1-aminobenzotriazole modulates oral drug pharmacokinetics through cytochrome \textit{P}450 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, Wimblehurst Road, Horsham, West Sussex, RH12 5AB, UK – RS, EW, PL, SM, BS

Global Imaging Group, Novartis Pharma AG, P.O. Box, CH-4002, Basel, Switzerland – BT
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Corresponding Author:
Rowan A. Stringer,
Novartis Institutes for Biomedical Research
Wimblehurst Road
Horsham
West Sussex
RH12 5AB
UK
Tel: +44 (0) 1403 272827
Fax: +44 (0) 1403 323307
Email: rowan.stringer@novartis.com

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Non-standard abbreviations:
ABT, 1-aminobenzotriazole; AUC, area under the concentration-time curve; C\text{max}, Highest drug concentration observed in plasma after administration of extravascular dose; P450, cytochrome P450; LC-MS/MS, liquid chromatography tandem mass spectrometry; T\text{max}, Time at which highest concentration occurs after extravascular dose.
Abstract

The simultaneous effect of the cytochrome P450 inhibitor 1-aminobenzotriazole (ABT) on inhibition of in vivo metabolism and gastric emptying was evaluated with the test compound NVS-CRF38, a novel CRF₁ antagonist with low water solubility, and the reference compound midazolam with high water solubility in rats. Pre-treatment of rats with 100 mg/kg oral ABT administered 2 hours prior to a semi-solid caloric test meal, markedly delayed gastric emptying. ABT increased stomach weights by 2-fold, this is likely to be attributed to a pro-secretory effect because stomach concentrations of bilirubin were comparable in ABT and control groups. ABT administration decreased the initial systemic exposure of orally administered NVS-CRF38 and increased T\text{max} 40-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 co-administered compounds can be expected due to a disturbance of gastrointestinal transit.
Introduction

1-aminobenzotriazole (ABT) is a well-established time dependant 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 (Furnes and Schlenk, 2005; Schulz-Utermoehl, et al., 2010; Dalmadi, et al., 2003). ABT is generally considered as a non-specific 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 benzyne which forms an NN bridged adduct on the P450 porhyrin 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 (e.g. changes in body weight, food consumption or clinical appearance) being observed after 100 mg/kg/day dosing for 13-weeks to male Sprague-Dawley rats (Meschter, et al., 1994). The effects of repeat dosing ABT at a dose which 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 pre-treatment as a method to increase the exposure of co-administered molecules. 2 hour pre-treatment 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 chloroxazone 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 pre-treatment has been expanded to pharmacology and toxicology studies. In mice intra-peritoneal (i.p.) administration of ABT increases brain levels of flurazepam, offering protection against pentylenetetrazole induced convulsions (Capello, et al., 1990). Co-administration of ABT with the bisphosphonate U-91502 increases drug exposure by 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).

Despite many literature studies which have investigated the utility of ABT to block drug metabolism in vivo, few describe 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, Figure 1), a P450-metabolized novel low molecular weight CRF₁ antagonist with excellent physicochemical (MW = 351, CLOGP = 2.7, PSA = 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., in press). A non-invasive 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. 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) (>99% as free base) and 4-(7-3,5-dimethyl-1H-1,2,4-triazol-1-yl-2,6-dimethylpyrazolo[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, UK. Animals were housed at 24°C in a 12-hour light–dark cycle. Female Wistar rats (210 to 266 g) and male Sprague-Dawley rats (229 to 419 g) were purchased from Charles River laboratories. 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 pre-treatment 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 via the p.o. route. 2 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. 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, fed versus fasted animals. A second NVS-CRF38 study was conducted to test the effect of 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).

