**Regular Article**

*In Vitro Inhibitory Effect of 1-Aminobenzotriazole on Drug Oxidations in Human Liver Microsomes: a Comparison with SKF-525A*

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**Summary:** 1-Aminobenzotriazole (ABT) is extensively used as a non-specific cytochrome P450 (CYP) inhibitor. In this study, the inhibitory effect of ABT on CYP-dependent drug oxidations was investigated in human liver microsomes (HLM) and compared with that of SKF-525A, another non-specific inhibitor. The following probe activities for human CYP isoforms were determined using pooled HLM: phenacetin O-deethylation (CYP1A2); diclofenac 4'-hydroxylation (CYP2C9); S-mephenytoin 4'-hydroxylation, (CYP2C19); bufuralol 1'-hydroxylation (CYP2D6); chlorzoxazone 6-hydroxylation (CYP2E1); midazolam 1'-hydroxylation, nifedipine oxidation, and testosterone 6β-hydroxylation (CYP3A). ABT had the strongest inhibitory effect on the CYP3A-dependent drug oxidations and the weakest effect on the diclofenac 4'-hydroxylation. SKF-525A potently inhibited the bufuralol 1'-hydroxylation, but weakly inhibited chlorzoxazone 6-hydroxylation. The inhibitory effects of ABT and SKF-525A were increased by preincubation in some probe reactions, and this preincubation effect was greater in ABT than in SKF-525A. The remarkable IC₅₀ shift (> 10 times) by preincubation with ABT was observed on the phenacetin O-deethylation, chlorzoxazone 6-hydroxylation, and midazolam 1'-hydroxylation. In conclusion, ABT and SKF-525A had a wide range of IC₅₀ values in inhibiting the drug oxidations by HLM with and without preincubation.

**Key words:** Cytochrome P450 (CYP); human liver microsomes; 1-aminobenzotriazole (ABT); SKF-525A

**Introduction**

The cytochrome P450 (CYP) enzymes constitute a superfamily of heme-containing monoxygenase, and multiple forms of CYP enzymes exist in mammals. These CYP enzymes are responsible for the oxidation of many drugs, environmental chemicals and endogenous substrates. CYP enzymes mainly exist in the liver as well as in the extrahepatic tissues, such as the intestines, lungs and kidneys. In humans, xenobiotics are metabolized primarily by three CYP families; namely, CYP1, CYP2, and CYP3. CYP enzymes play an important role in the drug metabolism, one of the major clearance routes in the body. Therefore, estimation of the contribution of CYP enzymes to metabolic reactions in human liver microsomes (HLM) is very important in the drug development process.

1-Aminobenzotriazole (ABT) shows the non-specific CYP inhibition, while it does not inhibit other enzymes such as cytochrome b₅, NADPH cytochrome c reductase, glutathione S-transferase, and UDP-glucuronosyl transferase. The selectivity of ABT in inhibiting the drug oxidations catalyzed by human CYP enzymes had not been fully investigated. Therefore, we investigated previously the inhibitory effect of ABT on probe drug oxidations catalyzed by several human recombinant CYP isoforms and found that ABT is a potent inhibitor for CYP3A4-dependent drug oxidations, but weakly inhibits CYP2C9-dependent drug oxidations. ABT is also known as a mechanism-based inactivator of CYP enzymes by N-alkylation of heme moiety. Recently,
Balani et al. have reported that the preincubation of recombinant CYP enzymes with ABT increases the inhibitory effect of this inhibitor on the CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4-dependent oxidations of fluorescent substrates in a dose-dependent manner. Taking advantage of the non-specificity in inhibiting the activities of CYP enzymes, ABT has been used in the inhibition study using liver and lung microsomes from mice, rats, rabbits, guinea pigs, and humans for the purpose of assessing the contribution of CYP enzymes to overall metabolism of a compound in question. However, the methods employed in the inhibition studies using ABT varied depending on the laboratories. Some laboratories used ABT without a preincubation, but others used it with the preincubation. Therefore, we investigated the inhibitory effect of ABT with and without preincubation in this study. With respect to CYP3A, it is recommended to use multiple CYP3A probes for the in vitro evaluation of CYP3A-dependent drug interactions, and we used midazolam, nifedipine, and testosterone. In addition, these inhibitory effects of ABT were compared with those of SKF-525A, another non-specific inhibitor.

