1-Aminobenzotriazole Modulates Oral Drug Pharmacokinetics through Cytochrome P450 Inhibition and Delay of Gastric Emptying in Rats

Rowan A. Stringer, Eckhard Weber, Bruno Tigani, Paul Lavan, Stephen Medhurst, and Bindi Sohal

Novartis Institutes for Biomedical Research, Horsham, West Sussex, United Kingdom (R.A.S., E.W., P.L., S.M., B.S.); and Global Imaging Group, Novartis Pharma AG, Basel, Switzerland (B.T.)

ABSTRACT

The simultaneous effects of the cytochrome P450 inhibitor 1-aminobenzotriazole (ABT) on inhibition of in vivo metabolism and gastric emptying were evaluated with the test compound 7-(3,5-dimethyl-1H-1,2,4-triazol-1-yl)-3-(4-methoxy-2-methylphenyl)-2,6-dimethylpyrazolo[5,1-b]oxazole (NVS-CRF38), a novel corticotropin releasing factor receptor 1 (CRF1) antagonist with low water solubility, and the reference compound midazolam with high water solubility in rats. Pretreatment of rats with 100 mg/kg oral ABT slowed gastric emptying by 2 hours before a semisolid caloric test meal markedly delayed gastric emptying. ABT increases stomach weights by 2-fold; this is likely attributable to a prosecretory effect because stomach concentrations of bilirubin were comparable in ABT and control groups. ABT administration decreased initial systemic exposure of orally administered NVS-CRF38 by ∼10-fold, suggesting gastric retention and delayed oral absorption. ABT increased the initial systemic exposure of midazolam, however, for orally (but not subcutaneously) administered midazolam, extensive variability in plasma-concentration time profiles was apparent. Careful selection of administration routes is recommended for ABT use in vivo, variable oral absorption of coadministered compounds can be expected due to a disturbance of gastrointestinal transit.

Introduction

1-Aminobenzotriazole (ABT) is a well-established time-dependent inhibitor of cytochrome P450 (P450) enzymes, often used in conjunction with in vitro drug metabolism systems such as microsomes and hepatocytes to determine the relative contribution of P450 enzymes to the metabolism of a drug (Dalmadi et al., 2003; Furnes and Schlenk, 2005; Schulz-Utermoehl et al., 2010). ABT is generally considered as a nonspecific inhibitor of P450; the mechanism of P450 inactivation is likely to be attributed to oxidation of ABT’s 1-amino group and decomposition of the molecule yielding reactive benzene, which forms an NN-bridged adduct on the P450 porphyrin ring (Ortiz de Montellano et al., 1984).

ABT’s physiochemical properties make it highly amenable as a tool compound for in vivo studies. The compound is highly soluble in aqueous solvents, enabling convenient administration in either saline or water at doses up to 100 mg/kg. ABT is well tolerated in rodents with no overt toxicity (i.e., changes in body weight, food consumption, or clinical appearance) observed after 100 mg/kg per day dosing for 13-weeks to male Sprague-Dawley rats (Meschter et al., 1994). The effects of repeat dosing of ABT at a dose that significantly reduces oxidative metabolism are considered to be of minimal short-term significance; therefore ABT has potential as a tool compound to inhibit P450 metabolism in vivo.

A large amount of literature evidence supports the effectiveness of ABT pretreatment as a method to increase the exposure of coadministered molecules. Two-hour pretreatment of rats, dogs, and monkeys with 100 mg/kg oral ABT increases the area under the concentration-time curve (AUC) of intravenous antipyrine 14-fold in rats, 12-fold in dogs, and 9-fold in monkeys (Balani et al., 2002). In rats ABT has been shown to increase the oral exposure of midazolam by ∼100-fold (Strelevitz et al., 2006) and chlorzoxazone by 3- to 4-fold (Muzeeb et al., 2005). For phenacetin the inhibitory effects elicited by a single dose of ABT to rats amounted to ∼80% and were maintained to 24 hours after administration of ABT; by 36 hours the degree of inhibition subsided to ∼50% (Mico et al., 1988).

The application of ABT pretreatment has been expanded to pharmacology and toxicology studies. In mice, intraperitoneal administration of ABT increases brain levels of flurazepam, offering protection against pentylenetetrazole-induced convulsions (Capello et al., 1990). Coadministration of ABT with the bishophosphonate 1-(1,6-dihydro-1-methyl-1-oxo-4-phenyl-2-pyrimidinyl)-propylyliden (U-91502) increases drug exposure between 3- and 4-fold; increased exposure was associated with a worsening of cardiovascular side effects, implicating the parent drug rather than metabolites as the toxic compound (Leong et al., 1997).

