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1-AMINOBENZOTRIAZOLE, A KNOWN CYTOCHROME P450 INHIBITOR, IS A SUBSTRATE AND INHIBITOR OF *N*-ACETYLTRANSFERASE

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Running Title: Interaction of ABT with *N*-acetyltransferase

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Abbreviations: ABT, 1-aminobenzotriazole; AUC, area under the curve; CL, clearance; CoA, coenzyme A; LC/MS/MS, liquid chromatography-tandem mass spectrometry; NAT, *N*-acetyltransferase; P450, cytochrome P450; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; PK, pharmacokinetics; RLMs, rat liver microsomes; RLS9, rat liver 9000g supernatant fraction; SULT, sulfotransferase; UDPGA, uridine-diphosphoglucuronic acid; UGT, UDP-glucuronosyltransferase.

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Abstract

1-Aminobenzotriazole (ABT) has been used widely as a non-selective *in vitro* and *in vivo* inhibitor of cytochrome P450 enzymes. To date, however, it has not been evaluated as an inhibitor of UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT) and *N*-acetyltransferase (NAT). In the present study, ABT was shown not to inhibit UGT and SULT activity (acetaminophen and 7-hydroxycoumarin as substrates) in rat liver microsomes and rat liver 9000g supernatant fraction (RLS9), respectively. However, it did inhibit the RLS9-catalyzed *N*-acetylation of procainamide (IC_{50} ~ 30 μM) and no preincubation time dependency was evident. In agreement, oral ABT (100 mg/kg; 2 hours pre-dose) decreased the clearance of intravenous procainamide (45%) in rats, accompanied by a decreased *N*-acetylprocainamide-to-procainamide ratio in urine (0.74 vs. 0.21) and plasma (area under the curve ratio 0.59 vs. 0.11). Additional studies with human forms of NAT (hNAT1 and hNAT2), revealed that ABT is a more potent inhibitor of hNAT2 compared to hNAT1 (IC_{50} 158 μM vs. > 1 mM). Consistent with the IC_{50} estimate, formal inhibition studies revealed that inhibition of hNAT2 was competitive with an inhibition constant (K_i) of 67 μM. In accordance with the competitive inhibition, ABT was shown to undergo *N*-acetylation in the presence of both human NAT forms, with hNAT1 exhibiting less activity under the same assay conditions (~40% of hNAT2). In summary, the results described herein indicate that ABT is a substrate and inhibitor of NAT. Such an interaction should be considered when employing ABT as a non-selective inhibitor of P450, especially when NAT-dependent metabolism is also involved.

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Introduction

1-Aminobenzotriazole (ABT) is a well known non-specific mechanism-based inhibitor of both human and non-human P450 enzymes. For example, it has been used widely to distinguish P450-mediated metabolism from non-P450-mediated metabolism *in vitro* (Dalmadi et al., 2003; Wang et al., 2006; Kenneke et al., 2008). Recently, ABT has been increasingly used *in vivo* to investigate the poor pharmacokinetic profile of new drug candidates, especially when oral bioavailability is low and first-pass metabolism is suspected. Specifically, ABT can be used to distinguish P450-dependent first pass from other factors such as solubility, permeability, and active efflux (Tang et al., 2008; EI-Kattan et al., 2008). Depending on the dose route chosen, ABT can also be used to elucidate the contribution of the gut (vs. liver) to first pass metabolism and systemic clearance (Strelevitz et al., 2006). ABT has been demonstrated to be safe in rats following an acute high dose and upon multiple dosing (Mico et al., 1988; Meschter et al., 1994), making it an attractive agent for differentiating parent- or metabolite-based toxicities in safety assessment studies. In addition, ABT can potentially be used to boost concentrations of metabolically unstable compounds in rodents, enable proof-of-concept studies and support pharmacological and toxicological profiling of a drug candidate when metabolic stability varies across species (Eng et al., 2010).

ABT has been shown not to inhibit NADPH cytochrome c reductase (Mugford et al., 1992), flavin-containing monooxygenase (FMO) (Cashman et al, 1987; Furnes et al., 2005) and transporters such as organic anion transporting peptides and P-glycoprotein (Strelevitz et al, 2006). However, its effect on phase II drug-metabolizing enzymes has not been studied extensively and only a single report has described the lack of inhibition

of UGT activity in liver microsomes of rats pretreated with ABT (50 mg/kg i.p. 2 h before sacrifice) (Mugford et al., 1992). Therefore, *in vitro* studies were conducted with rat liver subcellular fractions in order to evaluate ABT as an inhibitor of UGT, SULT and NAT activity therein. Because ABT was found to inhibit NAT activity catalyzed by RLS9, the inhibition study was expanded to include two forms of human NAT (hNAT1 and hNAT2). Furthermore, ABT is known to undergo *N*-acetylation in the rat and so it was evaluated as a human NAT substrate also. Overall, it is concluded that interactions with NAT should be considered when using ABT as a tool to assess P450-dependent metabolism. This is especially true in a drug discovery setting, when routes of metabolism have not been studied in detail, and when chemotypes contain nitrogen centers that are susceptible to *N*-acetylation in addition to P450-catalyzed *N*-oxidation.

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Materials and Methods

Materials. ABT, acetaminophen, acetaminophen glucuronide, acetaminophen sulfate, acetyl CoA (sodium salt), alamethicin, 4-aminosalycylic acid, DL-dithiothreitol, 7-hydroxycoumarin, *N*-acetylprocainamide, PAPS, procainamide and UDPGA were purchased from Sigma-Aldrich (St. Louis, MO). 4-(acetylamino)-2-hydroxybenzoic acid was orded from Ryan Scientific, Inc. (Mt. Pleasant, SC) and *N*-acetyl aminobenzotriazole was obtained from ArrayChem, Inc. (Monmouth Jct, NJ). RLS9 was purchased from XenoTech (Lenexa, KS). RLM, hNAT1 and hNAT2 were purchased from BD Gentest (Woburn, MA). Fresh rat whole blood was obtained from Bioreclamation Inc. (Westbury, NY).