**Methods**

**Materials:** Phenacetin was obtained from Sigma-Aldrich (Steinheim, Switzerland). Testosterone, tolbutamide, 4-hydroxyphenytoin, 1'-hydroxybufuralol, 6-hydroxychlorzoxazone, 6β-hydroxytestosterone, oxidized nifedipine, and 1'-hydroxymidazolam were from Ultrafine (Manchester, UK). 4'-Hydroxydiclofenac was from BD Gentest Corp. (Woburn, MA, USA). S-mephenytoin was from Biomol (Plymouth Meeting, PA, USA). ABT, SKF-525A, midazolam, alprazolam, 4-acetamidophenol, buspirone, caffeine, and diclofenac were from Sigma Chemical Co. (St. Louis, MO, USA). Chlorzoxazone and nifedipine were from Wako Pure Chemical (Osaka, Japan). Bufuralol and [D3] 4-acetamidophenol were synthesized in-house. NADPH was from Oriental Yeast (Japan). Bufuralol and [D7] 4-acetamidophenol were obtained from a Pfizer Global R&D in-house supply. Other reagents were of HPLC grade or better.

**Inhibition study without preincubation:** As probe reactions, the following oxidations were selected: phenacetin O-deethylation (POD) for CYP1A2, diclofenac 4'-hydroxylation (DFOH) for CYP2C9, S-mephenytoin 4'-hydroxylation (S-MPOH) for CYP2C19, bufuralol 1'-hydroxylation (BFOH) for CYP2D6, chlorzoxazone 6-hydroxylation (CZXOH) for CYP2E1, and midazolam 1'-hydroxylation (MDZ1'OH), nifedipine oxidation (NIFOX), and testosterone 6β-hydroxylation (TESOH) for CYP3A. For each of the above oxidations, the standard incubation mixture consisted of 1.4 mM NADPH, 3.3 mM MgCl2, 100 mM phosphate buffer (pH = 7.4), HLM, and a probe substrate in the presence or absence of inhibitor (ABT or SKF-525A) in a final volume of 200 μL. After a preincubation for 5 min on the air incubator at 37°C, the reaction was initiated by adding NADPH. The reaction was terminated by the addition of acetonitrile, which contained an internal standard. The final HLM concentrations and the incubation time were 0.03 mg/mL and 10 min, respectively, except POD (0.10 mg/mL and 20 min) and S-MPOH (0.15 mg/mL and 30 min). For the kinetic analysis, the range of substrate concentration was 9.4–300 μM for POD, 1.6–50 μM for DFOH, 9.4–300 μM for S-MPOH, 0.16–50 μM for BFOH, 7.8–250 μM for CZXOH, 0.05–100 μM for MDZ1'OH, 2.2–70 μM for NIFOX, and 7.8–250 μM for TESOH. After the removal of proteins by centrifugation at 2,000 rpm for 15 min at 4°C, the supernatants were subjected to high-performance liquid chromatographic separation with tandem mass spectrometric detection (LC/MS/MS). In the NIFOX, incubation was performed in a dark room to avoid the photodegradation of nifedipine.

**Inhibition study with preincubation:** The inhibitor (ABT or SKF-525A) was preincubated with 3.3 mM MgCl2, 100 mM phosphate buffer, and HLM in the presence of 1.3 mM NADPH. After a preincubation for 30 min in the presence of NADPH, an aliquot of the incubation mixture (165 μL) was added to the mixture of NADPH (final 1.4 mM) and the probe substrate (final volume, 200 μL). The final HLM concentrations and incubation time were the same as mentioned above. After the incubation, the reaction was terminated as described above.