Although many literature studies have investigated the ability of ABT to block drug metabolism in vivo, few have described a potential for altered gastrointestinal function. In the present study we used the reference P450 substrate midazolam maleate and the test compound 7-(3,5-dimethyl-1H-1,2,4-triazol-1-yl)-3-(4-methoxy-2-methylphenyl)-2,6-dimethylpyrazolo[5,1-b]oxazole (NVS-CRF38) (Fig. 1), a P450-metabolized novel low-molecular-weight corticotropin releasing-factor receptor 1 (CRF1) antagonist with excellent physicochemical (mol. wt. = 351; CLOGP = 2.7; polar surface area = 76; H-bond donors = 0; H-bond acceptors = 7) and pharmacokinetic
properties, to simultaneously test blockade of in vivo metabolism and gastric emptying on oral pharmacokinetics. NVS-CRF38 undergoes complete absorption in rats (F = 100%), elimination occurs almost entirely by hepatic metabolism catalyzed by P450 enzymes (Stringer et al., 2014). A noninvasive imaging approach, based on tracking the transit of fluorescent beads through the gastrointestinal tract, has been applied to test the hypothesis that ABT delays gastric emptying. On the basis of these findings recommendations are suggested for the most effective study design for the use of ABT as an in vivo tool compound to block P450 metabolism.

Materials and Methods

Chemicals. The compounds 7-(3,5-dimethyl-1H-1,2,4-triazol-1-yl)-3-(4-methoxy-2-methylphenyl)-2,6-dimethylypyrazolo[5,1-b]oxazole (NVS-CRF38) (> 99% as free base) and 4-(7,3,5-dimethyl-1H-1,2,4-triazol-1-yl-2,6-dimethylypyrazolo[5,1-b] oxazol-3-yl-3-methylphenol (O-desmethyl NVS-CRF38) were synthesized at the Novartis Institutes for Biomedical Research (Horsham, UK). All other solvents and chemicals were of analytical grade or better.

Pharmacokinetic Studies. Pharmacokinetic studies were conducted in accordance with the British Home Office regulations (Scientific Procedures) Act of 1986, United Kingdom. Animals were housed at 24°C in a 12-hour light-dark cycle. Female Wistar rats (210–266 g) and male Sprague-Dawley rats (229–419 g) were purchased from Charles River Laboratories (Margate, UK). On the first day of each study, each rat was surgically implanted with a tail vein cannula. After approximately 30 minutes treatment was started.

NVS-CRF38/ABT Interaction Studies. A preliminary interaction study between orally administered NVS-CRF38 (10 mg/kg) and orally administered ABT (100 mg/kg, 2-hour pretreatment time) was undertaken in female Wistar rats (n = 4). ABT was formulated in water at 20 mg/ml and administered by oral gavage; the dose volume was 5 ml/kg. Control rats received an equivalent volume of water by mouth. Two hours after ABT administration each rat received 10 mg/kg NVS-CRF38 via oral gavage. NVS-CRF38 was formulated as a suspension in 0.5% methylcellulose and 0.5% Tween 80; the dose volume was 5 ml/kg.

NVS-CRF38/ABT Interaction Studies, Fed versus Fasted Animals. A second NVS-CRF38 study was conducted to test the effect of the fed state on the ABT/NVS-CRF38 interaction. Female Wistar rats (n = 4 per group) were either fasted overnight or had free access to food and water. The study design was similar to the first study with the exception that ABT (100 mg/kg dose formulated in water at 50 mg/ml, dose volume 2 ml/kg) was dosed by i.p. injection 2 hours prior to NVS-CRF38 treatment; the control group received an equivalent volume of water via the i.p. route. Serial blood samples (0.15 ml) were taken via an implanted tail vein catheter at defined time points to 72 hours (0.25, 0.75, 1.5, 3, 6, 24, 29, 48, 53, and 72 hours).

NVS-CRF38/ABT Interaction Studies, 15-Hour ABT Pretreatment. This study was undertaken to consider a longer ABT pretreatment time on the observed ABT/NVS-CRF38 interaction; for this group (n = 4) NVS-CRF38 was administered 15 hours after ABT pretreatment. ABT (100 mg/kg dose formulated in water at 50 mg/ml, dose volume 2 ml/kg) was dosed by i.p. injection 15 hours prior to NVS-CRF38 treatment. Serial blood samples (0.15 ml) were taken via an implanted tail vein catheter at defined time points to 72 hours (0.25, 0.75, 1.5, 3, 6, 24, 29, 48, 53, and 72 hours).

