# Cytochrome P450 2E1/2A6-Selective Inhibition by Halogenated Anilines on Metabolic Activation of Dimethylnitrosamine in Human Liver Microsomes

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(Received April 25, 2006; Accepted May 30, 2006; Published online June 22, 2006)

**Nine halogenated anilines, consisting of di- and tri-substituted fluoro-, chloro-, and bromoanilines, were subjected to analysis of their cytochrome P450 (CYP) 2E1/2A6-selective inhibitory effects on metabolic activation of dimetylnitrosamine (DMN) in human liver microsomes. Inhibitory activities ($IC_{50}$) of these anilines on human recombinant CYP2E1 ranged from 8.0 to 549 $\mu$M. However, most of these anilines showed no remarkable inhibition of CYP1A2, while their $IC_{50}$ values on CYP2A6 ranged from 2.9 to 232 $\mu$M. The CYP2E1 selectivity of these anilines in terms of the ratio of the $IC_{50}$ values of these anilines on CYP2A6 to those on CYP2E1, ranged from 0.1- to 5.2-fold. Their CYP2E1 selectivity decreased in the following order: 3,4,5-trifluoroaniline (3,4,5-triF-A) > 3,5-diF-A >> 3,5-dichloroaniline (3,5-diCl-A) > 3,4-diCl-A > 2,6-diCl-A > 2,3,4-trichloroaniline (2,3,4-triCl-A) > 2,4,6-triCl-A > 2,6-dibromoaniline (2,6-diBr-A) > 3,4,5-triCl-A. The inhibitory effects of these anilines on metabolic activation of DMN were analyzed using human liver microsomes. The $IC_{50}$ values of these anilines on demethylation metabolism of DMN correlated with those of these anilines on CYP2E1. These results suggest that these halogenated anilines may be useful as indicators of CYP-selectivity involved in metabolic activation of nitrosamines.**

**Key words** —— inhibitor, halogen-substitution, cytochrome P450 2E1, alkylnitrosamine, carcinogenicity

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## INTRODUCTION

Carcinogenic nitrosamines, such as dimethylnitrosamine (DMN), are metabolized at the $\alpha$-carbon by cytochrome P450 (CYP) to form the active metabolite (methyldiazohydroxide) by demethylation accompanied with the formation of formaldehyde.[1–3] It is well-known that CYP2E1 and CYP2A6 are responsible for activation of carcinogenic nitrosamines, and CYP2E1 may be the major enzymes catalyzing the metabolic *N*-demethylation of DMN in human liver microsomes.[4–8]

Our previous study indicated that 3,4-dichloroaniline (3,4-diCl-A) and 3,5-diCl-A showed potent inhibition of recombinant human CYP2E1 ($IC_{50}$ = 8.0 and 9.2 $\mu$M, respectively) and no inhibition of CYP1A2 ($IC_{50}$ > 200 $\mu$M).[9] Moreover, the inhibitory effects of the anilines on CYP2E1 were widely affected by the substituted halogen species, number, and positions.[9] In this study, nine halogenated anilines, 3,5-difluoroaniline (3,5-diF-A), 3,4,5-trifluoroaniline (3,4,5-triF-A), 2,6-diCl-A, 3,4-diCl-A, 3,5-diCl-A, 2,3,4-trichloroaniline (2,3,4-triCl-A), 2,4,6-triCl-A, 3,4,5-triCl-A, and 2,6-dibromoaniline (2,6-diBr-A), were subjected to analysis of their inhibitory effects on metabolic activation of DMN in human liver microsomes to investigate the structure-CYP2E1/2A6 selectivity relationships and CYP-selective metabolic activation of DMN.

## MATERIALS AND METHODS

**Materials** —— 7-Fluoro-4-methylquinoline and 7-fluoro-3-hydroxy-4-methylquinoline were synthesized as described in our previous report.[10] Pooled and individual human liver microsome preparations (donor Nos. HG3, HK25, HK37, HG43, HG56, HG64, HG89, HG93, and HG95) and insect microsome preparations from bacurovirus-infected insect cells expressing CYP2E1, CYP2A6, and CYP1A2, 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, Japan); DMN and Formaldehyde-Test-Wako from Wako Pure Chemicals (Osaka, Japan); and halogenated anilines (3,5-diF-A, 3,4,5-triF-A, 2,6-diCl-A, 3,4-diCl-A, 3,5-diCl-A, 2,3,4-triCl-A, 2,4,6-triCl-A, 3,4,5-triCl-A, and 2,6-diBr-A) and all the other chemicals from Aldrich.