**Time-dependent inhibition assay:** ABT was preincubated with 3.3 mM MgCl2, 100 mM phosphate buffer, and HLM in the presence of 1.0 mM NADPH. At selected time interval, aliquots of the preincubation mixture (30 μL) were collected and added to an incubation mixture containing the substrate, 1.0 mM NADPH, 3.3 mM MgCl2, and 100 mM phosphate buffer (final volume, 300 μL). The final HLM concentration and incubation time were the same as mentioned above. After the incubation, the reaction was terminated as described above.

**LC/MS/MS analysis:** All measurements were performed by LC/MS/MS in the multiple reaction monitoring (MRM) mode according to the methods of Emoto et al. except the LC condition for the determination of 4'-hydroxydiclofenac, 6-hydroxychlorzoxazone, 1'-hydroxy bufuralol, oxidized nifedipine, and 1'-hydroxymidazolam.

In the detection of 4'-hydroxydiclofenac, 6-hydroxychlorzoxazone, 1'-hydroxy bufuralol, and oxidized nifedipine, the supernatants of the reaction mixtures were analyzed by LC/MS/MS.
were injected into a YMC ODS L-80 column (2 × 35 mm; 4 μm, YMC) and eluted at a flow rate of 0.35 mL/min by a linear gradient with the mobile phase, which consisted of a mixture of A (10% (v/v) acetonitrile containing 10 mM ammonium acetate) and B (80% (v/v) acetonitrile containing 10 mM ammonium acetate). The gradient conditions for elution were as follows: 0 to 100% B (0.0–1.0 min); 100% B (1.0–2.0 min); 100 to 0% B (2.0–2.5 min); and 0% B (2.5–6.0 min).

In the detection of 1'-hydroxymidazolam, the supernatants of the reaction mixture were injected into a Phenomenex Luna C18 column (2 × 50 mm; 3 μm, Phenomenex) and eluted at a flow rate of 0.35 mL/min by a linear gradient with the mobile phase, which consisted of a mixture of C (95% 20 mM acetic acid, pH adjusted to 4.0 with ammonia/5% acetonitrile) and D (5% 20 mM acetic acid, pH adjusted to 4.0 with ammonia/5% acetonitrile). The gradient conditions for elution were as follows: 0 to 50% D (0.0–2.5 min); 50% D (2.5–3.0 min); 50 to 0% D (3.0–3.5 min); and 0% D (3.5–6.0 min).

Data analysis: The kinetic parameters and the IC50 values were calculated from the following curves using WinNonlin™ 4.1 (Pharsight Corporation, Mountain View, CA).

Michaelis-Menten plots;

\[ v = \frac{C \times V_{\text{max}}}{C + K_m} \]

where \( v \) is the velocity, \( C \) is the concentration of substrate, \( V_{\text{max}} \) is the maximum velocity, and \( K_m \) is the Michaelis constant.

IC50 determination;

\[ \% \text{ Control activity} = 100A \times \left( 1 - \frac{1}{1 + IC_{50}} \right) \]

where \( A \) is the maximum activity, IC50 is the inflection point on the curve, and \( I \) is the concentration of inhibitor.

In the data analysis of the time-dependent inhibition assay, the \( k_{\text{obs}} \) value, which is an initial rate constant of inactivation, was calculated first by linear regression analysis, and obtained as a slope of the regression line generated by plotting logarithm of the residual activity against the preincubation time. The kinetic parameters, namely \( k_{\text{inact}} \) and \( K_i \) values, were determined as the y-intercept \((1/k_{\text{inact}})\) and the x-intercept \((-1/K_i)\), respectively, in the double reciprocal plots of \( k_{\text{obs}} \) versus inhibitor concentration by linear regression analysis. The \( k_{\text{inact}} \) and \( K_i \) values are the maximal rate constant of inactivation and inhibitor concentration required for half-maximal rate of inactivation, respectively.