Oral Midazolam/Oral ABT Interaction Studies. Pharmacokinetic studies in fed male Sprague-Dawley rats (n = 4) were undertaken to explore interactions between ABT and midazolam maleate in rats. Animals were pretreated with oral ABT (100 mg/kg formulated in phosphate-buffered saline at 25 mg/ml, dose volume 4 ml/kg); 2 hours later the rats received 2 mg/kg oral midazolam maleate (formulated as a solution in 0.5% methylcellulose and 0.5% Tween 80, dose volume 5 ml/kg). Control animals, which received no ABT treatment, received 7.4 mg/kg oral midazolam using the same formulation excipients; a higher midazolam dose for the control group was required to ensure that drug levels in plasma were detectable by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Serial blood samples (0.15 ml) were taken via an implanted tail vein catheter at defined time points to 102 hours (0.25, 0.75, 1.5, 3, 6, 24, 28, 48, 52, 72, 78, 96, and 102 hours).

Midazolam s.c./ABT i.p. Interaction Studies. To determine the potential for ABT to interact with midazolam administered by the s.c. route, male Sprague-Dawley rats (n = 4) were pretreated with i.p. ABT (100 mg/kg formulated in phosphate-buffered saline at 25 mg/ml, dose volume 4 ml/kg). Two hours after ABT treatment the rats received 0.5 mg/kg s.c. midazolam maleate (formulated as a solution in 0.5% methylcellulose and 0.5% Tween 80, dose volume 5 ml/kg). Serial blood samples (0.15 ml) were taken via an implanted tail vein catheter at defined time points to 102 hours (0.25, 0.75, 1.5, 3, 6, 24, 28, 48, 52, 72, 78, 96, and 102 hours).

Collection of Blood Samples. For all studies serial blood samples (0.15 ml) were taken via an implanted tail vein catheter into a collection tube containing EDTA crystals. Blood samples were briefly mixed and kept on ice; samples were then centrifuged at 2,000g for 10 minutes. A 100-µl sample of plasma supernatant was transferred to a microtiter plate and frozen at −80°C prior to bioanalysis. After removal of each blood sample an equal volume of hep- arinized saline was injected into the animal via the sampling catheter.

Sample Extraction and LC-MS/MS Analysis. Calibration samples were prepared for each analyte by dissolving an appropriate amount of compound in dimethylsulfoxide and adding to control plasma. Twelve calibration standards were prepared between 2.4 nM and 5,000 nM. To ensure that levels of ABT in plasma were within range of the calibration curve, higher concentration standards were used, ranging between 0.7 µM and 500 µM. We dispensed 50 µl of either plasma sample or calibration sample into a 96-well plate containing 150 µl of acetonitrile; the precipitated samples were mixed at room temperature for 10 minutes and centrifuged at 2,000g for 15 minutes. Next, 120 µl of supernatant was removed into a separate 96-well plate and diluted with 120 µl of water and 30 µl of the internal standard. For analysis of NVS-CRF38 and O-desmethyl NVS-CRF38 glyburide (2 µM) was used as an internal standard; for analysis of midazolam and 1’-hydroxymidazolam, triazolam (2 µM) was used. We injected 5 µl of sample into the LC-MS/MS system. LC-MS/MS analysis was performed using a Quattro Premier mass spectrometer and an Acquity UPLC system (both from Waters; Manchester, UK). NVS-CRF38, O-desmethyl NVS-CRF38, ABT, and the internal standard glyburide were analyzed using an Acquity UPLC BEH C18 analytical column (1.7-µm particle size; 50 × 2.1 mm; Waters). The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (methanol). A linear gradient was programmed from 5% to 95% B in 4.5 minutes; the mobile phase was maintained at 95% B for 1 minute and then returned to the initial conditions. The flow rate was 0.5 ml/min into the electrospray source. The desolvation temperature was 300°C and source block temperature 100°C. Analytes were detected using multiple reaction monitoring. For midazolam/ABT studies parent-to-daughter mass

![Fig. 1. Structure of NVS-CRF38.](image-url)
Due to the high levels of ABT present in plasma samples, it was necessary to quantify ABT from a diluted sample in a second analytical run. A 20-μl aliquot from the final sample analysis plate was dispensed into a second sample plate containing 180 μl of water. This dilution was necessary for two reasons: 1) to keep peak responses within the linear range of the LC-MS/MS system, and 2) to minimize the amount of organic solvent in the final sample—this was required to maintain the chromatographic peak shape for ABT. The lower limit of quantification for ABT was 0.7 μM; the dynamic range for the assay was 0.7–500 μM. For all analytes coefficients of determination (R²) describing the calibration curves remained above 0.99. The performance of the analytical methods was considered acceptable when at least two-thirds of quality control samples fell within 15% of their nominal values.

