Guanfu base A, an antiarrhythmic alkaloid of *Aconitum coreanum* is a CYP2D6 inhibitor of human, monkey, and dog isoforms

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LC–MS/MS, liquid chromatography–tandem mass spectrometry; HLM, human liver microsome; MKLM, monkey liver microsome; DLM, dog liver microsome; RLM, rat liver microsome; MLM, mouse liver microsome; IS, internal standard; AUC, area under the curve; Cmax, maximum concentration; DDI, drug–drug interaction; CYP, cytochrome P450; rCYP, recombinant cytochrome P450; DM, dextromethorphan; DXO, dextrorphan; BL, bufuralol; 1OH-BL, 1-hydroxybufuralol; GFA, Guanfu base A
Abstract

Guanfu base A (GFA) is a novel heterocyclic antiarrhythmic drug isolated from Aconitum coreanum (Lèvl.) rapaics and is currently in a phase IV clinical trial in China. However, no study has investigated the influence of GFA on cytochrome P450s (CYP450s) drug metabolism. We characterized the potency and specificity of GFA CYP2D inhibition based on dextromethorphan O-demethylation, a CYP2D6 probe substrate of activity in human, mouse, rat, dog, and monkey liver microsomes. In addition, (+)-bufuralol 1′-hydroxylation was used as a CYP2D6 probe for the recombinant form (rCYP2D6), 2D1 (rCYP2D1), and 2D2 (rCYP2D2) activities. Results show that GFA is a potent noncompetitive inhibitor of CYP2D6, with Ki = 1.20 ± 0.33 μM in human liver microsomes (HLMs), and Ki = 0.37 ± 0.16 μM for human recombinant form (rCYP2D6). GFA is also a potent competitive inhibitor of CYP2D in monkey (Ki = 0.38 ± 0.12 μM) and dog (Ki = 2.4 ± 1.3 μM) microsome. However, GFA has no inhibitory activity of mice or rats CYP2Ds. GFA did not exhibit any inhibition activity on human recombinant CYP1A2, 2A6, 2C8, 2C19, 3A4 or 3A5, but has slightly inhibition of 2B6 and 2E1. Preincubation of HLMs and rCYP2D6 resulted in the inactivation of the enzyme, which was attenuated by GFA or quinidine. Beagle dogs treated intravenously with dextromethorphan (2 mg/ml) after pretreatment with GFA injection showed reduced CYP2D metabolic activity, with Cmax of dextrorphan one third that of the saline-treated group and AUC half that of the saline-treated group. This study suggests that GFA is a specific CYP2D6 inhibitor that might play a role in CYP2D6 medicated drug-drug interaction.
Introduction

Guanfu base A (Liu J H 1981) (GFA; Fig. 1) is a novel heterocyclic antiarrhythmic drug isolated from *Aconitum coreanum* (Lèvl.) rapaics (“Guanbaifu” in Chinese). In 2005, injectable GFA was approved in China for the treatment of ventricular arrhythmias (SFDA, or CFDA since 2013). In addition to being equally effective in controlling premature ventricular contraction as that of propafenone (an active comparator), intravenous GFA was shown to be more effective and safer than propafenone in a 24 hr post-infusion in phase II/III clinical studies (Yang Y.M, 2006; Yang et al., 2006). As a product approved under regulatory pathway for Chinese medicine, no information is available about GFA effects on metabolic enzyme activities or its role in drug-drug interactions. GFA was demonstrated to convert to Guanfu base I, Guanfu alcohol-amine, and GFA glucuronide and sulfate conjugates in rats (A et al., 2002). In human urine, except the former metabolites, an additional metabolite, GFA oxide was also detectable (A et al., 2003). However, metabolism of GFA and its effects on metabolic enzymes such as cytochrome P450 (CYP) isoenzymes are yet to be fully understood. In a preliminary CYP450 inhibition study, GFA appeared to be a potent inhibitor of CYP2D6.

As CYP2D6 is a key CYP isoenzyme that metabolizes multiple antiarrhythmic drugs, including propafenone (Kroemer et al., 1991), and quinidine (Ai et al., 2009; Bramer and Suri, 1999; Kobayashi et al., 1989), it is essential to understand the selectivity and extent of GFA inhibitory effects on CYP enzymes and in particular CYP2D6. CYP2D6 is one of the best studied cytochrome P450 superfamily initially reported as the enzyme responsible for the debrisoquine/sparteine polymorphism. Since the discovery in 1977 that debrisoquine and sparteine produced unexpected adverse effects in several patients at the recommended doses (Bertilsson et al., 1980; Mahgoub et al., 1977), drug–drug interactions (DDIs) and the clinical impacts have been well-documented. Some
DDI may affect elimination while other may impact overall drug exposure (de la Torre et al., 1999). Fatal toxicity could also occur due to DDIs. CYP2D6 mediated Venlafaxine-propafenone interaction may lead to hallucinations and psychomotor agitation (Gareri et al., 2008). Combination use of serotonergic agents that are substrates or metabolites of CYP2D6 can also lead to severe adverse effects (Jagestedt and von Bahr, 2004).

As CYP450 metabolism of this antiarrhythmic drug GFA is not clear, and its potential to play a role in CYP450 mediated drug-drug interactions, we have characterized the interactions of GFA with CYP450 enzymes. We found that GFA is a selective inhibitor for CYP450 2D6 in human, monkey and dog microsomes, but not rats and mice. The in vitro CYP2D6 selectivity data were confirmed with in vivo CYP2D6 probe inhibition studies in beagle dogs, in which the Cmax of main metabolite DXO was found to be one third that of the saline-treated group and AUC half that of the saline-treated group.