Incubation Conditions. Acetaminophen and 7-hydroxycoumarin have been reported to be metabolized primarily through glucuronidation and sulfation in the rat (Davis et al., 1976; Wang et al., 2005). UGT activity was determined in RLM (4 mg/mL), employing acetaminophen (2 μM) or 7-hydroxycoumarin (50 μM) as substrates. Incubations contained $MgCl_2$ (10 mM), alamethicin (0.025 mg/mL) and ABT (1mM) in Tris-HCl buffer (pH 7.5, 100 mM). The mixture was pre-warmed at 37ºC for 3 min before the addition of UDPGA (5 mM). The final concentration of organic solvent was no greater than 1% (v/v). Control incubations were performed exactly as described above, except that no ABT was added. Sulfation of acetaminophen and 7-hydroxycoumarin was performed as above, except that assay employed RLS9 (4 mg/mL) as the enzyme source and PAPS (1mM) as the cofactor.

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N-acetylprocainamide was reported to be the major metabolite for procainamide in the rat (Schneck et al., 1977). NAT activity in RLS9 (2 mg/mL) was measured after incubation of procainamide (2 μM) in triethanolamine buffer (pH 7.5, 50 mM), containing EDTA (1mM), DL-dithiothreitol (1 mM), ABT (1mM) and acetyl CoA (2 mM). Typically, the mixture was pre-warmed at 37ºC for 3 min and then the reaction was initiated by addition of cofactor. The control experiment was performed in a similar means without ABT.

Aliquots were taken at different time points and the reaction was quenched with an equal volume of ice-cold acetonitrile containing the internal standard, vortex-mixed, and centrifuged at 14000g for 5 min. The resulting supernatant was used for LC/MS/MS analysis except for 7-hydroxycoumarin which was monitored by UV at 320 nm.

Assessment of Time-Dependent Inhibition of NAT Activity in RLS9 by ABT. *N*-acetylation of procainamide was measured in presence of ABT with and without pre-incubation. The incubation conditions were as described above, except that ABT was added at various concentrations (1000, 333, 111, 37, 12.3, 4.1, 1.37, 0.46, 0.15, 0.05, 0 μM). The mixture was pre-incubated for 0 min or 15 min with acetyl CoA and then the substrate procainamide (2 μM) was added. The reaction was continued at 37 ºC for 30 min before quenching by the addition of ice-cold acetonitrile containing the internal standard and the samples were analyzed by LC/MS/MS.

Blood-Plasma Partition. Procainamide was added to the fresh rat whole blood to obtain a final concentration of 10 μM and gently shaken in an incubator at 37°C for 2 h. Two

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aliquots of blood were removed and the first aliquot was centrifuged to obtain plasma. The second aliquot of blood was treated with a half volume of water to hemolyze the blood cells. Both plasma and hemolyzed blood samples were stored at -20°C until analysis. The blood-to-plasma concentration ratio was calculated as concentration in blood relative to plasma.

Animal Pharmacokinetic Studies. All studies were approved by the Bristol-Myers Squibb Co. Animal Care and Use Committee (Hopewell, NJ) and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

Male Sprague Dawley rats (0.26-0.30 kg, n = 3 per treatment) received an oral dose of ABT (100 mg/kg, 2 mL/kg) 2 h before a single intravenous bolus of procainamide (10 mg/kg, 2 mL/kg). The control group received only the intravenous bolus of procainamide without ABT pretreatment. The vehicle for both ABT and procainamide was 10% DMAC/90% water (v/v). Rats were fed 4 h after dosing and serial blood samples were collected at 0.03, 0.17, 0.25, 0.5, 1, 2, 4, and 6 h post-dose. Blood samples were centrifuged using tubes containing K_3-EDTA as the anticoagulant to obtain plasma. Urine samples were also collected over 24 h post-dose. Plasma and urine samples were frozen at -20 ºC until analysis.

Assessment of ABT as a hNAT1 and hNAT2 Substrate. Preparations of commercially available hNAT1 and hNAT2 (0.1 mg/mL) were individually incubated with ABT (2 μM) as the substrate. The assay employed acetyl CoA as described above. The negative

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control experiment was performed in the absence of hNAT1 or hNAT2. Additional assays employed hNAT1-selective 4-aminosalicylic acid (2 μM), and hNAT2-selective procainamide (2 μM) as the substrate and served as positive controls.

ABT as an Inhibitor of hNAT1 and hNAT2. A series of ABT concentrations (1000, 333, 111, 37, 12.3, 4.1, 1.37, 0.46, 0.15, 0.05, 0 μM) were used to determine the IC_{50} values for hNAT1 and hNAT2. The hNAT1 assay employed 4-aminosalicylic acid (2 μM) as substrate, whereas hNAT2 assay utilized procainamide (2 μM) as the substrate (assay conditions described above).

To determine the mode of inhibition and inhibition constant (K_i), various concentrations of procainamide (0.6, 0.8, 1, 1.5, 2 and 3 mM) and ABT (0, 100, 200 and 300 μM) were used. The incubation conditions were as described above, except that the final protein concentration of hNAT2 preparation was 0.005 mg/mL. The reaction was quenched after 10 min within which the product formation was linear. The mode of inhibition and K_i value were determined from triplicate measurements.