**In Vivo Assessment of Gastric Emptying.** In vivo and ex vivo assessments of gastric emptying procedures were conducted in Basel Switzerland in accordance with the Swiss animal welfare laws. For these studies animals were group-housed under standard conditions (constant temperature and humidity). Male Wistar rats (weight range 180–250 g) were maintained on a 12-hour light/12-hour dark cycle (lighting from 6 AM to 6 PM) and had access to food and water. They were allowed to acclimatize for 1 week. Prior to the experiments, animals were fasted for 8 hours, with water available ad libitum resulting in almost complete emptying of the stomach. To avoid circadian variations, fasting was always started between 5:30 and 7:30 AM and all measurements were carried out between 1:30 and 3:30 PM. On the experimental day, ABT (100 mg/kg) or vehicle (water, 10 ml/kg) was administered orally 2 or 15 hours before the rat received 1.5 ml of a viscous nutrient meal. This test meal, containing fluorescent beads, was administered via gavage to the rats (meal: Novasource, 1 kcal/ml, 7.5% methylcellulose; beads: 50 mg/ml TentaGel, 1% Cy5.5, diameter 0.1 mm). The Novasource meal was supplied by Nestle (Vevey, Switzerland), TentaGel beads were supplied by Rapp Polymere GmbH (Tuebingen, Germany). In a second series of experiments, ABT (100 mg/kg) or vehicle (saline, 5 ml/kg) was given i.p. 2 or 15 hours before rats received 1.5 ml of a viscous nutrient meal. Near-infrared fluorescence (NIRF) measurements of gastric emptying were performed 30 minutes after the meal. For NIRF imaging, the animals were temporarily anesthetized with isoflurane (1.5% vapor concentration in nitrous oxide:oxygen 2:1) (Forene, Abbott Laboratories, Baar, Switzerland). Anesthesia was induced in a chamber and maintained during the imaging time with a facemask. For gastric emptying measurements, animals were placed in right lateral position, exposing the left side (where the stomach is localized) to the excitation/detection system. To detect signal coming from the small intestine, an additional imaging data set was collected; with the animals placed in left lateral position to expose the right side. The ratio of fluorescence activity measured in the stomach and small intestine reflected the gastric emptying rate. The imaging procedure lasted about 10 minutes. For in vivo NIRF imaging of gastric emptying, an xPlor Optix (GE Medical Systems, Montreal, Canada) small-animal imager was used. A pulsed laser at 670 nm with a power of 4.4 mW at 80 MHz repetitive rate and pulse durations of 60 psec was used for fluorescence excitation. The fluorescent light emitted from the sample was detected by a photomultiplier tube (time-domain detector) at 700 nm. Data were acquired using the GE eXplore Optix acquisition software. NIRF images were analyzed quantitatively using the GE eXplore Optix OptiView data analysis software tool.

**Ex Vivo Assessment of Gastric Emptying and Small Intestinal Transit.** Upper gastrointestinal transit (gastric emptying and intestinal transit) was performed in fasted male Wistar rats (n = 5 to 6 and weight range 180–210 g). At 1 hour after oral administration of ABT (100 mg/kg) or vehicle (water, 10 ml/kg), rats received 1.5 ml of the viscous nutrient meal. Animals were decapitated under isoflurane anesthesia 1 hour after the test meal, and the stomach and small intestine from the gastric pylorus to the cecum were removed immediately. The gastrointestinal tract was then cut into 12 segments of equal length and the fluorescent signal in each sample was determined with an optical imaging system (Biospace Laboratory Photon Imager; Paris, France). Data were expressed as the percentage of fluorescence intensity per segment (stomach; small bowel segments 1–10; cecum) and plotted in a histogram.

**In Vivo Model of Gastrointestinal Reflux.** Gastrointestinal reflux was determined by the relative concentration of bilirubin in the stomach of ABT-treated and vehicle-treated rats. Gastric bilirubin is a validated natural marker to assess enterogastric reflux and is routinely used in clinical studies (Fein et al., 2002) and in preclinical studies in rats (Inoue et al., 2007). Groups of male nonfasted Sprague-Dawley rats (n = 6, weight range 200–250 g) were dosed with either 50 mg/kg ABT or vehicle (0.5% methyl cellulose and 0.5% Tween 80 (v/v) in saline via the s.c. route at a dose volume of 2 ml/kg. At 1 hour after ABT administration, animals were culled by exposure to carbon dioxide gas in a rising concentration. Death was ensured by neck dislocation, and plasma and stomach samples were removed. Stomachs were weighed and homogenized in 15 ml 1 M sodium hydroxide. Stomach and plasma samples were assayed for bilirubin using the diazonium method and a Cobas 6000 Analyzer (Roche Diagnostics, Mannheim, Germany). We added 2 μl of either plasma or
homogenized stomach sample to 124 µl of hydrochloric acid (120 mM) and 25 µl of 3,5-dichlorophenyldiazonium salt (1.5 mM). The sample was incubated at room temperature for 10 minutes and the level of azobilirubin was quantified photometrically at 546 nm. Bilirubin levels were determined against a standard curve over the range 2–650 µM.

