 mM) as a standard chelator in the case of DNA extraction. This concentration, 5 mM, is higher than that used in this study of 10–100 μM quinoline or derivatives. As a conclusion, we found coumarin 7-hydroxylation activity in bovine liver microsomes, and the inhibition of coumarin 7-hydroxylation with quinoline and some fluoro-quinolines, except 3-fluoro-quinoline. Analysis of the direct metabolites of quinoline with CYP2A6 is planned, and the inhibition of CYP2E1 by quinoline must also be studied in the future.

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