**Midazolam p.o./ABT p.o. 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). 2 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 72 hours (0.25, 0.75, 1.5, 3, 6, 24, 29, 48, 53 and 72 hours).
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 2000 (xg) for 10 minutes. 100 μL of plasma supernatant was transferred to a microtitre plate and frozen at -80°C prior to bioanalysis. Following removal of each blood sample an equal volume of heparinized 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 DMSO and adding to control plasma. 12 calibration standards were prepared between 2.4 nM and 5000 nM. To ensure 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. 50 μL of either plasma sample or calibration sample were dispensed into a 96-well plate containing 150 μL of acetonitrile, the precipitated samples were mixed at room temperature for 10 minutes and centrifuged at 2000 xg for 15 minutes. 120 μL of supernatant was removed into a separate 96-well plate, 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. 5 μL of sample was injected onto the LC-MS/MS system. LC-MS/MS analysis was performed using a Quattro Premier mass spectrometer and Acquity UPLC system (Waters, 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 x 2.1 mm; Waters, UK). 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 transitions were 326.1→291.1 m/z (Cone voltage 40 V and collision energy 25 eV) for midazolam, 342.1→324.2 m/z (Cone voltage 40 V and collision energy 25 eV) for 1'-hydroxymidazolam, 134.8→79.6 m/z (Cone voltage 30 V and collision energy 15 eV) for ABT and 343.0→308.2 m/z (Cone voltage 40 V and collision energy 25 eV) for the internal standard triazolam. The lower limit of quantification for midazolam and 1'-hydroxymidazolam were 2 nM. The dynamic range for both compounds was 2 to 5000 nM. For NVS-CRF38/ABT studies parent to daughter mass transitions were 352.2→255.1 m/z (Cone voltage 45 V and collision energy 25 eV) for midazolam, 338.2→241.1 m/z (Cone voltage 40 V and collision energy 25 eV) for O-desmethyl NVS-CRF38, 134.8→79.6 m/z (Cone voltage 30 V and collision energy 15 eV) for ABT and 494.2→369.1 m/z (Cone voltage 25 V and collision energy 15 eV) for the internal standard glyburide. The lower limit of quantification for NVS-CRF38 and O-desmethyl NVS-CRF38 were 8 and 7 nM respectively, the dynamic range for the assay was 8 to 16667 nM for NVS-CRF38 and 7 to 550 nM for O-desmethyl NVS-CRF38.

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, firstly to keep peak responses within the linear range of the LC-MS/MS system and secondly to minimize the amount of organic solvent in the final sample – this was required to maintain 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 to 500 µM. For all analytes coefficients of determination (R²) describing the calibration curves did not fall below 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**

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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 to 210 g) were maintained on a 12 hour light / 12 hour dark cycle (lighting from 6 a.m. to 6 p.m.) 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). 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 infra-red fluorescence (NIRF) measurements of gastric emptying were performed 30 minutes after the meal. For NIRF imaging, the animals were temporarily anaesthetized with isoflurane (1.5% vapor concentration in nitrous oxide:oxygen 2:1; Forene®, Abbott, 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; the animals being placed in left lateral position exposing 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, a GE ‘eXplore Optix™’ 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 (TD-detector) at 700 nm. Data was acquired using the GE eXplore Optix™ acquisition software. NIRF-images have been analyzed quantitatively using the GE eXplore 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 to 210 g). 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 test meal, and the stomach and small intestine from the gastric pylorus to the caecum were removed immediately. The gastrointestinal tract was then cut into 12 segments of equal length and the fluorescent signal in each sample was determined by using an optical imaging system (Biospace Lab Photon Imager, France). Data were expressed as the percentage of fluorescence intensity per segment (stomach; small bowel segments 1–10; caecum) 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 non-fasted Sprague-Dawley rats (n=6, weight range 200 to 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. 1 hour after ABT administration animals 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 Roche Cobas 6000® Analyzer. 2 µL of either plasma or homogenized stomach sample were added to 124 µL of hydrochloric acid (120 µM)
and 25 µL of 3,5-dichlorophenyl diazonium salt (1.5 mM). The sample was incubated at room temperature for 10 minutes and the level of azobilirubin quantified photometrically at 546 nm. Bilirubin levels were determined against a standard curve over the range 2 to 650 µM.
Results