**Results**

Treatment of rats with ABT was evaluated as a method to inhibit metabolism of NVS-CRF38. Two-hour oral ABT pretreatment profoundly impaired oral absorption of NVS-CRF38 (Fig. 2A, Table 1). NVS-CRF38 plasma levels in ABT-treated rats were markedly lower compared with the control group for the first 6 hours and then increased steadily, reaching maximal levels at ~30 hours post dose. Drug levels were only marginally reduced at 72 hours. For the ABT-treated group, blood concentrations at the last time point (72 hours) were ~6,000 nM. Due to the variable nature of the concentration-time profile it was not possible to extrapolate from the last time point to infinity. Therefore AUC values for NVS-CRF38 in this group are likely to be underestimated. In ABT-treated rats plasma concentrations for O-desmethyl NVS-CRF38 were just detectable between 0 and 6 hours of the study (range 1–3 nM), then increased over the time-course, reaching maximum levels in plasma at 72 hours. The AUC_{0→72} hour for the phenolic metabolite was 3.6-fold lower compared with the control group (Fig. 2B). Extrapolation of AUC values for the metabolite was not possible because levels were still increasing at 72 hours; therefore, the AUC for this metabolite is likely to be underestimated in ABT-treated rats.

The effect of ABT pretreatment, using a 2-hour pretreatment time, on NVS-CRF38 pharmacokinetics in fed and fasted rats was examined. For

**Figure 4.** Dose-normalized pharmacokinetic profiles for midazolam maleate in male Sprague Dawley rats. Chart (A) compares concentration-time profiles for 7.4 mg/kg oral midazolam in control rats (filled circles) with a 2 mg/kg oral midazolam dose in the group treated 2 hours previously with 100 mg/kg oral ABT (open circles). (B) Comparison of concentration-time profiles for 0.5 mg/kg s.c. midazolam in control rats (filled circles) with a group treated 2 hours previously with 100 mg/kg i.p. ABT (open circles). Data are the mean and standard deviation of four rats.

**Table 1.** Effect of 1-aminobenzotriazole administration on the oral pharmacokinetics of NVS-CRF38 in female Wistar rats.

| Parameters | Oral NVS-CRF38
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No Inhibitor</td>
<td>ABT (100 mg/kg)</td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>10</td>
</tr>
<tr>
<td>T_{max} (hr)</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>C_{max} (nM)</td>
<td>8263 ± 823</td>
</tr>
<tr>
<td>AUC_{0→t} (nM*hr)</td>
<td>157,107 ± 22,787</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameters</th>
<th>O-Desmethyl NVS-CRF38 Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>T_{max} (hr)</td>
<td>22 ± 14</td>
</tr>
<tr>
<td>C_{max} (nM)</td>
<td>428 ± 43</td>
</tr>
<tr>
<td>AUC_{0→t} (nM*hr)</td>
<td>15,102 ± 1,577</td>
</tr>
</tbody>
</table>

* P < 0.05 (Student’s t test) versus values in vehicle-treated group.
** P < 0.01 (Student’s t test) versus values in vehicle-treated group.
In this study, NVS-CRF38 was administered via the oral route and ABT was administered via the i.p. route to ensure that ABT did not inhibit its own absorption. For the fed animal group the impact of ABT on NVS-CRF38 was comparable to that observed in our first study (for which ABT was administered via the oral route). NVS-CRF38 plasma levels were depressed for the first 6 hours and steadily increased to Cmax by 32 hours.

In the fasted group, highly comparable results were observed (Fig. 3). We considered whether the effects of ABT treatment on oral NVS-CRF38 pharmacokinetics could be mitigated by applying a longer pretreatment time. Rats were pretreated with ABT (100 mg/kg via the i.p. route) 15 hours prior to oral NVS-CRF38 administration. Comparing the 15-hour ABT pretreatment (Fig. 3C) to the 2-hour treatment (Fig. 3B), it is apparent that the use of the longer pretreatment time mitigates retention of drug in the stomach to some extent. Extensive interanimal variability and delayed absorption are still apparent in ABT-treated rats.