Analytical Methods. Except for 7-hydroxycoumarin, samples from *in vitro* incubations and pharmacokinetic studies were analyzed using LC/MS/MS. In all cases, a HPLC system, Shimadzu LC20ADvp pumps (Columbia, MD) and a HTC PAL autosampler (Leap Technologies; Cary, NC) was interfaced to an API4000 Qtrap mass spectrometer (Toronto, Canada) with a turboionspray source. Mass spectrometer parameters were as follows: interface temperature, 400 °C; ionspray voltage, 5.5 kV; nebulizer gas flow rate (nitrogen), 30 arb; auxiliary gas flow rate (nitrogen), 30 arb; declustering potential, 50 V;

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collision energy, 20-35 V. Data acquisition utilized multiple reaction monitoring (MRM). Ions representing the positive ion mode $(M+H)^+$ species were selected in MS1 and collisionally dissociated with nitrogen under optimized collision energies to form specific product ions that were subsequently monitored by MS3. The MRM transitions were 328.1 → 152.1 for acetaminophen glucuronide, 232.1 → 152.1 for acetaminophen sulfate, 236.4 → 163.2 for procainamide, 278.1 → 205.0 for *N*-acetylprocainamide, 196.2 → 154.2 for 4-(acetylamino)-2-hydroxybenzoic acid and 177.2 → 135.2 for *N*-acetyl aminobenzotriazole, respectively.

The HPLC conditions used a flow rate of 0.2 mL/min with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B. The separation column used for 7-hydroxycoumarin was Phenomenex Luna C_{18} (150mm × 2mm, 100 Å) and the separation column for all other analytes was Phenomenex Luna C_{18} (50mm × 2mm, 100 Å). The separation for 7-hydroxycoumarin was achieved using the following gradient: 0 min, B: 5%; 2 min, B: 5%; 12 min, B: 100%; 16 min, B: 100 %; 17 min, B: 5 %; 20 min: 5 %. The gradient used for all other analytes was: 0 min, B: 20%; 2 min, B: 90%; 3 min, B: 90%; 3.1 min, B: 5%; 3.5 min, B: 5%. UV data was processed and quantitated using Shimadzu Class-VP 7.4 and MS data were processed and quantitated using PEBiosystems Analyst™ 1.4.2. 7-Hydroxycoumarin was monitored by UV at 320 nm and enzyme activity was monitored by measuring the disappearance of 7-hydroxycoumarin.

Data Analysis. Pharmacokinetic parameters of procainamide were obtained by noncompartmental analysis of plasma concentration vs. time data (KINETICA™

software, Version 4.2, InnaPhase Corporation, Philadelphia, PA). The area under the curve from time zero to infinity (AUC_{total}) was calculated using a combination of linear and log trapezoidal summations. The total plasma or blood clearance (CL), steady-state volume of distribution (Vss), apparent elimination half-life ($T_{1/2}$) and mean residence time (MRT) were estimated. Estimations of AUC and $T_{1/2}$ were made using a minimum of 3 time points with quantifiable concentrations. Urinary recovery was calculated as the cumulative amount of unchanged drug recovered in urine divided by the dose administered. Renal clearance was calculated by multiplying the % recovery in urine by CL. Metabolite-to-parent AUC ratio ($AUC_{metabolite/parent}$) was calculated by dividing $AUC_{N\text{-acetylprocainamide}}$ by $AUC_{procainamide}$ with and without ABT pretreatment.

The IC_{50} values, K_i and inhibition mode were evaluated by fitting the data using Prism Software (GraphPad Software, Inc., San Diego, CA).

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Results

Inhibition of Glucuronidation and Sulfation by ABT. Acetaminophen is metabolized predominantly by glucuronidation and sulfation in multiple species including the rat (Miners et al., 1984; Mrochek et al., 1974; Price et al., 1984). Therefore, ABT was evaluated as a SULT and UGT inhibitor employing acetaminophen as substrate; using PAPS-fortified RLS9 and UDPGA-fortified RLM, respectively. Enzyme activity was monitored by measuring the formation of the corresponding metabolites, acetaminophen glucuronide or acetaminophen sulfate (Fig 1A). ABT, at 1 mM, did not inhibit acetaminophen glucuronidation and sulfation, compared to the control without ABT. The lack of effect of ABT on glucuronidation and sulfation was further confirmed using 7-hydroxycoumarin as substrate, which is also metabolized via glucuronidation and sulfation in multiple species including the rat (Wang et al., 2005) (Fig 1B).

Inhibition of *N*-acetylation by ABT. The principal metabolite of procainamide in the rat is *N*-acetylprocainamide (Schneck et al., 1977). Inhibition of *N*-acetylation was investigated by incubation of procainamide with acetyl CoA-fortified RLS9 with and without ABT. The enzyme activity was monitored by measuring the formation of *N*-acetylprocainamide (Fig 1A). ABT (1 mM) showed pronounced (~95%) inhibition of the formation of *N*-acetylprocainamide compared to the control without ABT.

Assessment of Time-Dependent Inhibition of NAT Activity in RLS9 by ABT. The IC_{50} for ABT inhibition of *N*-acetylation of procainamide in RLS9 was measured using ABT concentrations generated by serial 1:3 dilutions from 1 mM. The time dependency

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of inhibition was evaluated by comparing the IC_{50} values with and without a 15 min pre-incubation with ABT. As shown in Fig. 2, the IC_{50} values were 25 μM without pre-incubation of ABT and 31 μM with pre-incubation of ABT. This result suggests pre-incubation of ABT has no influence on the inhibition of NAT-catalyzed procainamide *N*-acetylation. Unlike P450, therefore, inhibition of NAT is not mechanism-based.

Blood-Plasma Partition. The blood-to-plasma concentration ratio of procainamide based on 2 h incubation was 1.01 in the rat, suggesting procainamide is distributed equally in plasma and blood cells. Hence no correction is needed, in terms of the blood-to-plasma ratio, when calculating systemic CL of procainamide from plasma CL. The term CL in Table 1 describes either blood CL (CLb) or plasma CL (CLp) for procainamide.

Inhibition of *N*-acetylation of Procainamide in the Rat. ABT has been reported to inhibit 93% of P450 activity when administered as a 100 mg/kg oral dose 2 h prior to an antipyrine dose in rats (Balani et al., 2002). To evaluate the inhibitory effect of ABT on *N*-acetylation *in vivo*, under the established ABT regime commonly used for effective P450 inhibition, procainamide was used as the *in vivo* probe substrate and PK parameters of procainamide and its metabolite *N*-acetylprocainamide were compared with and without ABT pretreatment in the rat.