We report for the first time that GFA is a potent and specific noncompetitive inhibitor of CYP2D6 and has no effect on other CYP isoenzyme types. A predicted human drug-drug interaction model of GFA on dextromethorphan is presented.
Materials and Methods

Materials. Guanfu base A (GFA, 98.58%, acehytisine, CID 121446 or CID6917802) was generously provided by Prof. Liu Jinghan, Department of Phytochemistry, China Pharmaceutical University. Phenacetin, acetaminophen, coumarin, 7-hydroxycoumarin, paclitaxel, diclofenac, 4-hydroxy diclofenac, tolbutamide, 4-hydroxytolbutamide, S-mephenytoin, 4′-hydroxy mephenytoin, bufuralol, 1-hydroxybufuralol (1OH-BL), dextrorphan, chlorzoxazone, 6-hydroxy chlorzoxazone, testosterone, 6β-hydroxy testosterone, tryptamine, sertraline hydrochloride, quinidine, quercetin, sulfaphenazole, and nootkatone were purchased from Sigma-Aldrich (St. Louis, MO). Bupropion hydrochloride, hydroxybupropione, and 6α-hydroxy paclitaxel were obtained from Toronto Research Chemicals Inc. (Ontario, Canada). Dextromethorphan hydrobromide monohydrate was purchased from Adamas-beta (Shanghai, China). Ketoconazole and α-naphthoflavone were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). NADP (N0505), glucose 6-phosphate (G7250), and glucose-6-phosphate dehydrogenase (G6378) were obtained from Sigma-Aldrich. Mephenamine was obtained from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, PR China). All other chemicals and solvents used were of the highest quality and analytical grade or higher.

Human and animal liver microsomes and recombinant CYP enzymes. Supersome preparations containing recombinant CYP2D6, CYP1A2, 2A6, 2B6, 2C8, 2C19, 2D1, CYP2D2, 2E1, 3A4, or 3A5 (expressed in baculovirus-infected insect cells) plus NADPH-P450 reductase were purchased from BD Gentest (Woburn, MA). Pooled human and animal microsomes were
purchased from the Research Institute for Liver Diseases (Shanghai) Co. Ltd. (Shanghai, PR China).

**Enzyme incubation.** Incubation mixtures contained pooled liver microsomes from human or different animal species (0.2 mg protein/ml) or recombinant supersones containing rCYP2D6 (10 pmol P450/ml) and various concentrations of dextromethorphan (DM) (0-200 μM, pooled microsome systems) or bufuralol (BL) (rCYP2D6 system) in 100 mM potassium phosphate buffer (pH 7.4), containing 10 mM or 3.3 mM magnesium chloride, respectively. After incubating at 37 °C for 5 min, the reactions were initiated by the addition of an NADPH-generating system (0.5 or 1.3 mM NADP, 10 or 3.3 mM glucose 6-phosphate, and 1 or 0.4 U/ml glucose-6-phosphate dehydrogenase for pooled liver microsomes or rCYP2D6, respectively). The reaction mixtures (in 200 μl) were incubated at 37 °C for 20 min for pooled liver microsomes or 10 min for CYP2D6. The reaction was terminated by addition of 200 μl acetonitrile containing 5 ng/ml mephenamine (internal standard [IS]). Samples were subsequently centrifuged at 30,065 × g (Thermo Sovall Biofuge Stratos, Osterode, Germany) 10 min to remove protein precipitates. The products in supernatant are analyzed by liquid chromatography–tandem mass (LC–MS/MS) spectrometry.

**Inhibition studies.** To evaluate the effects of GFA on CYP isozyme selectivity, a cocktail of probe substrates was incubated with the pooled microsomes from different species and simultaneous LC–MS/MS detection was performed (please see LC–MS/MS methods below). To confirm the inhibitory effects, HLMs, purified rCYP2D6 in supersomes, or other rCYPs (rCYP2D1, rCYP2D2) were incubated with DM or BL in the presence of the test compounds, including GFA or
quinidine (up to 200 μM), as described in *Enzyme incubations* methods. All compounds used were dissolved in acetonitrile and added to the incubation mixture at a final acetonitrile concentration of ≤ 1%. The acetonitrile in the mixtures exhibit less than 5% inhibition of the DM O-demethylation to dextrorphan (DXO) and BL hydroxylation activities. To determine the Ki values for GFA and quinidine, the reactions were incubated with three substrate concentrations of BL (2, 4, and 10 μM for rCYP2D6), DM (1, 5, 20 μM) for the pooled human liver microsomes (HLMs), DM (2, 6, 15 μM) for the pooled monkey liver microsomes (MKLMs), DM (2, 5, 15 μM) for the pooled dog liver microsomes (DLMs), and serial concentrations of GFA or quinidine.

The inhibition by two concentrations of GFA (10, 100 μM) was evaluated in a panel of other recombinant CYPs, including CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2E1, 3A4, and 3A5. The incubation conditions for each enzyme were according to the instructions of BD Gentest, based on their respective probe substrates. The selective inhibitors used of each CYP isoform are as follows: α-naphthoflavone, 1A2; tryptamine, 2A6; sertraline, 2B6; quercetin, 2C8; sulfaphenazole, 2C9; nootkatone, 2C19; Clomethiazole, 2E1 and ketoconazole, 3A4/5. There were used as the positive controls. Table 1 summarizes the substrates and positive inhibitors of these enzymes.