Treatment of rats with ABT was evaluated as a method to inhibit metabolism of NVS-CRF38. 2 hour oral ABT pretreatment profoundly impaired oral absorption of NVS-CRF38 (Figure 2a, Table 1). NVS-CRF38 plasma levels in ABT treated rats were markedly lower compared to the control group for the first 6 hours then increased steadily reaching maximal levels ~30 hours post dose, and drug levels were only marginally reduced by 72 hours. For the ABT treated group blood concentrations at the last time point (72 hours) were ~6000 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 the O-desmethyl NVS-CRF38 were just detectable between 0 and 6 hours of the study (range 1 to 3 nM), then increased over the time-course reaching maximum levels in plasma at 72 hours. The $AUC_{0→72\ hr}$ for the phenolic metabolite was 3.6-fold lower compared to the control group (Figure 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 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 $C_{\text{max}}$ by ~32 hours. In the fasted group highly comparable results were observed (Figure 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.
the 15 hour ABT pretreatment (Figure 3C) to the 2 hour treatment (Figure 3B) it is apparent that the use of the longer pretreatment time mitigates retention of drug in the stomach to some extent. Extensive inter-animal variability and delayed absorption is 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 (T½ = 1 hour). 2 hour pretreatment of rats with oral ABT increased the oral exposure of midazolam ~60-fold, however marked inter-animal variability in terms of T_max was apparent for ABT treated animals (Figure 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 rationalised that for this study we would only observe pharmacokinetic changes attributed to inactivation of P450 enzymes. After s.c. administration midazolam was rapidly absorbed (T_max = 15 minutes) and eliminated (T½ = 1 hour). After normalization for dose, s.c. midazolam AUC values were ~10-fold higher compared to 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 inter-animal variability was low (Figure 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 4 hours after i.p. administration, after which the drug was eliminated in a mono-exponential fashion (T½ = 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 are observed for ABT (after p.o. and i.p. dosing) in male Sprague-
Dawley rats. A summary of pharmacokinetic data for ABT in these studies is shown in Figure 5 and Table 3.

Results from a non-invasive imaging method indicate marked inhibition of gastric emptying 2 hours after oral administration of ABT (Table 4). 56% of the nutrient meal containing fluorescent beads was retained in the stomachs of ABT treated rats compared to 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 (Figure 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 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 stomach 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 Figure 7 and depict stomach enlargement (~2-fold increase by wet weight) 1 hour after s.c. administration of ABT.
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 $^{14}$C-ABT remains in the stomach of rats 6 hours after oral administration (Town, et al., 1993). Our gastric emptying studies, using a non-invasive technology (Gremlich, et al., 2004), demonstrate that ABT inhibits gastric emptying in rats. Despite many literature reports describing co-administration of ABT with drugs or research compounds, ABT induced gastric retention has not been widely reported. For instance ABT treatment has been reported not to alter on the absorptive properties of propranolol, metoprolol and cimeditine in rats (Caldwell, et al., 2005). Consistently findings (i.e. no impact on drug absorption) have been reported for ABT interactions with midazolam and fexofendine 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 use 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 rationalised that in fasted animals a liquid formulation may be less retained and pass into the upper intestine relatively easily. However since the NVS-CRF38/ABT interaction was reproduced in both the fed and fasted state, 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 pre-incubation 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 to the control group delayed NVS-CRF38 absorption was still apparent with the longer pretreatment time and inter-animal 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 reproduce our findings 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 increased inter-animal variability. One potential application for in vivo ABT is as a tool compound to maximise the exposure for metabolically labile tool compound. This approach would be most applicable to compounds which 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 pharmacological response. 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 and low variability was observed between each
of the animals, overall midazolam exposure was increased ~140-fold when compared to the vehicle treated group. For rat pharmacokinetic studies with combined administration of p.o. 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 a combined effect of by passing 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 between 1 and 5 mg/kg p.o. which attenuated the degree of inhibition by ketoconazole (Vuppugalla, et al., 2012). Potentially non-linear 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 p.o. doses ranged between 1 to 20 mg/kg and plotted dose normalized AUC against dose (Supplemental Table 1). For a given dose level midazolam AUC is highly variable. The majority of literature studies have been conducted at doses between 5 and 20 mg/kg. In this range there does not appear to be an obvious trend for non-linear 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 non-linear 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, comparison 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.

