Phenotype Analysis of Human Cytochrome P450 2C9 Polymorphism Using a Panel of Fluorine-Substituted Benzo[h]quinolines as Inhibitors of Tolbutamide Hydroxylation

Noriko Matsuyama, Taka-aki Kato, Kazunori Kimura, Takaharu Mizutani, and Ken-ichi Saeki*

Graduate School of Pharmaceutical Sciences, Nagoya City University, Tanabedori, Mizuho-ku, Nagoya 467–8603, Japan
(Received July 24, 2006; Accepted September 1, 2006; Published online September 4, 2006)

To investigate the inhibitory effect of aza-polycyclic aromatic compounds on cytochrome P450 (CYP) 2C9 activity and analyze the fluorine-substitution effects on the CYP2C9 inhibition, benzo[h]quinoline (BhQ) and its fluorinated derivatives, 3-F-, 5-F-, 6-F-, 9-F-, 10-F-, 3,6-diF-, 5,6-diF-, 7,10-diF-BhQ, were subjected to analysis of their inhibitory effects on recombinant human CYP2C9.1-catalyzed tolbutamide hydroxylation. Although the inhibitory activity of BhQ itself on tolbutamide hydroxylation was not very strong (IC50 = 157 µM), the inhibitory activities of BhQ derivatives on tolbutamide hydroxylation varied with the substituted fluorine positions. Their inhibitory activities decreased in the following order; (BhQ) > 7,10-diF ≥ 3,6-diF, 9-F ≥ 10-F ≥ 6-F ≥ 5-F, 3-F ≥ 5,6-diF-BhQ. Moreover, the fluorine-substitution effect on the inhibition of tolbutamide hydroxylation catalyzed by wild-type CYP2C9 (CYP2C9.1) was different from the effects on the hydroxylation catalyzed by its polymorphic isozymes. CYP2C9.2 and CYP2C9.3. The inhibitory activities of BhQs on tolbutamide hydroxylation by CYP2C9.2 decreased in the following order; 5-F ≥ 10-F, 9-F ≥ (BhQ) ≥ 7,10-diF > 6-F ≥ 5,6-diF, 3,6-diF > 3-F-BhQ, and those on the hydroxylation by CYP2C9.3 decreased in the following order; 7,10-diF, 10-F, (BhQ) ≥ 9-F, 5-F > 3-F ≥ 5,6-diF, 6-F, 3,6-diF-BhQ. The results thus showed that the position-specific substitution by fluorine atom(s) altered the CYP2C9 inhibition by BhQ derivatives in different manners depending on the polymorphic isozymes involved. These results suggest that inhibitory profiles obtained with fluorine-substituted analogs of the key inhibitor molecule may be useful as a new tool for phenotyping the polymorphic CYP isoforms.

Key words — inhibitor, fluorine-substitution, cytochrome P450 2C9, polymorphism, aza-polycyclic aromatic hydrocarbon

INTRODUCTION

Cytochrome P450 (CYP) 2C9 is a major CYP isoform responsible for the metabolism of many drugs including S-warfarin, phenytoin, and tolbutamide,1–4) and some environmental mutagens such as benzo[a]pyrene.5) Human CYP2C9 consists of three major isozymes, CYP2C9.1 (wild-type), 2C9.2, and 2C9.3, due to its genetic polymorphism.6) Generally, it is suggested that CYP2C9.2 shows moderate activity and CYP2C9.3 shows poor activity compared with CYP2C9.1 for metabolism of most CYP2C9 substrates such as tolbutamide.6) However, it is well-known that the difference in catalytic activity among the CYP2C9 isoforms may vary with substrates: for example, it was reported that CYP2C9.1, 2C9.2, and 2C9.3 showed equal metabolic activities for diclofenac 4′-hydroxylation.7) These facts suggest that the degree of substrate recognition by each CYP2C9 isoform may vary with substrates.

In this study, we attempted to investigate the fluorine-substitution effects on CYP2C9 inhibition with special attention to the difference in substrate recognition by each CYP2C9 isozyme. Fluorine-substitution instead of a hydrogen atom often exerts so-called mimic effects on interactions with biological molecules, because the covalent and van der Waals' radii of the fluorine atom are similar to those of the hydrogen atom. On the other hand, fluorine-substitution on an aromatic compound results in not only changes in electron-density distribution within the molecule but also in electric repulsive/attractive interactions with intra/intermolecular environments. These changes may significantly affect the interactions between an enzyme and its substrate. In fact, our previous study indicated that the inhibitory effects of the anilines on CYP2E1 were widely affected by the substituting fluorine’s number and position.8,9) In the present study, benzo[h]quinoline (BhQ) and its fluorinated derivatives, 3-F-, 5-F-, 6-F-, 9-F-, 10-F-,
F-, 3,6-diF-, 5,6-diF-, and 7,10-diF-BhQ (Fig. 1), were subjected to analysis of their inhibitory effects on the metabolism of tolbutamide by recombinant human CYP2C9.1, 2C9.2, and 2C9.3 to investigate the structure-inhibition selectivity relationships.

**MATERIALS AND METHODS**

**Materials** — All the fluorinated BhQs were synthesized as described in our previous report. The microsome preparations from baculovirus-infected insect cells expressing CYP2C9*1, *2, and *3, each coexpressed with NADPH-CYP oxidoreductase, were purchased from Gentest Co. (Woburn, MA, U.S.A.); NADP, glucose-6-phosphate (G6P), and G6P dehydrogenase from Oriental Yeast Co. (Tokyo); and BhQ, tolbutamide, hydroxytolbutamide, and all the other chemicals from Aldrich.