**Testing for mechanism-based inhibition of CYP2D6 activity by GFA.** To evaluate mechanism of GFA inhibition on CYP2D6, HLMs and rCYP2D6 inhibition experiments were performed with varying concentration of GFA (0, 0.2, 0.4, 1.0, 2, or 4 μM) while fixing HLM concentration at 0.2 mg/ml. Other conditions remained the same. For the rCYP2D6 supersome system, varying GFA (0, 1, or 10 μM) concentrations were incubated with a fixed 10 pmol/ml rCYP2D6. For the HLM system, after preincubation with GFA for 0, 5, 10, 20, or 30 min, the 20 μl
mixture was mixed with 180 μl DM in 100 mM potassium phosphate buffer (pH 7.4). For the rCYP2D6 system, after preincubation with GFA for 0, 10, 20, or 30 min, the 50 μl mixture was mixed with 50 μl BL containing the NADPH-generating system (as described in Enzyme incubation) 100 mM potassium phosphate buffer (pH 7.4). The final substrate concentrations were 50 μM for DM and 20 μM BL and the inhibitor concentrations were 0, 0.02, 0.04, 0.1, 0.2 and 0.4 μM for the HLM system and 0, 0.5, and 5 μM for the rCYP2D6 system. CYP2D6 activities are estimated based on degrees of dextromethorphan O-demethylation and bufuralol hydroxylation per minutes. To evaluate whether enzyme activity reduction is caused by the denaturation of the enzyme, phenacetin (70 μM) is added to the incubation system to probe the activity of CYP1A2.

**Drug-drug interaction study in Beagle dogs.** Equal number male and female Beagle dogs, [Beijing Marshall Biotechnology Co. Ltd (Beijing, China)] enrolled in this study, were housed in a standard animal laboratory (temperature 22–25 °C, humidity 30%–70%) with a 12 h light/dark cycle. All experimental were performed under an protocol approved by the China Pharmaceutical University Animal Ethics Committee in accordance with the Guidelines for Animal Experimentation of Southeast University (Nanjing, China).

DM was used as substrate in vivo study(VandenBrink et al., 2012). Blood samples were obtained before drug administration as a baseline measure. After the dogs were fasted overnight for at least 12 h, with free access to water, they were intravenously administered 10 mg/kg (1 ml / kg) GFA or sodium saline. After 30 min, the dogs were treated with 2 mg/ml (1 ml / kg) DM to determine the change in CYP2D activity in vivo. Blood samples were collected at 2, 5, 15, 20, 30, 45 min and 1, 2, 4, 6, 8, 12, 24, and 36 h. The blood samples were immediately centrifuged at
1485 × g for 5 min. The plasma samples in polypropylene tubes were stored at –20 °C until LC–MS/MS analysis.

**LC–MS/MS assay.** The drugs in plasma and microsomes were analyzed with an LC-MS/MS system fitted with a SPD-20A UFLC System (Shimadzu, Tokyo, Japan) coupled to an API 4000 tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an electro spray ionization interface. Aliquots were injected onto a reversed-phase Phenomenex Luna C18 column (2.0 × 150 mm, 5 μm; Torrance, CA, USA) at a flow rate of 0.5 ml/min. The mobile phase consisted of aqueous 0.01% acetic acid with 5 mM ammonium acetate (A) and 50:50 (v/v) methanol:acetonitrile (B). The following gradient elution was applied: 0–0.5 min, 2% B; 0.5–4.0 min, increase B to 45%; 4.0–6.5 min, increase B to 60%; 6.5–6.8 min, increase B to 80%; 6.8–7.2 min, 80% B; 7.2–7.5 min, reduce B to 2%; for a run total time of 10.0 min. The mass spectrometer conditions were as follows: 5500 V (positive mode) or –4500 V (negative mode); gas 1 (N2), 65 arbitrary units (arb); gas 2 (N2), 70 arb; collision gas (N2), 10 arb; curtain gas (N2), 30 arb; entrance potential, 10 V; collision cell exit potential, 12 V. Quantitation was performed in the multiple reaction monitoring mode. The optimized transitions and parameters of the analytes are shown in **Table 2**. The assay is robust, reproducible and day-to-day variation is validated to be less than 10% covariance. (Chromatograms were shown in **Supplemental Figure 1**, validation of cocktail incubation were shown in **Supplemental Table 1, Table 2**). To detect DXO and DM in the dog plasma, the LC–MS/MS method was similar to that used *in vitro*, with some modifications, and with m/z transitions of 272.1→215.0 for DM, 258.1→157.0 for DXO, and 180.0→110.0 for IS.
Enzyme kinetics analysis. Based on the two concentrations of CYP enzyme product detected by LC-MS/MS assay, the enzyme activity was calculated and expressed in picomoles of DXO or 1-OH-BL formed per picomole of CYP2D6 per minute. The Michaelis-Menten kinetics parameters were calculated according to the relating reaction rate $V$ to $[S]$, the concentration of a substrate.

$$V = \frac{V_{\text{max}}[S]}{K_m + [S]} \quad \text{eq 1}$$

The velocity data for dextromethorphan O-demethylation or bufuralol 1'-hydroxylation in the absence and presence of GFA were estimated by derivative-free iterative nonlinear least-squares regression. IC50 were determined graphically using curves of mean enzyme activity vs. inhibitor concentration by Microsoft Excel (Redmond, WA).