Interestingly higher exposure of 1’-hydroxymidazolam was observed in ABT treated rats compared to the control group, we speculate that ABT inhibits both the formation of 1’-hydroxymetabolism and then 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 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 faeces. Bilirubin levels in stomach contents of treated and control rats were highly comparable ruling out the involvement of a reflux process. An ABT induced pro-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 metabolism and the subsequent impact of water retention in the stomach is a potential mechanism for ABT induced stomach enlargement and altered gastric emptying rate, further studies are required to explore this mechanism.

Guidance for application of ABT as a tool compound for pharmacology/toxicology studies. For these studies different rat strains and gender 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 CRF₁ antagonists our primary interest was to use the ABT approach to design mechanistic safety studies, Wistar rats were selected to match strain/gender 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 between the pharmacokinetic
profiles for ABT between Sprague-Dawley and Wistar rats and 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 maximise 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 since 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 pharmacological readout.
Acknowledgements

We sincerely appreciate the *in vivo* studies group for their assistance in animal studies. In addition we Dr David Ledieu, Dr Martina Stirn and Mr. Hansjoerg Zeller from the preclinical safety group for analytical support with respect to bilirubin analysis.
Authorship contributions

Participated in research design: Stringer, Weber, Tigani, Lavan, Medhurst, Sohal

Conducted experiments: Stringer, Weber, Tigani, Lavan, Medhurst

Performed data analysis: Stringer, Weber, Tigani, Lavan, Medhurst, Sohal

Wrote or contributed to the writing of the manuscript: Stringer, Weber, Tigani, Sohal
References


vascular endothelial growth factor tyrosine kinase inhibitor: interspecies comparison and human enzymology. *Drug Metab Dispos* **38**:1688-1697.


Legends for Figures

FIG. 1. Structure of NVS-CRF38

FIG. 2. Plasma concentration time profiles for A) NVS-CRF38 and B) its O-desmethyl metabolite after administration of NVS-CRF38 to either ABT treated (open circles) or vehicle treated (filled circles) female Wistar rats. 100 mg/kg ABT was administered p.o. 2 hours prior to NVS-CRF38 administration at 10 mg/kg as a suspension in 0.5% methylcellulose and 0.5% Tween 80, dose volume 5 mL/kg. Data are the mean and standard deviation of four rats.

FIG. 3. Either A) fasted or B) fed female Wistar rats were treated with 100 mg/kg i.p. ABT 2 hours prior to oral NVS-CRF38 administration C) fed female Wistar rats were pretreated with 100 mg/kg i.p ABT 15 hours prior to NVS-CRF38 administration. NVS-CRF38 was formulated as a suspension in 0.5% methylcellulose and 0.5% Tween 80. Serial plasma samples were taken to 72 hours. Data are the mean and standard deviation of four rats.

FIG. 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) compared to a 2 mg/kg oral midazolam dose in the group treated 2 hours previously with 100 mg/kg oral ABT (open circles). B) Compares concentration-time profiles for 0.5 mg/kg s.c. midazolam in control rats (filled circles) compared to 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.

FIG. 5. Plasma concentration time profiles for ABT after 100 mg/kg oral (filled circles) or 100 mg/kg i.p. (open circles) administration to either A) female Wistar rats or B) male Sprague-Dawley rats. Data are taken from interaction studies with either NVS-CRF38 or midazolam maleate and expressed as the mean and standard deviation of four rats.

FIG. 6. Transit histograms for the distribution of fluorescent marker along the GI tract. Rats were treated p.o. with either 100 mg/kg ABT or vehicle (equivalent dose volume of water) 1 hour prior to test meal. After 1 hour, animals were sacrificed, and the stomach and small intestine from the gastric pylorus to the cecum removed. The GI tract was then cut into 12
segments of equal length and the fluorescent signal in each sample was determined. Results are expressed as the percentage of fluorescence intensity per segment (stomach; SB, small bowel segments 1–10; caecum) and plotted in a histogram. Data is the mean and standard deviation of 5 to 6 rats.** Denotes a significant difference (students t-test) versus values in the vehicle treated group at the P<0.01 level.