**Inhibition of CYP1A2 Activity Determined by 7-Fluoro-4-Methylquinoline 3-Hydroxylation** —— In our previous study,[9] it was demonstrated that the fluorescence mothod for determination of quinoline 3-hydroxylation might be useful for monitoring CYP2E1 activity. Human CYP1A2 was reported as a secondly major isoform involved in the formation of 3-hydroxyquinoline.[11] Among several quinoline derivatives, 7-fluoro-4-methylquinoline was effectively metabolized by CYP1A2 to a fluorescent metabolite, 7-fluoro-3-hydroxy-4-methylquinoline (460 nm emission and 355 nm excitation). The incubation mixture (50 $\mu$l 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 $MgCl_2$, 0.08 units of G6P dehydrogenase, 0.5 mM 7-fluoro-4-methylquinoline, inhibitor (0 to 1000 $\mu$M), and 1.0 pmol CYP (1.7 $\mu$g protein). After incubation at 37°C for 60 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 200 $\mu$l of 0.5 M Tris base-10% dimethyl sulfoxide (DMSO) solution and added to wells in a 96-well plate. The production of fluorescent 7-fluoro-3-hydroxy-4-methylquinoline (460 nm emission and 355 nm excitation) was recorded with an ARVO1420 Multilabel Counter and the relative fluorescence was compared to the calibration curve. The inhibitory effect was calculated by $IC_{50}$. At least three independent experiments were performed.

Inhibition of CYP2A6 activity by halogenated anilines was determined by coumarin-7-hydroxylation as in the previous report.[12,13]

**Metabolism of Dimethylnitrosamine in Human Liver Microsomes** —— The incubation mixture (320 $\mu$l in a 1.5 ml microtube) contained 0.1 M potassium phosphate buffer (pH 7.4), 1.3 mM $\beta$-NADPH, 3.3 mM $MgCl_2$, 5 mM DMN, inhibitor (0 to 500 $\mu$M), and 0.16 mg human liver microsomes. After incubation at 37°C for 60 min, the production of formaldehyde was measured by Formaldehyde-Test-Wako. Briefly, the resulting mixture was mixed with an equal volume of 5 M NaOH and centrifuged at 10,000 $\times$ $g$ for 10 min. The supernatant (600 $\mu$l) was allowed to react with 300 $\mu$l of the reacting reagent (4-amino-3-hydrazino-5-mercapto-1,2,4-triazol) at room temperature for 15 min and the oxidizing reagent (potassium periodate) was added under vortex mixing for 20 second. The production of 7-mercaptotriazinotetrazine was recorded at the wavelength of 550 nm, and relative absorbance was compared to the calibration curve. At least three independent experiments were performed.

## RESULTS AND DISCUSSION

The metabolism of DMN in human liver microsomes was assayed to evaluate CYP-selectivity in DMN metabolic activation, using 9 individual human liver microsomes (HG3, HK25, HK37, HG43, HG56, HG64, HG89, HG93, and HG95) and pooled human liver microsomes. The results shown in Table 1 demonstrate the inter-individual diversity of the metabolic activity with DMN measured by formaldehyde production. The metabolic activity with DMN ranged from 0.14 to 1.07 nmol/min/mg

**Table 1.** Catalytic Activities of 9 Individual Donors' and Pooled Human Liver Microsome Preparations with CYP Isoform-Selective Substrates and DMN

| Human liver microsomes | Chlorzoxazone 6-hydroxylase[a)] (CYP2E1) (nmol/min/mg protein) | Coumarin 7-hydroxylase[a)] (CYP2A6) (nmol/min/mg protein) | DMN metabolism (HCHO production) (nmol/min/mg protein) |
|---|---|---|---|
| HG3 | 1.80 | 2.00 | $0.45 \pm 0.01$ |
| HK25 | 2.70 | 0.21 | $0.59 \pm 0.03$ |
| HK37 | 1.70 | 0.46 | $0.50 \pm 0.02$ |
| HG43 | 1.20 | 0.67 | $0.14 \pm 0.02$ |
| HG56 | 1.90 | 1.40 | $0.63 \pm 0.05$ |
| HG64 | 3.00 | 1.50 | $1.07 \pm 0.04$ |
| HG89 | 1.70 | 0.65 | $0.54 \pm 0.01$ |
| HG93 | 1.50 | 0.35 | $0.51 \pm 0.04$ |
| HG95 | 1.20 | 0.20 | $0.50 \pm 0.01$ |
| pooled | 2.00 | 1.00 | $0.61 \pm 0.02$ |

*a*) Data from the catalog (Gentest).