The modes of inhibition and the apparent Ki values were determined by nonlinear regression using equations below for competitive (eq2), noncompetitive (eq3), or mixed competitive (eq4) with GraphPad (Prism® Version 4.03, GraphPad Software, Inc.; San Diego, CA) (Webb, 1963):

$$V = \frac{V_{\text{max}}[S]}{K_m(1 + I/K_i) + [S]} \quad \text{eq 2}$$

$$V = \frac{V_{\text{max}}[S]}{K_m(1 + I/K_i) + [S](1 + I/K_i)} \quad \text{eq 3}$$

$$V = \frac{V_{\text{max}}[S]}{K_m(1 + I/K_i) + [S](1 + I/K_i)} \quad \text{eq 4}$$

where $V$, $V_{\text{max}}$, $[S]$, $K_m$, $K_i$, $K_i'$ and $[I]$ are the velocity, maximal velocity, substrate concentration, Michaelis constant, dissociation constant for the enzyme-inhibitor complex, dissociation constant for the enzyme-substrate-inhibitor complex, and inhibitor concentration, respectively.
Akaike’s information criterion was used as a measure of the goodness of fit. The mode of inhibition was verified by visual inspection of Lineweaver–Burk plots, Dixon plot and Cornish–Bowden plot of the enzyme kinetics data.

**Modeling and Molecular Docking.** The human CYP2D6 with the crystal structures was downloaded from the Protein Data Bank (PDB ID:3TBG, DOI:10.2210/pdb3tbg/pdb). Homology modeling and molecular docking were used to predict the modes of GFA and quinidine binding to animal and human CYP2D. The homology models of CYP2D from rat(CYP2D1,2), were produced using SWISS-MODEL workspace based on the template of 3TBGA(chain A) (Arnold et al., 2006; Guex and Peitsch, 1997; Schwede et al., 2003). Active site of the protein was predicted with SiteMap module (version 2.3, Schrödinger LLC, New York, NY, 2009) using identity top-ranked potential receptor binding sites to generate 15 possible binding orientations. Docking studies were performed using the program CDOCKER with the highest SiteScore for GFA and quinidine.

**Pharmacokinetic prediction and Simcyp ADME Simulator.**

The pharmacokinetic profiles of GFA at 10 mg/kg was simulated to predict possible drug-drug interaction based on our previous study at 4 mg/kg in dog and human (Sun Jianguo, 2012; Wang G.G, 2000; Yang XJ, 2000). The pharmacokinetic parameters of DM and DXO in dogs were calculated using WinNonlin version 6.1 (Pharsight, Mountain View, CA, USA), according to a noncompartmental model. The possible of DDIs is roughly evaluated according to the predictive function [I]/Ki, where [I]/Ki > 1 suggests a high DDI risk (Ito et al., 2004; Obach et al., 2006). According to the metabolism pathway of DM by CYP2D6, only one pathway subject to inhibition and the DDI was predicted with the following equation(eq 5) (Ito et al., 2005).
A direct measure of the importance of CYP2D6 (that is, \( f_mCYP2D6 \)) can be made by comparison of the poor and extensive phenotypes (Schmid et al., 1985; Yeh et al., 2003), and in this case, \( f_mCYP2D6 \) is 0.96 for DM (Nakashima et al., 2007). \( K_i \) is the inhibition constant, and \([I]\) is the maximum inhibitor concentration, and the unbound concentration was used here for prediction.

The commercially available PBPK software packages SimCYP (Version 13.1.61.0, SimCYP Ltd., Sheffield, United Kingdom) was widely used for drug DDI prediction (McGinnity et al., 2008; Shardlow et al., 2013; Xu et al., 2009; Youdim et al., 2008). In this study, the DDI potential of GFA on substrate of CYP2D6 in human was predicted based on a physiologically based pharmacokinetics (PBPK) model simulated by SimCYP at 4 mg/kg according to clinical dose.

SimCYP was used to simulate a drug interaction trial involving 1 trial of 10 subjects per trial, with 2 mg/kg DM i.v coadministered with 4 mg/kg or 10 mg/kg of GFA by iv bolus or infusion. The major metabolite DXO was also simulated in the trail. The parameters used in the simulation was listed in results (Table 3).
Results

Effects of Guanfu base A on different CYP450 isoenzymes

To assess its interaction potential, the inhibitory effects of GFA on different CYPs in HLM or rCYP2D6 were measured. The formation of DXO from DM or the 1-hydroxylation of BL was determined with LC–MS/MS. As presented in Figure 2, GFA is a potent inhibitor of CYP2D6, with a 50% inhibitory concentration (IC₅₀) recorded at ∼0.46 μM in HLM (DM 5 μM, Fig 2A) and 0.12 μM in rCYP2D6 (BF 5 μM, Fig 2B). However, GFA showed no inhibition on other CYPs (Fig. 2A).

To assess whether and to what extent Guanfu base A (GFA) inhibit specific cytochrome P450 isoenzyme activity, specific recombinant human CYP isoenzymes were incubated further with either 10 or 100 μM. The human recombinant CYP450 isoenzymes 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2E1, 3A4 and 3A5 were evaluated with respective probe substrates as well as probe inhibitors to validate the specific CYP isoenzyme assay. As shown in Figure 3, other than CYP2B6 and CYP2E1, none of the isoenzymes’ activity was inhibited by GFA. At high concentration, 100 μM GFA, 40% of CYP2B6 and CYP2E1 activity was inhibited (Figure 3).

Collectively, these data indicate that GFA is a potent CYP2D6 inhibitor and can partially inhibit 2B6 and 2E1 at 100 μM(high concentration), and no significant inhibition at 10μM(low concentration). Therefore, the following experiments focus on GFA inhibitory effects on CYP2D6 isoenzyme.