Plasma concentration-time profiles of procainamide and *N*-acetyl procainamide in the rat with and without ABT pretreatment are shown in Fig 3. Table 1 summarizes the PK parameters of both procainamide and *N*-acetylprocainamide after intravenous bolus dosing of procainamide (10 mg/kg) with and without ABT pretreatment. Procainamide

has been reported to have high CL in the rat, ranging from 83 mL/min/kg to 128 mL/min/kg when dosed at 20 mg/kg intravenously (Pang et al., 1984). In our study, the CL was higher, ranging from 143 mL/min/kg to 234 mL/min/kg (mean ± SD: 193 ± 46 mL/min/kg) at a lower dose of 10 mg/kg. We measured the blood cell partitioning of procainamide and confirmed that the high CL was not due to extensive partitioning in blood cells. The high CL suggests contribution of hepatic and extrahepatic (e.g. renal) pathways in the rat. The volume of distribution (Vss) of procainamide and urine recovery of both procainamide and *N*-acetylprocainamide without ABT pre-treatment were similar to the results reported in the literature (Schneck et al., 1977; Pang et al., 1984). The overall CL of procainamide decreased ~45% with ABT, which behaved as a NAT inhibitor; reflected by the decrease in *N*-acetylprocainamide AUC (66%) and the 5-fold decrease in the metabolite-to-parent AUC ratio. The half-life of procainamide did not change significantly despite the decrease in procainamide CL. The urinary recovery of procainamide increased from 18% to 30%, whereas the recovery of *N*-acetylprocainamide in urine decreased from 13.3% to 6.5% with ABT. Therefore, the ratio of metabolite to parent amount in urine was decreased (0.74 vs. 0.21) (Table 1). The renal CL of both procainamide and *N*-acetylprocainamide was not influenced by ABT pretreatment. These *in vivo* results corroborated our *in vitro* findings and strongly suggested that ABT can significantly inhibit *N*-acetylation under the commonly used regime for P450 inhibition in the rat.

ABT as a Substrate and Inhibitor of hNAT1 and hNAT2. Since ABT is known to undergo *N*-acetylation in the rat (Town et al., 1993), it was incubated with two different

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forms of human NAT (hNAT1 and hNAT2) and the formation of *N*-acetyl aminobenzotriazole was monitored. The reported hNAT1 selective substrate 4-aminosalicylic acid (Goodfellow et al., 2000) and hNAT2 selective substrate procainamide (Rawal et al., 2008) were also included as controls. The result (Fig. 4) suggests that activity of hNAT1 is five times greater than hNAT2 towards 4-aminosalicylic acid, while the activity of hNAT2 is ten times greater than hNAT1 towards procainamide. ABT was metabolized by both forms of human NAT, although the activity of hNAT2 was ~3-fold greater than that of hNAT1.

IC_{50} values for inhibition of hNAT1- and hNAT2-mediated *N*-acetylation were measured and compared using 4-aminosalicylic acid (hNAT1) and procainamide (hNAT2) as substrates. The substrate was tested at 2 µM which represented the approximate exposure level in the rat. As shown in Fig. 5, ABT was a more potent inhibitor of hNAT2-mediated procainamide *N*-acetylation than hNAT1-mediated 4-aminosalicylic acid *N*-acetylation (IC_{50} of 158 µM vs. >1 mM).

To evaluate the mode of inhibition and determine an inhibition constant (K_i) value, five concentrations of procainamide as the substrate and three concentrations of ABT as the inhibitor were tested. The Lineweaver-Burk plot (Fig. 6. A) revealed competitive inhibition and the enzyme kinetic parameters were determined by Prism software using the nonlinear regression mode with competitive inhibition. The inhibition constant (K_i) was determined to be 67 ± 6 µM and the procainamide Michaelis constant (K_m) value for hNAT2 was determined to be 2.0 ± 0.3 mM (V_{max} was 1.5 ± 0.1 µmole/min per mg) (Fig. 6. B).

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Discussion

ABT is a well-known non-specific P450 inactivator and historically its application as a mechanistic tool has focused on oxidative metabolism. Furthermore, the effective P450 inhibition dose regimen for ABT has been published for rat, dog, monkey, mouse and guinea pig using antipyrine as a non-specific P450 probe substrate (Balani et al., 2002; Balani et al., 2004). ABT is generally considered not to inhibit phase II drug-metabolizing enzymes. However, to date, there are no data addressing its effect on NATs and SULTs, and only limited data describing inhibition of rat UGTs (Mugford et al., 1992). Full understanding of ABT inhibition on phase II enzymes is essential for understanding the mechanism of poor pharmacokinetics or toxicity of drug candidates.

The studies presented herein evaluated the effect of ABT on UGTs, SULTs and NATs *in vitro* using RLMs and RLS9 preparations. The results revealed that ABT did not inhibit UGT or SULT activity at 1 mM using both acetaminophen and 7-hydroxycoumarin as substrates. However, ABT (1 mM) was found to significantly inhibit the *N*-acetylation of procainamide in RLS9. ABT is a non-specific suicide inhibitor of P450 and its effect is time-dependent either via covalent modification of the heme prosthetic group or via modification of the enzyme active site following bioactivation to form a reactive benzyne (Ortiz et al., 1981). Studies to investigate ABT as a time-dependent inhibitor of NAT indicated no time-dependent shift in IC_{50} following a 15 min pre-incubation of ABT with CoA-fortified RLS9. Although rat NAT1 and NAT2 are widely expressed in many tissues, the former is dominant in the rat liver (NAT1 to NAT2 ratio >7) (Barker et al., 2008). Furthermore, procainamide has been demonstrated to be metabolized selectively by rat recombinant NAT1 compared to rat NAT2 (Walraven et

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al., 2006). Therefore, the effective inhibition of RLS9-catalyzed procainamide *N*-acetylation suggests that ABT serves as an effective inhibitor of rat NAT1.