FIG. 7. Male non-fasted 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. 1 hour after ABT administration animals were culled via Schedule 1 method, stomachs were ligated and surgically removed.
Table 1

Oral pharmacokinetic parameters for NVS-CRF38 in female Wistar rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>p.o. NVS-CRF38</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No inhibitor</td>
</tr>
<tr>
<td></td>
<td>ABT (100 mg/kg p.o.)</td>
</tr>
<tr>
<td>NVS-CRF38 parameters</td>
<td></td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>10</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (hr)</td>
<td>0.9±0.4</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (nM)</td>
<td>8263±823</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0→∞&lt;/sub&gt; (nM*hr)</td>
<td>157107±22787</td>
</tr>
<tr>
<td></td>
<td>††390985±48100</td>
</tr>
<tr>
<td>O-desmethyl NVS-CRF38 parameters</td>
<td></td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (hr)</td>
<td>22±14</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (nM)</td>
<td>428±43</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0→∞&lt;/sub&gt; (nM*hr)</td>
<td>15102±1577</td>
</tr>
<tr>
<td></td>
<td>††4209±1274</td>
</tr>
</tbody>
</table>

Effect of 1-aminobenzotriazole administration on the oral pharmacokinetics of NVS-CRF38 in female Wistar rats. ABT treated animals received 100 mg/kg oral ABT dissolved in water, the control animals received an equivalent dose of water. After 2 hours 10 mg/kg NVS-CRF38, formulated as a suspension in 0.5% methylcellulose and 0.5% Tween 80 was orally administered to all rats. The dose volume for both compound and inhibitor was 5 mL/kg. Blood samples were taken to 72 hours and analysed by LC-MS/MS. Data are the mean and standard deviation of four rats. †, ††Denote significant differences (students t-test) versus values in vehicle treated groups at the P<0.05 and P<0.01 levels respectively.
**Table 2**

*Effect of 1-aminobenzotriazole on midazolam pharmacokinetics in rats*

<table>
<thead>
<tr>
<th>Parameters</th>
<th>p.o. midazolam</th>
<th>s.c. midazolam</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inhibitor</td>
<td>ABT (100 mg/kg p.o.)</td>
<td>No inhibitor</td>
</tr>
<tr>
<td><strong>Midazolam parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Route</td>
<td>p.o.</td>
<td>p.o.</td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>7.4</td>
<td>2.0</td>
</tr>
<tr>
<td>d.n. $C_{\text{max}}$ (nM)</td>
<td>53±40</td>
<td>†298±183</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (hr)</td>
<td>0.25±0.00</td>
<td>7±12</td>
</tr>
<tr>
<td>d.n. AUC$_{0\rightarrow t}$ (nM*hr)</td>
<td>52±29</td>
<td>††2975±855</td>
</tr>
<tr>
<td>d.n. AUC$_{0\rightarrow \infty}$ (nM*hr)</td>
<td>52±29</td>
<td>*—</td>
</tr>
<tr>
<td>$T_{1/2}$ (hr)</td>
<td>1.0±0.1</td>
<td>*—</td>
</tr>
</tbody>
</table>

**$1'$-Hydroxymidazolam parameters**

| d.n. $C_{\text{max}}$ (nM) | 20±14 | 28±11 | *— | 46±20 |
| $T_{\text{max}}$ (hr) | 0.25±0.00 | *— | **— | 3.9±2.6 |
| d.n. AUC$_{0\rightarrow t}$ (nM*hr) | 15±8 | ††344±127 | **— | 572±58 |
| d.n. AUC$_{0\rightarrow \infty}$ (nM*hr) | 15±8 | *— | **— | 704±160 |
| $T_{1/2}$ (hr) | 0.6±0.1 | *— | **— | 12±7 |