The impact of oral ABT treatment on the oral pharmacokinetics of midazolam maleate was tested to determine whether we could repeat our findings with NVS-CRF38. After oral administration to the control group, midazolam was rapidly absorbed, reaching maximum concentrations in plasma by 15 minutes, after which the compound was rapidly eliminated (T1/2 = 1 hour). Two-hour pretreatment of rats with oral ABT increased the oral exposure of midazolam 60-fold; however, marked interanimal variability in terms of Tmax was apparent for ABT-treated animals (Fig. 4A and Table 2).

To reinforce our hypothesis that ABT has a dual role both as an inhibitor of P450 enzymes and gastric emptying, we examined the effect of ABT treatment on s.c. midazolam pharmacokinetics. We rationalized that for this study we would only observe pharmacokinetic changes attributed to inactivation of P450 enzymes. After s.c. administration, midazolam was rapidly absorbed (Tmax = 15 minutes) and eliminated (T1/2 = 1 hour). After normalization for dose, s.c. midazolam AUC values were ~10-fold higher compared with oral administration. A further 10-fold increase in s.c. midazolam AUC was observed after i.p. application of ABT (2-hour pretreatment time), and interanimal variability was low (Fig. 4B, Table 2).

Both i.p. and oral administration routes were used in these experiments for administration of ABT. In female Wistar rats, ABT reached
maximum levels in blood at 4 hours after i.p. administration, after which the drug was eliminated in a mono-exponential fashion (T1/2 = 20 hours). After oral administration the plasma concentration-time profile for ABT was more variable. Initially, ABT was rapidly absorbed, drug levels then declined and a second peak in drug concentrations was observed between 24 and 48 hours post dose. Comparable ABT pharmacokinetic profiles were observed for ABT (after oral and i.p. dosing) in male Sprague-Dawley rats. A summary of pharmacokinetic data for ABT in these studies is shown in Fig. 5 and Table 3.

Results from a noninvasive imaging method indicate marked inhibition of gastric emptying 2 hours after oral administration of ABT (Table 4). Fifty-six percent of the nutrient meal containing fluorescent beads was retained in the stomachs of ABT-treated rats, compared with only 20% in the control group. Similar findings were observed after either i.p. or oral administration of ABT (Table 4). Comparable gastric emptying rates were observed in ABT-treated and control rats when the test meal was administered 15 hours after ABT administration.

We considered whether ABT could delay intestinal transit (Fig. 6). Our ex vivo data confirmed a marked delay in gastric emptying in ABT-treated rats. Effects on intestinal transit were also observed, but should be attributed to the delayed gastric emptying. To further elucidate the mechanism of ABT-induced inhibition of gastric emptying, we conducted a gastrointestinal reflux study, examining the concentration of stomach bilirubin 1 hour after administration of s.c. ABT. In instances of normal gastrointestinal function, stomach levels of bilirubin (which is secreted into the small intestine) should be low; higher levels of stomach bilirubin are a diagnostic measure of intestinal reflux. Levels of bilirubin in the stomachs of both ABT-treated and vehicle-treated groups were highly comparable, indicating no evidence of gastrointestinal reflux (Table 5). A marked increase in stomach weight was observed over the duration of the study. Representative stomachs from the ABT and control group are provided in Fig. 7 and depict stomach enlargement (~2-fold increase by wet weight) 1 hour after s.c. administration of ABT.

### TABLE 4

Effect of pretreatment on ABT-induced inhibition of gastric emptying in vivo

<table>
<thead>
<tr>
<th>Pretreatment Time (hr)</th>
<th>2</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABT 100 mg/kg (Oral)</strong></td>
<td><em>80 ± 5</em></td>
<td><em>61 ± 19</em></td>
</tr>
<tr>
<td><strong>Vehicle</strong></td>
<td><em>44 ± 19</em></td>
<td><em>52 ± 14</em></td>
</tr>
<tr>
<td><strong>ABT-Treated</strong></td>
<td><em>67 ± 16</em></td>
<td><em>55 ± 19</em></td>
</tr>
<tr>
<td><strong>ABT 100 mg/kg (i.p.)</strong></td>
<td><em>29 ± 14</em></td>
<td><em>68 ± 24</em></td>
</tr>
</tbody>
</table>