To investigate the inhibition of rat NAT *in vivo*, a study was conducted in which rats were pre-dosed with ABT (100 mg/kg orally) 2 hours prior to the administration of procainamide. The protocol was similar to that used when investigating P450-dependent metabolism *in vivo*. Since hepatic metabolism is known to be saturable in rats at higher doses of procainamide (Pang et al., 1984), a low intravenous dose of procainamide (10 mg/kg) was chosen in the present study. At such a dose, hepatic extraction is more significant. The *in vivo* results described herein agreed with the *in vitro* findings that ABT can inhibit the *N*-acetylation reaction effectively at a dose of 100 mg/kg in the rat, as evidenced by the 66% decrease in the AUC of *N*-acetylprocainamide and a 51% decrease in its recovery in urine. The total CL of procainamide was still high despite pronounced inhibition of *N*-acetylation. Such a result can be explained if one considers that acetylation only accounts for 38% of procainamide total CL; urinary excretion (34%) and other unknown metabolic pathways (28%) account for the remainder (Schneck et al., 1977).

Since ABT has been reported to be metabolized by *N*-acetylation and *N*-acetyl aminobenzotriazole is the major metabolite in rat plasma (Town et al., 1993), it was evaluated as a potential substrate of hNAT1 and hNAT2. ABT was metabolized selectively by hNAT2, although the selectivity was less than that reported for the well known hNAT2 substrate procainamide (Fig 4). Metabolism by hNAT2 is consistent with the competitive inhibition observed (Fig. 6). The effective inhibition of both hNAT2 and

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RLS9 (rNAT1)-catalyzed procainamide *N*-acetylation is in accordance with reports suggesting high homology between these two enzymes (Walraven et al., 2006).

In a discovery setting, if mechanistic investigation with ABT suggests involvement of P450-mediated metabolism, soft spots can be identified by elucidation of a compound's metabolic profile to guide further structural modifications. This approach may also be extended to NAT substrates if *N*-acetylation contributes to CL. Although most NAT substrates contain an aniline or hydrazine moiety, recent reports indicate that aliphatic unsubstituted piperazines can also be acetylated rapidly in the presence of rat liver cytosol, leading to high CL in the rat. It was further shown that small structural changes to the piperazine group can retain potency but prevent metabolism by NATs *in vitro* and thus reduce *in vivo* CL (Rawal et al., 2008). Furthermore, structural modifications to prevent *N*-acetylation of the most common NAT substrates (e.g., aromatic amines) are also possible (Liu et al., 2007). These results illustrate that the relative contribution of *N*-acetylation and oxidative metabolism can be evaluated *in vivo* through ABT inhibition, and further structural modification to avoid *N*-acetylation beyond P450-mediated oxidation is possible without deminishing potency.

In conclusion, the findings described herein extend our current understanding of the inhibition profile of ABT. ABT does not inhibit rat UGTs and SULTs, but can significantly inhibit NAT activity. *In vitro*, such inhibition is evident at concentrations used to inhibit P450 in both rat and human subcellular fractions. Therefore, it may be possible to expand the use of ABT as a tool inhibitor to encompass NAT substrates. Before its use *in vivo*, however, it is advisable to evaluate NAT-catalyzed metabolism and determine whether such activity is inhibited by ABT *in vitro*. Moreover, the optimal

dose for inhibition of rat NAT *in vivo* remains to be confirmed and investigated with additional substrates. For procainamide, it was possible to show an effect *in vivo* employing a P450 inhibition protocol. The results of the present study also imply that caution is needed when ABT is used as a diagnostic inhibitor of P450 in the absence of information related to NAT-dependent metabolism.

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Authorship Contributions.

Participated in research design: Sun, Rodrigues, Marathe, Harper, and Dierks.

Conducted experiments: Sun, Zhang, and Chang.

Contributed new reagents or analytic tools: Sun, Zhang, and Chang.

Performed data analysis: Sun.

Wrote or contributed to the writing of the manuscript: Sun, Rodrigues, Marathe, Dierks, and Harper.

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Figure Legends

Fig. 1. A. Effect of ABT on UGT, SULT and NAT activity in rat liver microsomes and 9000g supernatant fraction. Enzyme activity was monitored by measuring the corresponding products formed and normalized by the activity without ABT. The probe substrates were acetaminophen for UGT and SULT, and procainamide for NAT. **B.** Effect of ABT on UGT and SULT in rat liver microsomes and 9000g supernatant fraction employing 7-hydroxycoumarin as substrate. Enzyme activity was monitored by measuring the disappearance of parent compound and normalized by the activity without ABT.

Fig. 2. Inhibition of procainamide *N*-acetylation in the presence of rat liver 9000g supernatant fraction without pre-incubation (left) and with 15 min pre-incubation of ABT (right).

Fig. 3. Mean (± SD) plasma concentrations vs. time profile of procainamide (top) and *N*-acetylprocainamide (bottom) in male Sprague-Dawley rats after intravenous dosing of 10 mg/kg procainamide with (Δ) and without (□) ABT pretreatment. ABT was dosed orally at 100 mg/kg 2 h prior to procainamide i.v. bolus dosing.

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Fig. 4. Incubation of 4-aminosalicylic acid, procainamide and ABT with recombinant NATs (hNAT1 and hNAT2). Enzyme activity was monitored by measuring the formation of the corresponding *N*-acetylated product and normalized by the highest observed enzyme activity.

Fig. 5. Inhibition of hNAT1-mediated 4-aminosalicylic acid *N*-acetylation (A) and hNAT2-mediated procainamide *N*-acetylation (B) in presence of ABT.

Fig. 6. A. Lineweaver-Burk plot describing the competitive inhibition of hNAT2-mediated procainamide *N*-acetylation by ABT. **B.** Data were fitted simultaneously to an equation describing competitive inhibition (Prism Software, GraphPad Software, Inc., San Diego, CA):

$$V = \frac{V_{max} \cdot [S]}{K_m \cdot \left(1 + \frac{[I]}{K_i}\right) + [S]}$$

The following parameters were determined: K_i = 67 ± 6 µM, K_m = 2.0 ± 0.3 mM, and V_{max} = 1.5 ± 0.1 µmole/min per mg protein.