**Table 2.** Inhibitory Effect ($IC_{50}$) of Halogenated Anilines on Human Recombinant CYP2E1, CYP1A2, and CYP2A6 Activities

| Chemical | $IC_{50}$ ($\mu$M) | | | CYP2E1/2A6 selectivity[a)] |
|---|---|---|---|---|
| | CYP2E1 | CYP1A2 | CYP2A6 | |
| 3,5-diF-A | 46.3 ± 2.0[b)] | >500 | 231.6 ± 24.7 | 5.0 |
| 2,6-diCl-A | 251.7 ± 37.1 | >500 | 140.1 ± 14.1 | 0.6 |
| 3,4-diCl-A | 8.0 ± 1.2[b)] | 333.9 ± 21.1 | 7.6 ± 2.5 | 1.0 |
| 3,5-diCl-A | 9.2 ± 0.8[b)] | 349.1 ± 11.6 | 12.9 ± 2.1 | 1.4 |
| 2,6-diBr-A | 548.9 ± 84.8 | >500 | 84.6 ± 8.9 | 0.2 |
| 3,4,5-triF-A | 32.2 ± 2.1 | >500 | 167.6 ± 15.8 | 5.2 |
| 2,3,4-triCl-A | 35.5 ± 1.4[b)] | 96.9 ± 22.3 | 9.6 ± 0.4 | 0.3 |
| 2,4,6-triCl-A | 178.0 ± 15.6[b)] | >500 | 45.3 ± 2.6 | 0.3 |
| 3,4,5-triCl-A | 29.1 ± 4.0[b)] | 42.4 ± 1.7 | 2.9 ± 0.4 | 0.1 |

*a*) CYP2E1/2A6 selectivity was calculated as the ratio of the $IC_{50}$ values of anilines on CYP2A6 to those on CYP2E1. *b*) Data from our previous report.[9)]

protein among the 9 microsome samples tested. The metabolic activity obtained with the pooled human liver microsome preparation was approximately equal to the average level of the 9 individual samples.

Then, the metabolic activity with DMN was compared with the CYP2E1- and CYP2A6-selective catalytic activities of each microsomal preparation as given in the GENTEST catalog (Table 1). The metabolic activity significantly correlated with the CYP2E1-selective catalytic activity ($r = 0.81$, $p < 0.01$), but not with the CYP2A6-selective catalytic activity ($r = 0.26$) of each microsome preparation. The result confirmed that CYP2E1 is the principal enzyme responsible for the metabolic activation of DMN in human liver microsomes as shown in the previous studies.[4–8)]

Nine halogenated anilines, 3,5-diF-A, 3,4,5-triF-A, 2,6-diCl-A, 3,4-diCl-A, 3,5-diCl-A, 2,3,4-triCl-A, 2,4,6-triCl-A, 3,4,5-triCl-A, and 2,6-diBr-A, were subjected to analysis of their inhibitory effects on metabolic activation of DMN in human liver microsome (HG56) to evaluate their CYP2E1/2A6-selective inhibition. The inhibitory activities ($IC_{50}$) of these anilines on human recombinant CYP2E1, CYP1A2, and CYP2A6 activities are shown in Table 2. Most of these anilines showed no remarkable inhibition of recombinant CYP1A2 activity and only 3,4,5-triCl-A showed moderate inhibition of CYP1A2. The $IC_{50}$ values of these anilines on human recombinant CYP2E1 and CYP2A6 activities varied considerably with the substituted halogen species, number, and positions. The $IC_{50}$ values of these anilines on human recombinant CYP2E1 and CYP2A6 activity ranged from 8.0 to 549 $\mu$M and 2.9 to 232 $\mu$M, respectively. Our preliminary study showed that 3,4-diCl-A and 3,5-diCl-A inhibited CYP2E1 activity by the competition mechanism (Ki = 0.53 ± 0.01 and 0.92 ± 0.03 $\mu$M, respectively). The CYP2E1 selectivities of these anilines, the ratios of the $IC_{50}$ values of these anilines on CYP2A6 to those on CYP2E1, ranged from 0.1- to 5.2-fold. Although 3,5-diF-A and 3,4,5-triF-A showed moderate inhibition of recombinant CYP2E1 activity, the CYP2E1 selectivities of the fluorinated anilines were sufficiently higher than those of all other anilines. On the other hand, trichlorinated anilines, 2,3,4-triCl-A, 2,4,6-triCl-A, and 3,4,5-triCl-A, and 2,6-diBr-A showed very low CYP2E1 selectivities, and these lipophilic and large anilines were rather CYP2A6-selective. These results suggested that CYP2E1-selective inhibition by halogenated anilines were variable with the lipohilicity and molecular size, and oligofluorine substitution may be responsible for enhancement of CYP2E1 selectivity. The $IC_{50}$ values of these anilines on DMN metabolic activation in human liver microsomes are shown in Table 3. HG56 was used because it had the highest total P450 content (480 pmol/mg) and showed moderate activity in DMN metabolism. The $IC_{50}$ values of these anilines on DMN metabolic activation in human liver microsomes ranged from 3.6 to 338 $\mu$M. Surprisingly, the ranges of the $IC_{50}$ of these anilines on DMN metabolism in human liver microsomes, in which many CYP-isoforms exist at various levels, were very similar with those on human recombinant CYP2E1 and CYP2A6 activities. Moreover, as shown in Fig. 1, the $IC_{50}$ of these anilines on DMN metabolism in human liver microsomes significantly correlated with the $IC_{50}$ on human recombinant CYP2E1 activity ($r = 0.89$, $p < 0.01$) but not with