**Inhibition of CYP2C9 Activity Determined by Tolbutamide Hydroxylation** — The determination of tolbutamide hydroxylase activity catalyzed by a CYP2C9 enzyme was performed according to the previous report with slight modification. Briefly, the incubation mixture (50 µl in a 1.5 ml microtube) contained 0.1 M potassium phosphate buffer (pH 7.4), 1.3 mM NADP, 3.3 mM G6P, 3.3 mM MgCl₂, 0.08 units of G6P dehydrogenase, 100 µM tolbutamide, an inhibitor (200 µM), and 5.0 pmol CYP (ca. 8 µg protein). After incubation at 37°C for 40 min, the resulting metabolites were extracted with 2.5 volumes of ethyl acetate, and the organic solvent layer was evaporated. The residue was dissolved in 30 µl of 0.01 M HCl-20% AcCN solution and analyzed by HPLC. HPLC analysis was performed using a Shimadzu liquid chromatograph equipped with a Model LC-10A solvent delivery system, a Model SPD-10AV uv-vis spectrophotometric detector and a Wakosil II 5C18RS (ODS) column (2 × 150 mm). The solvent system consisted of 40% AcCN-H₂O. The flow rate was 0.15 ml/min. The increase of hydroxylated tolbutamide was quantified from the peak areas measured by UV absorption at 236 nm with reference to its authentic sample (retention time = 5.6 min). At least three independent experiments were performed.

**RESULTS AND DISCUSSION**

The metabolism of tolbutamide by recombinant human CYP2C9 (2C9.1, 2C9.2, and 2C9.3) was assayed to evaluate the fluorine-substitution effects on the CYP2C9 inhibition by the BhQs listed in Fig. 1. The metabolic activities of tolbutamide hydroxylation by each CYP2C9 without any inhibitor were 1.20 ± 0.06 (2C9.1), 1.29 ± 0.09 (2C9.2), and 0.22 ± 0.02 nmol product/min/nmol CYP (2C9.3). Because the tolbutamide hydroxylase activity obtained with CYP2C9.3 was weak, the amount of CYP contents in the incubation mixture was doubled (10 pmol/50 µl incubation mixture). Although the inhibitory activity of BhQ itself on tolbutamide hydroxylation was not very strong (IC₅₀ = 157 µM), BhQ inhibited CYP2C9.1 activity by the competition mechanism (Ki = 85 µM).
The inhibitory activities (relative % activity of control) of nine BhQs (200 µM) on recombinant human CYP2C9 isozymes are shown in Fig. 2. In the case of CYP2C9.1 (Fig. 2A), BhQ itself showed highest inhibitory activity (36% of control) among the tested BhQs and the inhibitory activities decreased in the following order; (BhQ) > 7,10-diF (47%) ≥ 3,6-diF (51%), 9-F (52%) ≥ 10-F (57%) > 6-F (65%) ≥ 5-F (72%), 3-F (73%) ≥ 5,6-diF-BhQ (77%). Almost the same tendency was obtained with 100 µM BhQs, but the degrees of inhibition were not enough to be compared with each other (data not shown). As previously reported, fluorine-substitution on the BhQ molecule (except for the position-10) decreased the pKa value probably because of the electron-withdrawing inductive effect of the fluorine atom. As shown in Fig. 1, the pKa values of BhQs decreased in the following order; 10-F (4.9) > BhQ (4.3), 7,10-diF (4.2) > 9-F (3.7), 6-F (3.6), 5-F (3.2) ≥ 5,6-diF (2.3), 3-F (2.0) > 3,6-diF-BhQ (1.5).

The results showed that the inhibitory activities of BhQs on CYP2C9.1 did not simply depend on the pKa values. On the other hand, the fluorine-substitution effect on the BhQ inhibition of the tolbutamide hydroxylation catalyzed by CYP2C9.2 was different from the effect on the hydroxylation by the other polymorphic isozymes CYP2C9.1 and CYP2C9.2. The inhibitory activities of BhQs on the tolbutamide hydroxylation by CYP2C9.2 decreased in the following order; 5-F (45%) ≥ 10-F (49%), 9-F (52%) ≥ (BhQ) (58%) ≥ 7,10-diF (63%) > 6-F (72%) ≥ 5,6-diF (79%), 3,6-diF (82%) > 3-F-BhQ (93%). Moreover, the fluorine-substitution effect on the BhQ inhibition of the tolbutamide hydroxylation catalyzed by CYP2C9.3 (Fig. 2C) was different from either of those on the hydroxylation by the other polymorphic isozymes CYP2C9.1 and CYP2C9.2. The inhibitory activities of BhQs on the tolbutamide hydroxylation by CYP2C9.3 decreased in the following order; 7,10-diF (42%), 10-F (44%), (BhQ) (46%) ≥ 9-F (51%), 5-F (51%) > 3-F (59%) ≥ 5,6-diF (65%), 6-F (66%), 3,6-diF-BhQ (68%). The fluorine-substitution effect on the BhQ inhibition of the tolbutamide hydroxylation catalyzed by CYP2C9.3 was not simply correlated with the pKa values either, because the substituent effects of fluorine atom(s) are ascribable to not only the electron-withdrawing inductive effect leading to a decrease in pKa but also to changes in electron-density distribution within the aromatic moiety leading to effects on such interactions as π-π and lipophilic-lipophilic interactions. Although the fluorine-substitution effects on CYP2C9 inhibition by BhQs were complex, these results showed that the position specific substitution by fluorine atom(s) altered the CYP2C9 inhibi-
tion by BhQ derivatives in different manners depending on the polymorphic isozymes.

In conclusion, these results suggest that inhibitory profiles obtained with fluorine-substituted analogs of the key inhibitor molecule may be useful as a new tool for phenotyping the polymorphic CYP isoforms.

Acknowledgements This work was partly supported by a Grant-in-Aid for Research in Nagoya City University.

REFERENCES