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Table 1

Pharmacokinetic parameters of procainamide and its *N*-acetyl metabolite in Sprague-Dawley rats with and without ABT pretreatment (100 mg/kg oral dose of ABT 2 h before 10 mg/kg i.v. bolus of procainamide).

		Procainamide		*N*-acetylprocainamide	
PK parameters	Units	with ABT	without ABT	with ABT	without ABT
AUC_{total}	nM*h	5873 ± 803	3308 ± 869	664 ± 259	1945 ± 339
$T_{1/2}$	h	0.7 ± 0.2	0.6 ± 0.2		
CL	mL/min/kg	106 ± 14	193 ± 46		
Vss	L/kg	3.8 ± 0.3	5.0 ± 0.7		
Urine recovery	%	30.4 ± 4.5	18.0 ± 2.7	6.5 ± 2.2	13.3 ± 2.7
Renal CL	mL/min/kg	32.0 ± 5.6	34.1 ± 4.0	61.7 ± 9.4	43.5 ± 15.9
$AUC_{metabolite/parent}$				0.11	0.59
Amount in $urine_{metabolite/parent}$				0.21	0.74

Figure 1

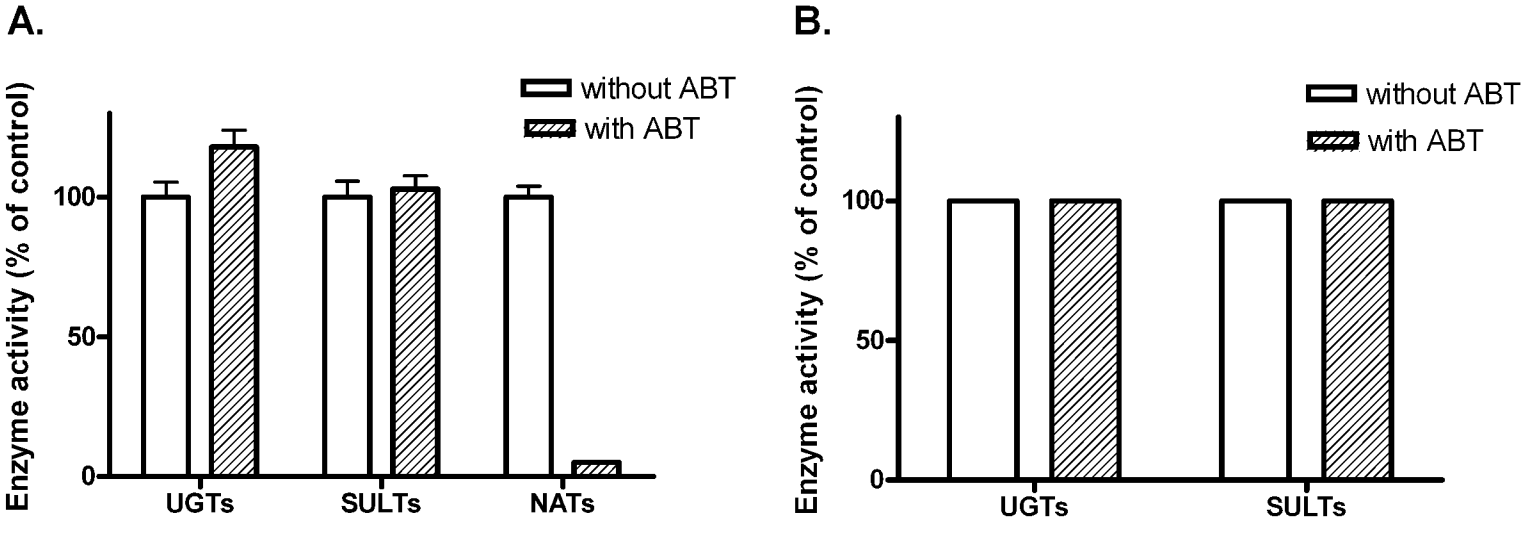
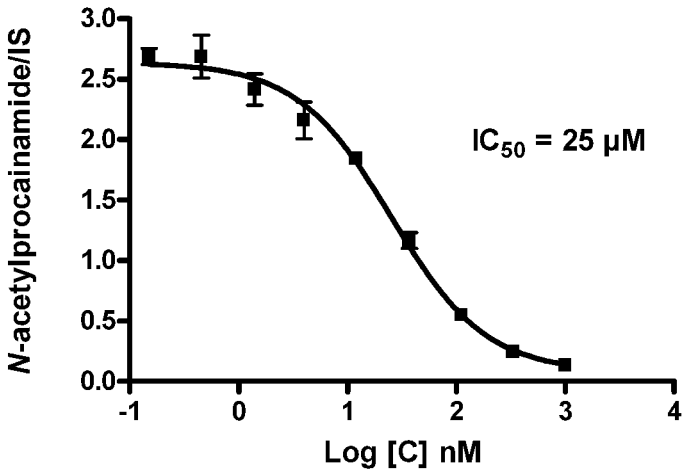

A.
without ABT
with ABT
Enzyme activity (% of control)
100
50
0
UGTs
SULTs
NATs
B.
without ABT
with ABT
Enzyme activity (% of control)
100
50
0
UGTs
SULTs

Figure 2


3.0
2.5
2.0
1.5
1.0
0.5
0.0
N-acetylprocainamide/IS
IC50 = 25 µM
-1
0
1
2
3
4
Log [C] nM