**Table 3.** Inhibitory Effect ($IC_{50}$) of Halogenated Anilines on Formaldehyde Production in DMN Metabolism by Human Liver Microsome (HG56)

| Chemical | $IC_{50}$ ($\mu$M) |
|---|---|
| 3,5-diF-A | 27.8 ± 5.4 |
| 2,6-diCl-A | 337.9 ± 33.5 |
| 3,4-diCl-A | 3.7 ± 0.9 |
| 3,5-diCl-A | 5.2 ± 0.5 |
| 2,6-diBr-A | 317.6 ± 57.0 |
| 3,4,5-triF-A | 4.6 ± 0.2 |
| 2,3,4-triCl-A | 3.6 ± 1.1 |
| 2,4,6-triCl-A | 89.9 ± 16.4 |
| 3,4,5-triCl-A | 4.2 ± 0.5 |

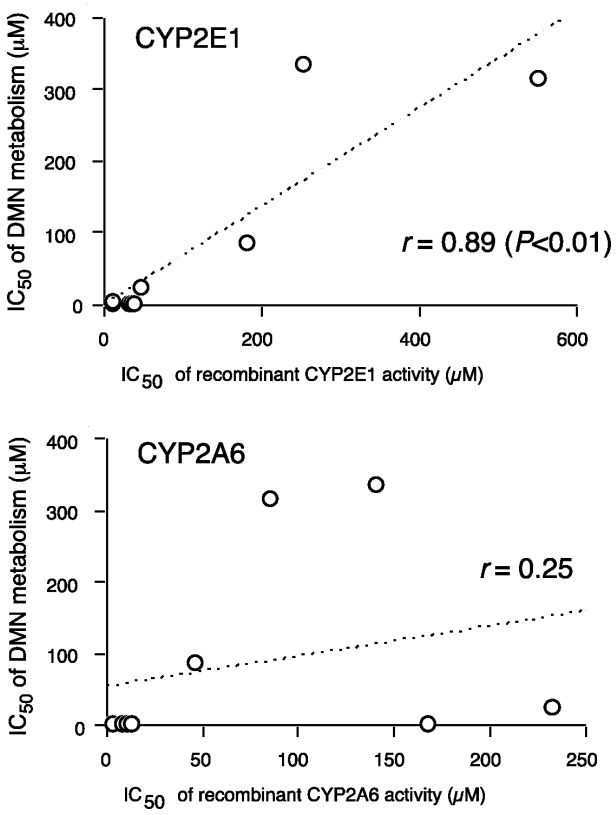


**Fig. 1.** Correlation between the $IC_{50}$ of Halogenated Anilines on Formaldehyde Production in DMN Metabolism by Human Liver Microsomes and Recombinant CYPs
*p*-Value was obtained by one-way analysis of variance (ANOVA).

the $IC_{50}$ on CYP2A6. The results suggested that 9 halogenated anilines inhibited CYP2E1 activity in human liver microsomes to the same degree as in human recombinant CYP2E1.

In conclusion, these results suggest that these halogenated anilines may be useful as indicators of CYP-selectivity involved in metabolic activation of nitrosamines in human liver microsomes.

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

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