Characterization of GFA inhibitory effects on CYP2D6 isoenzyme activity

We next determine whether there is specie specificity on GFA inhibitory effects on CYP2D6.

To do so, dose-dependent GFA CYP2D6 inhibitory effects in liver microsomes from different
animal species were also evaluated. As with human data, GFA potently inhibited the CYP2D6 orthologs in monkey and dog, with IC\textsubscript{50} recorded at \textasciitilde0.45 \textmu M and \textasciitilde9 \textmu M, respectively. However, we found that GFA did not inhibit rat or mouse orthologues of human CYP2D6 (Figure 4), but has slightly inhibition on CYP2E1, this might be due to the species different (Supplemental Figure 2). The CYP2D6 IC\textsubscript{50} values for GFA were further evaluated in purified recombinant proteins in supersomes. The IC\textsubscript{50} of GFA for rCYP2D6 was recorded at 0.1 \textmu M compared to 0.028 \textmu M for the probe drug quinidine (Figure 5). Rat CYP2D1 and CYP2D2 are considered to be orthologues of human CYP2D6 that expressed in the liver (Hiroi et al., 1998; Komori, 1993; Wyss et al., 1995). Unlike its potent inhibitory effect on human CYP2D6, GFA had no inhibitory effect on CYP2D1 or CYP2D2 (Figure 5).

Taken together, these data confirmed that GFA inhibitory effects are mainly restricted to CYP2D6 in human and similar degree potency for the orthologue in the dog and monkey, but not rat and mice.

**Kinetic analysis of GFA inhibit effects on CYP2D6 activity**

To characterize the CYP2D6 enzyme kinetics of GFA inhibition, the reaction velocities were assessed in the HLM system at three substrate concentrations (1, 5, and 20 \textmu M of DM) in the absence and presence of GFA (0, 0.2, 0.5, 1, 2, or 5 \textmu M). In the rCYP2D6 system, the reactions were performed with three substrate concentrations (2, 5, and 10 \textmu M of BL) in the absence and presence of GFA (0, 0.04, 0.2, 1, or 4 \textmu M). The data obtained for the dextromethorphan O-demethylation velocities mediated by HLMs (0.2 mg protein/ml) and the velocities of the 1-hydroxylation of BL mediated by rCYP2D6 in the presence of GFA both best described a
noncompetitive inhibition model. The initial rate of product formation in varying concentrations of GFA was analyzed with a Dixon plot (Fig. 6a and Fig. 7a) and a Cornish–Bowden plot (Fig. 6b and Fig. 7b). Based on Dixon and Cornish-Bowden analysis in Figure 5 and 6, GFA is a noncompetitive inhibitor of CYP2D6, with Ki estimate of $1.20 \pm 0.33 \mu M$ and $0.37 \pm 0.16 \mu M$ in HLMs and rCYP2D6, respectively. To compare the mode of GFA inhibition, the Ki values were further evaluated in DLMs and MKLMs. The results in table 4 showed that GFA is a competitive inhibitor of CYP2D in DLMs and MKLMs, with Ki = $2.4 \pm 1.3$ and $0.38 \pm 0.12 \mu M$, respectively. As the positive control, quinidine was shown to be a competitive inhibitor of HLMs (Ki = $0.19 \pm 0.079$) and MKLMs (Ki = $3.64 \pm 1.63$), and a noncompetitive inhibitor of DLMs (Ki =Ki’ at $2.5 \pm 1.29$) (Table 4).

**Evaluation of whether GFA inhibition on CYP2D6 is mechanism-based**

To determine whether the inhibition of CYP2D6 by GFA is mechanism based, GFA was preincubated with HLMs or rCYP2D6 in the presence or absence of NADPH. Compared with the negative control, which was preincubated without GFA, preincubation of HLMs or rCYP2D6 with GFA did not increase the inhibitory potency of the reaction (Fig. 8). Therefore, GFA seems to be a specific noncompetitive inhibitor of CYP2D6 and this inhibition is not mechanism based.

**Characterization of GFA inhibitory effect on CYP2D6 with dextromethorphan a probe drug substrate in beagle dogs**

To characterize GFA inhibitory effects on drugs that subjected to CYP2D6 dependent metabolic clearance, we evaluated pharmacokinetics of CYP2D6 probe drug dextromethorphan in
beagle dog in the presence and absence of GFA (10 mg/kg). Time-course plasma drug concentration data of O-demethylation product of DM were presented in Figure 9 and the pharmacokinetic parameters are presented in Table 5. The pharmacokinetic profile of the O-demethylation metabolite (DXO) differed significantly between the GFA- and placebo (saline) groups. After treatment with GFA, CYP2D activity was inhibited and the metabolite formed by CYP2D decreased, with Cmax one third that of the saline group and the area under the plasma concentration–time curve (AUC) half that of the saline group (Table 5, Fig. 9).

**CYP2D docking of GFA and quinidine**

To illustrate the mechanism underlying the observed specifies difference in inhibition by GFA and quinidine, molecular docking studies for homology models of CYP2D were further performed to confirm the inhibition modes for each CYP isoforms. As can be seen from the docking results, GFA and quinidine are well predicted to be located in the hydrophobic area of sitemap in CYP2D6, with the active-site residues Phe120, Glu216, Arg221, ASP301 and Phe483 (Figure 10A,B). In CYP2D2, GFA and quinidine are located outside the sitemap of hydrophobic area (Figure 10C,D). This partly showed the different binding style between human and rat CYP2D. But it can’t distinguish the difference of GFA and quinidine. Further docking with CYP2D6, 2D1 and 2D2 showed that quinidine located in the same area in CYP2D1,2D2,2D6, but GFA located in CYP2D6 quite different from CYP2D1 and 2D2 (Figure 10E,10F). Further study will be carried out to identify the species different inhibition ability of GFA and quinidine on CYP2D.