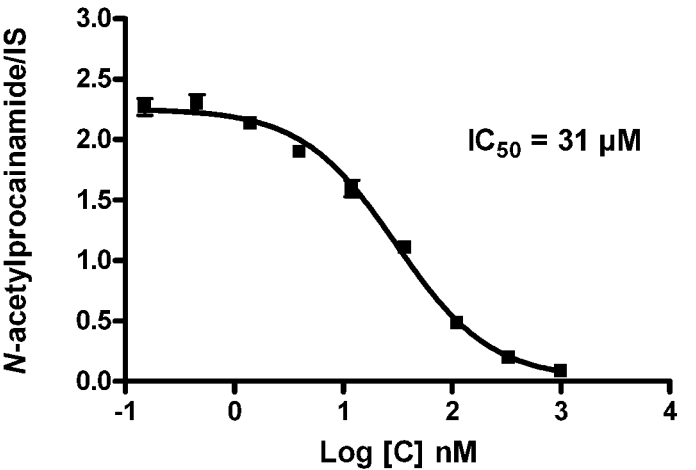
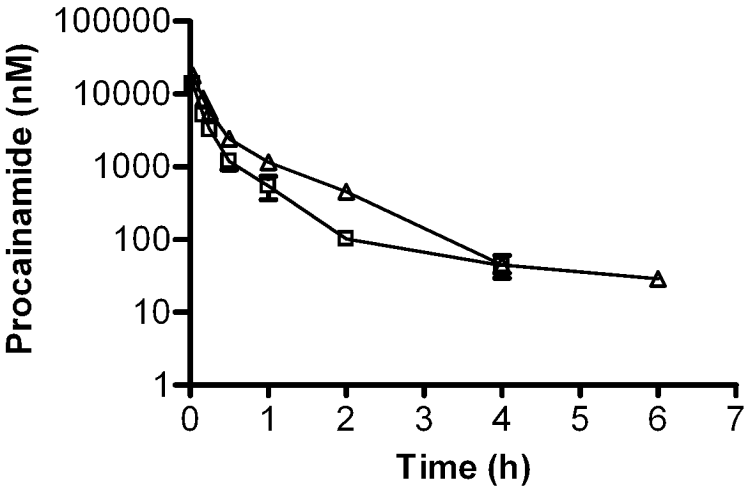

3.0
2.5
2.0
1.5
1.0
0.5
0.0
N-acetylprocainamide/IS
IC50 = 31 µM
-1
0
1
2
3
4
Log [C] nM

Figure 3


Procainamide (nM)
100000
10000
1000
100
10
1
0
1
2
3
4
5
6
7
Time (h)

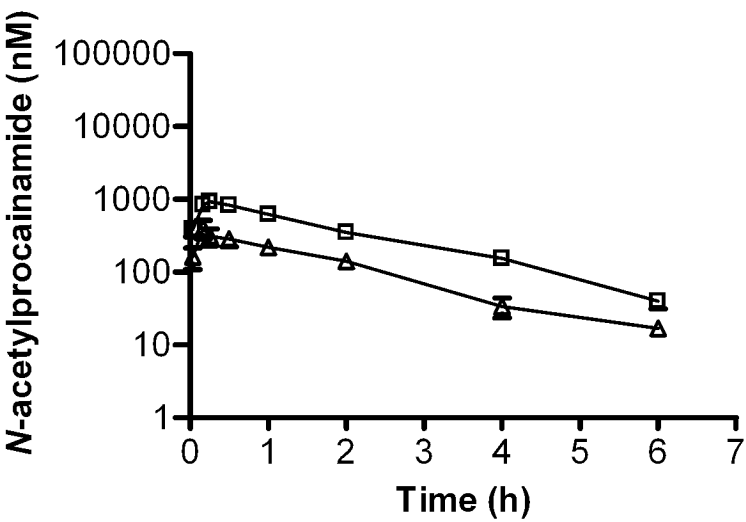

N-acetylprocainamide (nM)
100000
10000
1000
100
10
1
0
1
2
3
4
5
6
7
Time (h)

Figure 4

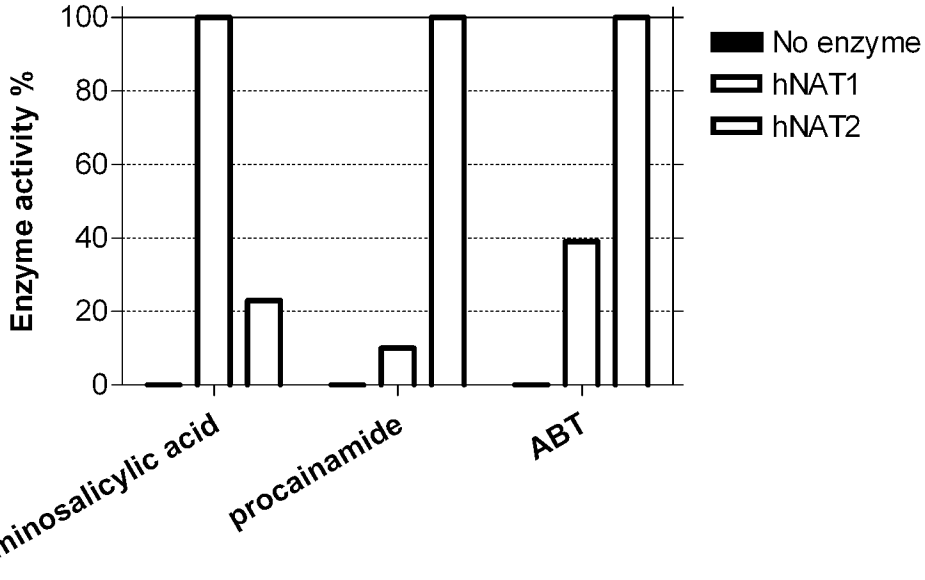

Enzyme activity %
100
80
60
40
20
0
No enzyme
hNAT1
hNAT2
4-aminosalicylic acid
procainamide
ABT