Results

\( K_m \) values of probe substrates: To obtain the activity specific to each CYP isofrom in the inhibition study using HLM, it is recommended to use the probe substrate concentration close to the \( K_m \) value. Therefore, the \( K_m \) values in all drug oxidations were determined prior to the inhibition study using HLM prepared in-house, and were as follows; POD, 36 μM; DFOH, 4.9 μM; S-MPOH, 21 μM; BFOH, 11 μM; CZXOH, 110 μM; MDZ1’OH, 0.89 μM; NIFOX, 5.5 μM; and TESOH, 18 μM (Table 1). These results were almost equivalent to the reported values in a two-fold range except NIFOX. The \( K_m \) for NIFOX was more than 4 times lower than the reported values.

Effect of preincubation on the inhibition by ABT and SKF-525A: Preincubation of ABT for 30 min in the presence of NADPH shifted the IC50 values in inhibiting the probe metabolic reactions to a low concentration (Table 2). The IC50 values of ABT with preincubation were as follows; POD, 21 μM; DFOH, 400 μM; S-MPOH, 37 μM; BFOH, 16 μM; CZXOH, 38 μM; MDZ1’OH, 0.71 μM; NIFOX, 0.99 μM; and TESOH,
Table 2. 
IC50 values of ABT and SKF-525A in inhibiting the CYP-dependent drug oxidations with and without preincubation

<table>
<thead>
<tr>
<th>Probe reactions</th>
<th>Substrate concentration (µM)</th>
<th>IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without</td>
</tr>
<tr>
<td>POD</td>
<td>40</td>
<td>340 ± 31</td>
</tr>
<tr>
<td>DFOH</td>
<td>5</td>
<td>860 ± 150</td>
</tr>
<tr>
<td>S-MPOH</td>
<td>20</td>
<td>110 ± 9</td>
</tr>
<tr>
<td>BFOH</td>
<td>10</td>
<td>120 ± 11</td>
</tr>
<tr>
<td>CZXOH</td>
<td>80</td>
<td>690 ± 170</td>
</tr>
<tr>
<td>MDZ1'OH</td>
<td>1</td>
<td>11 ± 0.9</td>
</tr>
<tr>
<td>NIFOX</td>
<td>5</td>
<td>3.3 ± 0.4</td>
</tr>
<tr>
<td>TESOH</td>
<td>20</td>
<td>0.58 ± 0.07</td>
</tr>
</tbody>
</table>

The inhibitory effects were investigated with and without preincubation for 30 min in the presence of NADPH. Data are the mean ± S.E. (n = 3).

a): The ratio of IC50 without preincubation to IC50 with preincubation.

Fig. 1. Inhibitory effects of ABT and SKF-525A on BFOH.
Preincubation of HLM with ABT or SKF-525A was conducted for 30 min in the presence of NADPH (●: preincubation conducted, ○: preincubation not conducted). After preincubation, bufuralol at 10 µM was added to the reaction mixture. Data points with bars represent the mean ± S.E. (n = 3).

0.70 µM. ABT exhibited the strongest inhibitory effect on the CYP3A-dependent drug oxidations (MDZ1’OH, NIFOX, and TESOH) and the weakest effect on the DFOH with and without preincubation. A remarkable IC50 shift (> 10 times) by preincubation was observed on the POD, CZXOH, and MDZ1’OH. On the other hand, the IC50 shift of ABT was not recognized on the TESOH.

SKF-525A had a wide range of IC50 values in inhibiting the probe drug oxidations by HLM as well as ABT (Table 2). SKF-525A potently inhibited the BFOH, but weakly inhibited the POD and the CZXOH with and without preincubation. However, the preincubation of SKF-525A did not cause a remarkable IC50 shift as the preincubation of ABT did. On the other hand, in BFOH, the inhibitory effect of SKF-525A was decreased by preincubation (Fig. 1). The IC50 value of SKF-525A with preincubation was 5 times higher than that without preincubation on BFOH.