**Prediction of GFA effects on CYP2D6 dependent DDI in human**

The concentration of GFA in dog and human at 10 mg/kg was simulated based on previous study,
and the parameters used were listed in table 3. Compared with the observed data, Cmax is generally predicted quite accurate, but the AUC is overestimated since metabolism of GFA is ignored. According to the prediction equation 5 by Ito, the AUC ratio of inhibited and controlled uninhibited group was predicted by $f_{\text{in}CYP}$, [I] and $k_i$ and the results were listed in Table 6. This equation only gives an average estimation of the parent substrate and gives no information of metabolite. With the help of SimCYP, both parent substrate (DM) and metabolite (DXO) pharmacokinetic profiles can be predicted after the interaction of inhibitor (table 6). Simulation results showed that the parent substrate is not influenced much by GFA but the AUC of metabolite DXO is reduced almost 2 times, which is validated by experimental data in dogs (the experimental data of $\text{AUC}_i/\text{AUC}$ in dogs is about 0.99 for DM and 0.52 for DXO).
Discussion

GFA is a novel antiarrhythmia drug approved for human use in China. It is currently under a phase IV post-approval clinical trial for safety evaluation. GFA is not extensively metabolized by liver microsomes in vitro (Supplemental Figure 3). The GFA sulfate and glucuronide are major metabolites found in vivo (A et al., 2002). Previous reported and the data presented confirmed that P450 is not the major enzyme that regulates GFA metabolism.

In this report we have evaluated how GFA interacts with CYP450 enzymes. We found for the first time that GFA is a potent CYP2D6-specific noncompetitive inhibitor, with Ki recorded at 1.20 ± 0.33 µM and 0.37 ± 0.16 µM in HLMs and rCYP2D6, respectively. GFA is a competitive inhibitor of CYP2D6 homologue in liver microsomes of dog and monkey (with Ki = 2.4 ± 1.3 and 0.38 ± 0.12 µM, respectively), but has no inhibitory effect on mouse and rat orthologue. However, another CYP2D6 inhibitor quinidine was shown to be able to inhibit rat CYP2D as demonstrated by 2 fold increase of DM in rats (Marier et al., 2004). The mechanism underlying the observed species difference in inhibition by GFA and quinidine was further investigated by homology modeling and molecular docking. According to the study of McLaughlinsd (McLaughlin et al., 2005), at least 100-fold decreased quinidine inhibition of bufuralol 1-hydroxylation and dextromethorphan O-demethylation was found after abolition of the negative charge at either or both residues Glu216 and Asp301. Our docking results showed a different binding style between human and rat CYP2D. GFA also showed a different position in CYP2D6 from that of CYP2D1, 2D2, but quinidine showed a similar position in CYP2D6,2D1 and 2D2. The docking results suggest that the binding style of quinidine in rat might be different from that of GFA, which partly illustrated the different inhibition mechanism of GFA and quinidine on CYP2D.
The effect of 2D6 inhibition by GFA was further evaluated in beagle dogs with DM as a probe drug for drug-drug interaction. A CYP2D6 probe DM can be metabolized into the O-demethylation metabolite (DXO) as a indicator of enzyme activity (Barnhart, 1980; Shou et al., 2003). After dosing of GFA 30 min prior to DM did not influence the AUC of the parent drug DM. However, the concentration of the active metabolite DXO decreased significantly. Two third reduction in Cmax and 50% AUC reduction was attributed to GFA inhibiton of CYP2D. The inhibition effect could lead to clinical impact for some drugs with narrow therapeutic index.

Since patients with heart disease commonly also suffer from other syndromes such as hypertension, dysthymia, a combination of drugs is often prescribed to manage the disease and symptom. In such cases, DDIs can be a significant problem when the patient takes GFA with drugs that are the substrates of CYP2D6. As CYP2D6 is polymorphic enzyme, addition precaution should be taken to avoid drug-drug interactions. Clinical DDI between CYP2D6 substrate and inhibitors have been reported previously. A significant inhibition in the O-demethylation of codeine to morphine in homozygous EM of CYP2D6 treated with low-dose levomepromazine was documented (Vevelstad et al., 2009). A reported clinical DDI between flecainide and paroxetine has lead to suggestion to monitor plasma flecainide concentrations for those who are on CYP2D6 inhibitors, such as paroxetine, quinidine and possibly GFA(Tsao and Gugger, 2009).

Prior to our report, there is no DDI information of GFA on substrate of CYP2D6 in human. While it is clear that GFA is a CYP2D6 inhibitor, it is important to understand whether at clinical dose, it may significantly impact CYP2D6 enzyme activity. In healthy volunteers at i.v dose at 4 mg/kg, the C5min was 6.71 ± 1.51 μg/ml (15.6 μM). Because the protein binding ratio of GFA is 78.6%–84.6% in human plasma (unpublished data), in light of Ki of GFA on CYP2D6, the
possibility of DDIs is quite high according to the predictive functions [I]/Ki, where [I]/Ki > 1 suggests a high DDI risk (Ito et al., 2004; Obach et al., 2006). It is likely that at clinical GFA dose, it may have significant impact on CYP2D6 substrate.