Figure 5

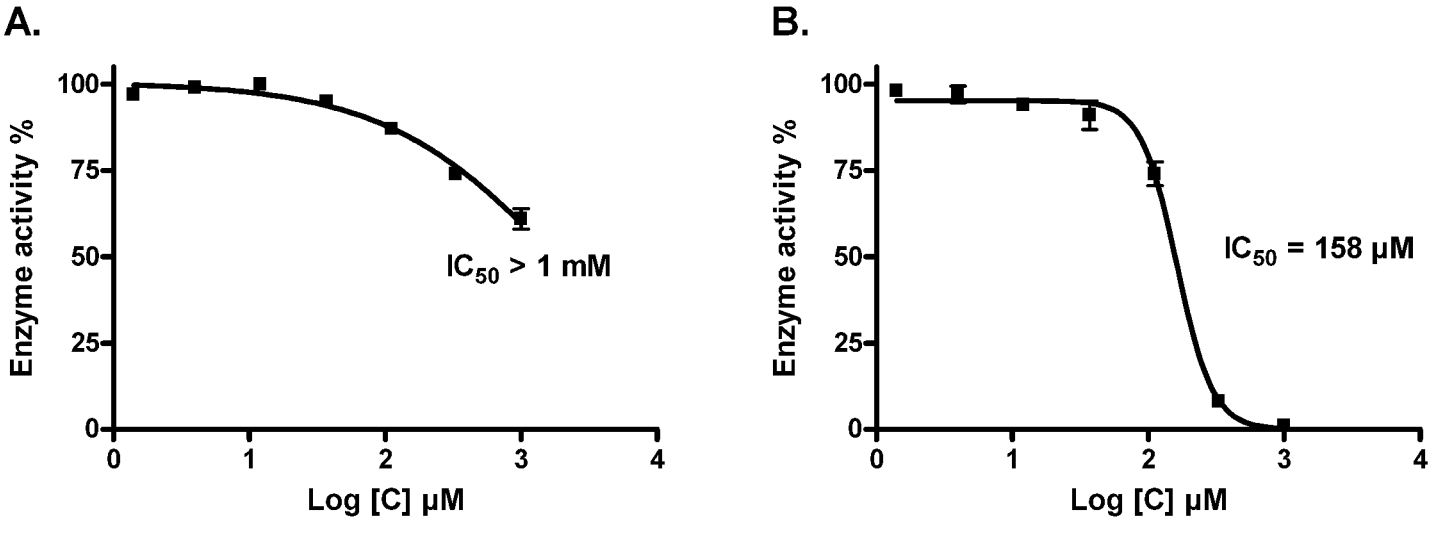
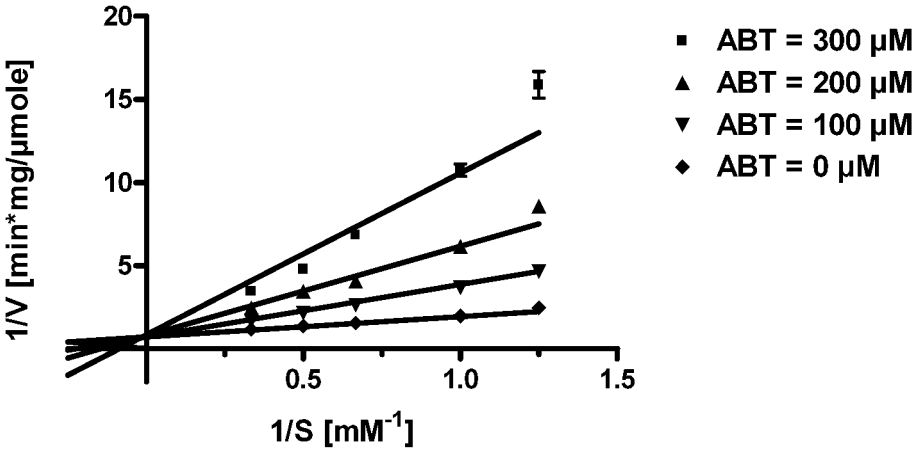
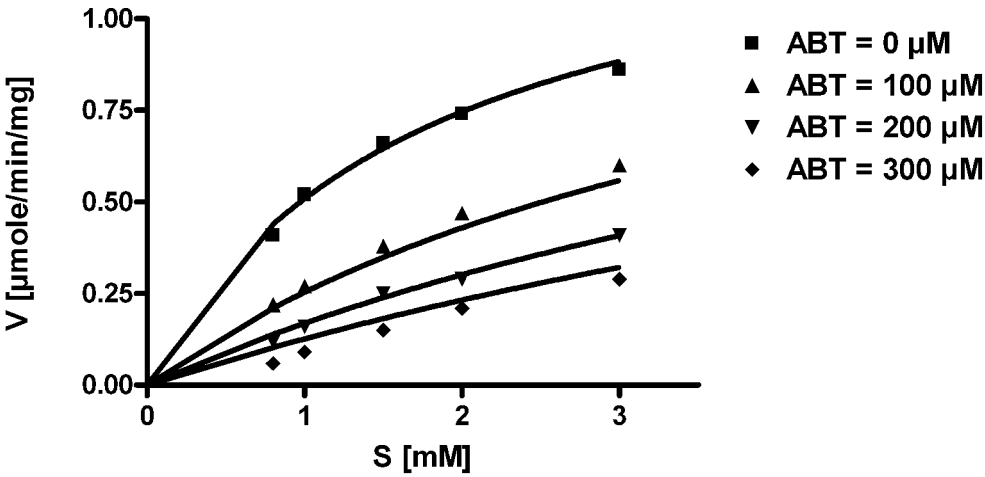

A.
100
75
50
25
0
Enzyme activity %
0
1
2
3
4
Log [C] µM
IC50 > 1 mM
B.
100
75
50
25
0
Enzyme activity %
0
1
2
3
4
Log [C] µM
IC50 = 158 µM

Figure 6

A.


20
15
10
5
1/V [min*mg/µmole]
0.5
1.0
1.5
1/S [mM-1]
ABT = 300 µM
ABT = 200 µM
ABT = 100 µM
ABT = 0 µM

B.


1.00
0.75
0.50
0.25
0.00
V [µmole/min/mg]
0
1
2
3
S [mM]
ABT = 0 µM
ABT = 100 µM
ABT = 200 µM
ABT = 300 µM