Time-dependent inhibition by ABT: As mentioned above, a remarkable IC50 shift by the preincubation was observed in the inhibition of the POD, CZXOH, and MDZ1’OH by ABT. Therefore, the time-dependent inhibition assay was conducted on these drug oxidations, preincubating varying concentrations of ABT with HLM for varying times. POD, CZXOH, and MDZ1’OH in HLM were inactivated by ABT in a time- and concentration-dependent manner in the presence of NADPH (Fig. 2). The initial linear phase in the plot of logarithm of the residual activity against the preincubation time was observed up to 10 min for POD, 30 min for CZXOH, and 5 min for MDZ1’OH. The Ki and k_inact

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Fig. 2. Time- and concentration-dependent inhibition of on POD (A), CZXOH (B), and MDZ1’OH (C) by ABT.

HLM was preincubated with ABT in the presence of NADPH. After preincubation, each substrate was added to the reaction mixture and the metabolite was determined. Phenacetine, chlorzoxazone, and midazolam concentrations were set at 40 µM, 80 µM, and 1.0 µM, respectively. ABT concentrations were as follows: POD (A), 0.0, 0.47, 0.94, 1.94, 3.8, 7.5, 15, 30, and 300 µM; CZXOH (B), 0.0, 4.7, 9.4, 19, 38, and 75 µM; MDZ1’OH (C), 0.0, 0.63, 1.3, 5.0, 10, and 20 µM. Data points represent the mean of duplicate determinations.

### Table 3. The apparent inactivation constants for ABT of each drug oxidation by HLM

<table>
<thead>
<tr>
<th>Probe reactions</th>
<th>$K_i^{a)} (µM)$</th>
<th>$K_{inact}^{b)} (/min)$</th>
<th>$K_{inact}/K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>POD</td>
<td>9.70</td>
<td>0.14</td>
<td>0.014</td>
</tr>
<tr>
<td>CZXOH</td>
<td>6.4</td>
<td>0.069</td>
<td>0.011</td>
</tr>
<tr>
<td>MDZ1’OH</td>
<td>0.85</td>
<td>0.12</td>
<td>0.14</td>
</tr>
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</table>

The apparent inactivation constants were calculated by the KitzWilson double reciprocal plot between ABT concentration and $K_{obs}$, which is the initial rate constant of the enzyme inactivation.

*a): Inhibitor concentration required for half-maximal rate of inactivation.

*b): Maximal rate constant of inactivation.

### Discussion

CYP enzymes play an important role in the drug oxidation, which is one of the major clearance processes in the body. Therefore, in the drug development process, it is useful to obtain the information on the contribution of CYP enzymes to the overall metabolism of drug candidate compounds. ABT is known as a suicide substrate for CYP isoforms and widely used as a non-specific CYP inhibitor. It is believed that benzene, which was produced from ABT, forms an $N,N$-bridged benzene-protoporphyrin IX adduct in rats. However, selectivity of ABT in inhibiting the drug oxidations catalyzed by various CYP isoforms in HLM has not been fully investigated. Therefore, we investigated the inhibition of CYP enzymes by ABT with and without preincubation using conventional CYP substrates and HLM prepared in-house. ABT had a wide range of IC$_{50}$ values in inhibiting the drug oxidations in HLM, 0.58–860 µM without preincubation and 0.70–400 µM with preincubation. ABT had the strongest inhibitory effect on the CYP3A-dependent drug oxidations, namely MDZ1’OH, NIFOX, and TESOH and the weakest effect on the DFOH with and without preincubation. The inhibitory effects of ABT using HLM in the present study are consistent with our previous results using human recombinant CYP enzymes. Balani et al. also investigated the inhibitory effects of ABT on the oxidations of fluorescence substrates by recombinant CYP1A2, 2B6, 2C9, 2C19, 2D6, and 3A4 after preincubation for 30 min in the presence of NADPH, and reported that the effect is highest in inhibiting the activity of CYP3A4. Therefore, our present and previous results with HLM and recombinant CYP enzymes using the probe substrates in combination with their results using the fluorescence substrates demonstrated that ABT shows the highest inhibition on the activity of CYP3A4 among the human CYP isoforms.