#### Discussion

**Effect of ABT on NVS-CRF38 Oral Pharmacokinetics.** In terms of its well-characterized P450 inhibitory activity, ABT performed as expected in vivo. NVS-CRF38 drug levels in plasma were maintained for much longer in ABT-treated animals, and plasma concentrations for the O-desmethyl metabolite were decreased. The impact of ABT-pretreatment on the oral absorption of NVS-CRF38 was unexpected, leading us to consider mechanisms by which ABT may impede drug absorption.
Inhibition of Gastric Emptying. We considered the hypothesis that ABT inhibits gastric emptying in the rat, thus delaying absorption of NVS-CRF38. Some evidence of ABT-induced retention of stomach contents has been reported, Town’s study reveals that ~50% of 14C-ABT remains in the stomachs of rats 6 hours after oral administration (Town et al., 1993). Our gastric emptying studies, using a noninvasive technology (Gremlich et al., 2004), demonstrate that ABT inhibits gastric emptying in rats. Despite many literature reports describing coadministration of ABT with drugs or research compounds, ABT-induced gastric retention has not been widely reported. For example, ABT treatment has been reported not to alter the absorptive properties of propranolol, metoprolol, or cimetidine in rats (Caldwell et al., 2005). Consistent findings (i.e., no impact on drug absorption) have been reported for ABT interactions with midazolam and fexofenadine in rats (Strelevitz et al., 2006). Comparison of our methodology with these literature reports highlights differences in study design. Animals used in our experiments had free access to food and water, whereas literature reports usually involve fasted animals. It is plausible that the ABT-induced delay of gastric emptying may be more apparent in fed animals, due to the presence of undigested food that may impede transit. We rationalized that in fasted animals a liquid formulation may be less retained and pass into the upper intestine relatively easily. However, because the NVS-CRF38/ABT interaction was reproduced in both the fed and fasted states, we can discount this hypothesis.

ABT-pretreatment time differs between our study and many literature reports. Largely based on observations with antipyrine/ABT interactions (Balani et al., 2002), we felt a preincubation time of 2 hours to be sufficient for ABT absorption, tissue distribution, and efficient inactivation of P450 enzymes. Longer pretreatment times are often reported in literature studies; administration of ABT on the afternoon/evening prior to a pharmacokinetic study the following morning is not uncommon. We hypothesized that ABT-induced delay of gastrointestinal transit may be more apparent with shorter pretreatment times. Our findings with NVS-CRF38 partially support this hypothesis, as the delayed absorption was partially recovered by increasing the pretreatment time from 2 to 15 hours. However compared with the control group, delayed NVS-CRF38 absorption was still apparent with the longer pretreatment time, and interanimal variability was extensive.

Potential Applications for ABT as an In Vivo P450 Inhibitor. We repeated ABT interaction studies with midazolam maleate to determine whether we could reproducibly find our results with a second compound. It should be noted that both the physiochemical and pharmacokinetic properties of NVS-CRF38 and midazolam are quite different. Midazolam maleate is highly water-soluble and undergoes extensive P450-mediated metabolism in the liver. NVS-CRF38 is less soluble in comparison and has lower P450-mediated hepatic extraction. Our studies have revealed that ABT treatment impairs the absorption of midazolam (characterized by a marked shift in T_max) and increases interanimal variability. One potential application for in vivo ABT is as a tool compound to maximize the exposure for metabolically labile tool compounds. This approach would be most applicable to compounds that are rapidly absorbed but undergo extensive (and P450-mediated) metabolism, such as midazolam. Results obtained with orally administered midazolam indicate that ABT treatment will contribute to variability in blood levels, potentially contributing to variability in drug effect. We rationalized that gastric effects of ABT could be mitigated by dosing both ABT and midazolam via administration routes other than oral. This study design proved highly effective; midazolam absorption was efficient, low variability was observed among animals, and overall midazolam exposure was increased ~140-fold when compared with the vehicle-treated group. For rat pharmacokinetic studies with combined administration of oral midazolam and ABT, we selected a midazolam dose of 2 mg/kg. This dose was selected to avoid excessive sedation from the anticipated interaction. The s.c. midazolam dose was minimized further to 0.5 mg/kg, as higher blood levels of midazolam were anticipated due to the combined effects of first-pass liver extraction and ABT inhibition. The midazolam dose in the control group was increased to 7.4 mg/kg to ensure that blood concentrations were detectable by LC-MS/MS analysis. In making these dose changes we assumed that exposure of midazolam would be linear over the dose range. Vuppugalla’s study reports over proportional increases in midazolam AUC with oral doses between 1–5 mg/kg, which attenuated the degree of inhibition by ketoconazole (Vuppugalla et al., 2012). Potentially nonlinear oral pharmacokinetics for midazolam could also attenuate the degree of inhibition observed in this study with midazolam and ABT. We tabulated oral AUC values in rats from several other studies for which oral doses ranged between 1–20 mg/kg and plotted dose-normalized AUC against dose (Supplemental Table 1). For a given dose level, the midazolam AUC is highly variable. The majority of literature studies have been conducted at doses between 5–20 mg/kg. In this range there does not

**Table 5**

<table>
<thead>
<tr>
<th>Group</th>
<th>Bilirubin Concentration (μM)</th>
<th>Stomach Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>Stomach</td>
</tr>
<tr>
<td>ABT-treated</td>
<td>0.87 ± 0.31</td>
<td>13.1 ± 3.7</td>
</tr>
<tr>
<td>Vehicle-treated</td>
<td>0.81 ± 0.25</td>
<td>10.3 ± 3.4</td>
</tr>
</tbody>
</table>

*P < 0.01 (Student’s t test) versus values in the vehicle-treated group.