To provide a tool to predict DDI, we evaluated two prediction simulation based on equation 5 and a dynamic computer based prediction of DDI in human by simCYP model. Both model assumed that an increase in the AUC of a substrate in the presence of an inhibitor of the substrate’s elimination pathway is a function of the ratio of the inhibitor concentration ([I]) to the inhibition constant (Ki) (Einolf, 2007; Obach et al., 2006; Shou, 2005). Equation 5 only gives an average estimation of the parent substrate and gives no information of metabolite. With simCYP computer simulation, both parent substrate (DM) and metabolite (DXO) pharmacokinetic profiles can be predicted with inhibitor. According to the dynamic simulation by simCYP and in vivo data in dogs, there was no apparent change in the parent drug(DM), but a significant reduction in the AUC of the metabolite(DXO) compared with that of the control(AUCi/AUC = 0.583 and 0.407 in human at 4 and 10 mg/kg, respectively) (Table 6). Therefore, in some cases, the change in the concentration of the specific metabolite might be a more accurate and sensitive index for evaluating DDI. As the metabolite from CYP2D6 is decreased after coadminstrated with GFA, activity of drugs which need to be activated by CYP2D6 will compromised.

CYP2D6 has been shown to be unstable in the presence of co-factor NADPH, detectable as a 50% activity reduction after 30 mins(Bertelsen et al., 2003; Madeira et al., 2004), which was confirmed in our experiment. We found that GFA reversed the NADPH dependent CYP2D inactivity. GFA reduced NADPH-dependent enzyme inactivity as much as 80% after 30 min preincubation. Cemitidine has been reported to have the same effect on CYP2D6 activity (Madeira
et al., 2004). This protective effect may be due to the drug’s antioxidant properties (unpublished data). In general, it has no impact on CYP1A2 (Fig. 11).

In conclusion, this study has demonstrated that GFA is a specific inhibitor of CYP2D6 and that this inhibition is noncompetitive and not mechanism based. It has therefore added to the growing list of specific CYP2D6 inhibitors that have been documented. Based on the recommended dose of GFA and its plasma drug concentration, it is likely to induce drug-drug interaction for those that are CYP2D6 substrate.
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Authors’ Contributions.

*Participated in research design:* JG Sun, Peng, Wang.

*Conducted experiments:* JG Sun, Peng, Wu, X Zhang, Zhong, Xiao, F Zhang, Qi, Shang, Zhu, Y Sun, K Liu.

*Contributed new reagents or analytic tools:* JH Liu.

*Performed the data analysis:* JG Sun, Peng, A, Ho

*Wrote or contributed to the writing of the manuscript:* JG Sun, X Zhang, Ho
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Yeh GC, Tao PL, Ho HO, Lee YJ, Chen JY and Sheu MT (2003) Analysis of pharmacokinetic parameters

Footnote

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Jianguo Sun and Peng Ying contributed equally to this research.
Figure Legends

Figure 1. Chemical structure of Guanfu base A (GFA).

Figure 2. Inhibition of CYP450 isoforms by GFA in HLMs (0–100 μM) (A), rCYP2D6 (0-20 μM) (B). The activities of each isoform were measured by isoform-specific substrate reaction probes at their approximate or lower respective Km values (Supplemental Table 1). The data represent the averages of four times (HLMs) or duplicated (rCYP2D6) experiments and are expressed as the percentages of the control activity remaining.

Fig 3. The inhibitory potency of GFA (10 and 100 μM) for different recombinant P450s (each data was duplicated). The respective isoenzyme probe inhibitor was used as positive control. The concentration used for specific probe inhibitor and substrate are listed in Table 1.

Fig. 4. Inhibition of CYP450 isoforms by GFA (0–100 μM) in MKLMs (A), DLMs (B), RLMs (C), and MLMs (D). The activities of each isoform were measured by isoform-specific substrate reaction probes at their approximate respective Km values with no apparent drug-drug interaction. The data represent the averages of four repeated experiments and are expressed as the percentages of the control activity remaining.

Fig. 5. Recombinant rat CYP2D1-, CYP2D2-, and human CYP2D6-catalyzed (+)-bufuralol 1′-hydroxylation. The 1′-hydroxylated (+)-bufuralol formation rate (pmol/min/pmol of P450) from 25 μM (+)-bufuralol by CYP2D1(triangle),
CYP2D2(circle), and CYP2D6(square) in the presence of GFA(closed) or quinidine(QND, open) (0–200 μM) is expressed as a percentage of the control velocity with no inhibitor present. The data represent the averages of duplicated experiments.

Fig. 6. Determination of Ki values and types of inhibition. A, Dixon plot (1/V against i); and B, Cornish–Bowden plot (S/V against i) of dextromethorphan O-demethylation velocities mediated by HLM (0.2 mg protein/ml) in the presence of various GFA concentrations. Each line represents a concentration of dextromethorphan (circles, 1 μM; squares, 5 μM; and diamonds, 20 μM) and their intersection points determine the Ki values.

Fig. 7. Determination of Ki values and types of inhibition. A, Dixon plot (1/V against [I]), and B, Cornish–Bowden plot (S/V against [I]) of (+)-bufuralol 1’-hydroxylation velocities mediated by rCYP2D6 (2 pmol/ml) in the presence of various GFA concentrations. Each line represents a concentration of (+)-bufuralol (circles, 2 μM; squares, 5 μM; and diamonds, 10 μM) and their intersection points determine the Ki values.

Fig. 8. Time-dependent inactivation of CYP2D6 by GFA.