A remarkable IC$_{50}$ shift (> 10 times) by preincubation with ABT was observed in the inhibition of POD, CZXPH, and MDZ1’OH. The $K_{inact}/K_i$ value of ABT, which indicates the inactivation potency, was 0.014 for...
POD in this assay. The $k_{\text{inact}}$ and $K_i$ values of furafylline, another known mechanism-based inactivator of CYP1A2, were 0.87 min$^{-1}$ and 23 $\mu$M, respectively, and the $k_{\text{inact}}/K_i$ value was 0.038.$^{18}$ For MDZ1’OH, on the other hand, the $k_{\text{inact}}/K_i$ value of ABT (0.14) was much higher than that for POD. Diltiazem, erythromycin, ritonavir, troleanodimycin, and verapamil, all known as a typical mechanism-based inactivator for CYP3A, showed the $k_{\text{inact}}/K_i$ values of 0.055, 0.0017, 1.11, 0.83, and 0.053, respectively.$^{19}$ According to the available data, ABT seems to be a comparable inactivator for CYP1A2 to furafylline and a moderate inactivator for CYP3A4.

SKF-525A inhibited the tested 8 probe drug oxidations with a wide range of the IC$_{50}$ values. SKF-525A potently inhibited the BFOH, but weakly inhibited POD and CZXOH. From the IC$_{50}$ values without preincubation in this study, SKF-525A as a CYP inhibitor was classified into three classes: potent inhibitor for BFOH by CYP2D6 (IC$_{50}$<1.0 $\mu$M); moderate inhibitor for DFOH by CYP2C9, S-MEOH by CYP2C19, and MDZ1’OH, NIFOX, and TESOH by CYP3A (1.0 $\mu$M<IC$_{50}$<20 $\mu$M); and relatively weak inhibitor for POD by CYP1A2, CZXOH by CYP2E1 (IC$_{50}$>20 $\mu$M). Ono et al. reported that the inhibitory effects of SKF-525A differ among CYP-dependent drug oxidations in HLM, in a rank order for the inhibition at 300 $\mu$M as CYP1A2<CYP2E1<CYP2C<CYP3A<CYP2C19<CYP2D6.$^{20}$ These results were equivalent with those in this study. In addition to the HLM studies, SKF-525A inhibited the drug oxidations by recombinant CYP enzymes in cDNA-expressed HepG2 cell lysate and bacuviroirus-infected insect cells in the same manner as it did in HLM.$^{5,20}$

The inhibitory effect of SKF-525A on BFOH in HLM was decreased by preincubation in the presence of NADPH, suggesting that SKF-525A is metabolized in HLM. In fact, it has been reported that SKF-525A is metabolized to a mono-deethylated form and minor metabolites in rat and swine liver microsomes.$^{21}$ The inhibitory effects of the metabolites are normally smaller than those of the parent compound as reported in several drugs such as azelastine, amiodarone, and troglitazone.$^{22-26}$ Therefore, it would be acceptable that the preincubation decreased the concentration of SKF-525A leading to a decreased inhibition.

As described above, ABT and SKF-525A showed a wide range of IC$_{50}$ values in inhibiting the drug oxidations in HLM with and without preincubation, and their inhibitory effects were increased by preincubation in some probe reactions. Therefore, we should be careful in interpreting the results of the inhibition study using single CYP inhibitor for estimation of the contribution of CYP enzymes to the overall metabolism of the compounds in question.

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References

(1997).