Pharmacokinetic parameters for midazolam maleate in male Sprague Dawley rats, data are the mean and standard deviation of four rats. *Due to a high degree of variability in this group half-life values and extrapolated AUC values could not be determined. **Metabolite concentrations below the limit of detection. d.n. = dose normalized to 1 mg/kg. †, ††Denote significant differences (students t-test) versus values in vehicle treated groups at the P<0.05 and P<0.01 levels respectively.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Female Wistar rat</th>
<th>Male Sprague-Dawley rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p.o. (n=4)</td>
<td>i.p. (n=4)</td>
</tr>
<tr>
<td></td>
<td>p.o. (n=4)</td>
<td>i.p. (n=4)</td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µM)</td>
<td>94±40</td>
<td>265±42</td>
</tr>
<tr>
<td></td>
<td>°268±11</td>
<td>°579±53</td>
</tr>
<tr>
<td>Tmax (hr)</td>
<td>16±16</td>
<td>4.1±1.1</td>
</tr>
<tr>
<td></td>
<td>2.7±0.6</td>
<td>°2.4±0.3</td>
</tr>
<tr>
<td>T½ (hr)</td>
<td>12.3±0.3</td>
<td>19.6±2.0</td>
</tr>
<tr>
<td></td>
<td>°7.0±0.7</td>
<td>°10.6±1.2</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0−t&lt;/sub&gt; (µM*hr)</td>
<td>3032±1557</td>
<td>7638±119</td>
</tr>
<tr>
<td></td>
<td>°5706±325</td>
<td>8265±1531</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0−∞&lt;/sub&gt; (µM*hr)</td>
<td>3153±1618</td>
<td>8241±1449</td>
</tr>
<tr>
<td></td>
<td>°5723±325</td>
<td>8359±1591</td>
</tr>
</tbody>
</table>

Pharmacokinetic parameters for ABT in rats were obtained as an integral part of midazolam/ABT and NVS-CRF38/ABT interaction studies. *2 hours prior to a 100 mg/kg p.o. dose of NVS-CRF38. **2 hour prior to a 2 mg/kg oral dose of midazolam maleate. °°2 hour prior to a 0.5 mg/kg s.c. dose of midazolam maleate. Data are expressed as mean and standard deviation of four rats. *, **Denotes significant differences (students t-test) versus values in Wistar rats at the P<0.05 and P<0.01 levels respectively.
**Table 4**

*Effect of pretreatment time on ABT induced inhibition of gastric emptying in vivo*

<table>
<thead>
<tr>
<th>Pretreatment time (hr)</th>
<th><em>ABT 100 mg/kg p.o.</em></th>
<th>*<em>ABT 100 mg/kg i.p.</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>ABT treated</td>
</tr>
<tr>
<td>2</td>
<td>80±5</td>
<td>44±19</td>
</tr>
<tr>
<td>15</td>
<td>61±19</td>
<td>52±14</td>
</tr>
</tbody>
</table>

*Rats were treated with either oral (p.o.) ABT (100 mg/kg) or vehicle (equivalent dose volume of water) 2 or 15 hours prior to test meal. **Rats were treated i.p. with either ABT (100 mg/kg) or vehicle (equivalent dose volume of water) 2 or 15 hours prior to test meal. Gastric emptying was examined 30 minutes after the meal as ratio of fluorescence activity measured in the stomach and small intestine. Data are provided as mean and standard of 6 to 8 rats. ††Denotes significant differences (students t-test) versus values in the vehicle group at the P<0.01 level.
1-aminobenzotriazole was administered via the s.c. route (dose 50 mg/kg) to male Sprague-Dawley rats. After 1 hour, animals were culled; stomachs and plasma were removed and quantified for bilirubin. Data are the mean and standard deviation of 6 rats. †Denotes a significant difference (students t-test) versus values in the vehicle treated group at the P<0.01 level.

Table 5

Gastrointestinal reflux study

<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>
Figure 1

NVS-CRF38
Figure 4

A

Dose normalized concentration (nM)

Time (hr)

0 5 10 15 20 25

B

Dose normalized concentration (nM)

Time (hr)

0 5 10 15 20 25
Figure 6

Distribution of fluorescent marker (%)

- Vehicle (Water, 5 mL/kg p.o., n=5)
- ABT (100 mg/kg p.o., n=6)