Fig. 7. Male nonfasted Sprague-Dawley rats were dosed either 50 mg/kg ABT or vehicle in saline via the s.c. route at a dose volume of 2 ml/kg. At 1 hour after ABT administration animals were culled via the Schedule 1 method; stomachs were ligated and surgically removed.
appear to be an obvious trend for nonlinear pharmacokinetics (just high variability between AUC values at a given dose). AUC values from lower dose levels (1 and 2.5 mg/kg) may reflect nonlinear pharmacokinetics, but given the high variability observed over the dose range it is difficult to be certain about this. Due to uncertainty about the linearity of oral midazolam pharmacokinetics, comparisons of oral AUC values for midazolam in ABT-treated rats and the vehicle-treated group should be treated with caution as an absolute measure of ABT-induced inhibitory activity.

Given that higher exposure of 1'-hydroxymidazolam was observed in ABT-treated rats compared with the control group, we speculate that ABT inhibits both the formation of 1'-hydroxymetabolism and the subsequent biotransformation of this species to the 4'-hydroxy-1'-hydroxymethyl derivative (Woo et al., 1981).

What is the Mechanism for Inhibition of Gastric Emptying? Short-term studies with ABT reveal profound effects on stomach physiology; s.c. application of ABT to rats doubles stomach weight by 1 hour after dosing. These animals were maintained on grids and had no access to food or water; stomach weight increases cannot therefore be associated with consumption of food, water, or feces. Bilirubin levels in stomach contents of treated and control rats were highly comparable, ruling out the involvement of a reflux process. An ABT-induced secretory effect in the stomach is worthy of consideration as a mechanism for the increase in stomach weight. Evidence in the kidney highlights a link between P450 inhibition and ion channels involved in water retention. Treatment of rats with ABT has been shown to reduce renal excretion of sodium in response to elevated renal tubule pressure. It is proposed that this effect is attributed to blockade of P450-mediated arachidonic acid metabolism, decreased Na+-K+-ATPase activity, and internalization of NHE-3 protein from the brush border of the proximal tubule (Dos Santos et al., 2004). We speculate that altered arachidonic acid metabolism, decreased Na+-K+-ATPase activity, and the subsequent impact of water retention in the stomach are potential mechanisms for ABT-induced stomach enlargement and the altered gastric emptying rate. Further studies are required to explore these mechanisms.

Guidance for Application of ABT as a Tool Compound for Pharmacology/Toxicology Studies. For these studies different rat strains and genders were used to explore interactions between the test compounds and ABT. Our rationale for using different strains was attributed to the potential application we envisaged in each case. For CRF, antagonists our primary interest was to use the ABT approach to design mechanistic safety studies, Wistar rats were selected to match the strains/genders used in toxicity studies. For studies with midazolam we explored the potential of the in vivo ABT approach to enable pharmacology studies for metabolically labile compounds. For this application we considered male Sprague-Dawley rats to be the most appropriate strain; in our experience they are most commonly used for pharmacology studies. Our results indicate some differences in the pharmacokinetic profiles for ABT between Sprague-Dawley and Wistar rats, and these may account for differences observed between the ABT interactions with NVS-CRF38 and midazolam.

The use of ABT as an in vivo tool compound to block P450-mediated drug metabolism offers promise as a method to either maximize the exposure of highly metabolized compounds or, alternatively, block the formation of drug metabolites in vivo. Based on our observations, we would recommend careful selection of administration routes for ABT and the test compound, because impaired and highly variable oral absorption can be expected, due to a disturbance of gastrointestinal transit. When applying this approach for pharmacology studies it is important to include appropriate controls to test the effect of ABT on a given pharmacokinetic readout.

Acknowledgments
The authors thank the in vivo studies group for assistance in animal studies. Thanks in addition to Dr David Ledieu, Dr Martina Stirn, and Hansjoerg Zeller from the preclinical safety group for analytical support with respect to bilirubin analysis.

Authorship Contributions
Participated in research design: Stringer, Weber, Tigan, Lavan, Medhurst, Sohal.
Wrote or contributed to the writing of the manuscript: Stringer, Weber, Tigan, Sohal.

References

Address correspondence to: Rowan A. Stringer, Novartis Institutes for Biomedical Research, Wimbleshurst Road, Horsham, West Sussex, RH12 5AB, UK. E-mail: rowan.stringer@novartis.com