A, HLMs (0.2 mg/ml), or B, rCYP2D6 (10 pmol/ml) was preincubated with buffer (control) or various concentrations of GFA (0.1, 0.2, 0.5, 1.0, or 20 μM) and an NADPH-generating system. Each point represents the mean of four measurements ± SD for HLMs and duplicate measurements for rCYP2D6. Please refer to Testing for mechanism-based inhibition of CYP2D6 activity by GFA in the text for details of the
sample processing.

Fig. 9. The inhibition effects of GFA on the metabolism of dextromethorphan (DM) in beagle dogs. Mean ± SD concentrations of DM (A) and DXO (B) after DM (2.0 mg/kg) was administered intravenously to six healthy adult beagle dogs pretreated with GFA (10 mg/kg, closed diamonds) or saline (open squares).

Fig10. Molecular modeling of GFA and CYP2D. A, the docking model of GFA and CYP2D6, the key residues and hydrophobic area of sitemap (yellow grid) are shown. B, the docking model of quinidine and CYP2D6. C, the docking model of GFA and CYP2D2. D, the docking model of quinidine and CYP2D2. E, the docking model of GFA and CYP2D1,2,6 (light blue for CYP2D6 and green for CYP2D1,2). F, the docking model of quinidine and CYP2D1,2,6 (light blue for CYP2D6 and green for CYP2D1,2).

Fig. 11. Time-dependent inactivation of CYP2D6 and CYP1A2 in HLMs. HLMs were preincubated with buffer (negative control) or an NADPH-generating system and aliquots (50 μl) were removed at 5, 10, 20, and 30 min after the initiation of the preincubation reaction and were added to reaction tubes containing DM (final concentration 5 μM) and the NADPH-generating system. The reactions (200 μl total volume) were incubated for another 15 min. Each point represents the mean ± SD of three measurements.
Table 1. Substrate and positive inhibitor of each CYP isoform.

<table>
<thead>
<tr>
<th>CYPs</th>
<th>Substrate (Concentration, µM)</th>
<th>Metabolite</th>
<th>Probe inhibitor (Concentration, µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>Phenacetin (50 µM)*</td>
<td>Acetaminophen</td>
<td>α-naphthoflavone (0.1 µM)</td>
</tr>
<tr>
<td>2A6</td>
<td>Coumarin (1 µM)</td>
<td>7-hydroxycoumarin</td>
<td>Tryptamine (2 µM)</td>
</tr>
<tr>
<td>2B6</td>
<td>Bupropion (5 µM)</td>
<td>2-hydroxybupropion</td>
<td>Sertraline (1 µM)</td>
</tr>
<tr>
<td>2C8</td>
<td>Paclitaxel (5 µM)</td>
<td>6α-hydroxypaclitaxel</td>
<td>Quercetin (0.5 µM)</td>
</tr>
<tr>
<td>2C9</td>
<td>Diclofenac (4 µM)</td>
<td>4'-hydroxydiclofenac</td>
<td>Sulfaphenazole (0.5 µM)</td>
</tr>
<tr>
<td></td>
<td>Tolbutamide (70 µM)*</td>
<td>4-hydroxytolbutamide</td>
<td>Sulaphenazol (0.5 µM)</td>
</tr>
<tr>
<td>2C19</td>
<td>Mephenytoin (1 µM)</td>
<td>4'-hydroxymephenytoin</td>
<td>Nootkatone (0.5 µM)</td>
</tr>
<tr>
<td></td>
<td>Oxyomeprazole (20 µM)*</td>
<td>5-hydroxyomeprazole</td>
<td>Ticlopidine (1.0 µM)</td>
</tr>
<tr>
<td>2E1</td>
<td>Chlorzoxazone (20 µM)*</td>
<td>6-hydroxychlorzoxazone</td>
<td>Clomethiazole (10 µM)</td>
</tr>
<tr>
<td>3A4/5</td>
<td>Midazolam (5 µM)*</td>
<td>1'-hydroxymidazolam</td>
<td>Ketoconazole (0.05 µM)</td>
</tr>
<tr>
<td></td>
<td>Testosterone (75 µM)*</td>
<td>6β-hydroxytestosterone</td>
<td>Ketoconazole (0.05 µM)</td>
</tr>
</tbody>
</table>

*: Cocktail substrate
Table 2. Optimized transitions and parameters of the analytes.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>MRM transition (m/z)</th>
<th>Declustering Potential (V)</th>
<th>Collision Energy (eV)</th>
<th>Polarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>152.0 → 110.0</td>
<td>60</td>
<td>24</td>
<td>ESI +</td>
</tr>
<tr>
<td>4-hydroxytolbutamide</td>
<td>287.0 → 188.0</td>
<td>60</td>
<td>18</td>
<td>ESI +</td>
</tr>
<tr>
<td>7-hydroxycoumarin</td>
<td>163.0 → 107.0</td>
<td>90</td>
<td>30</td>
<td>ESI +</td>
</tr>
<tr>
<td>2-hydroxybupropion</td>
<td>256.3 → 238.0</td>
<td>60</td>
<td>18</td>
<td>ESI +</td>
</tr>
<tr>
<td>6α-hydroxypaclitaxel</td>
<td>870.5 → 286.2</td>
<td>90</td>
<td>20</td>
<td>ESI +</td>
</tr>
<tr>
<td>4’-hydroxydiclofenac</td>
<td>312.0 → 266.0</td>
<td>70</td>
<td>19</td>
<td>ESI +</td>
</tr>
<tr>
<td>5-hydroxyomeprazole</td>
<td>362.0 → 196.0</td>
<td>60</td>
<td>42</td>
<td>ESI +</td>
</tr>
<tr>
<td>4’-hydroxymephenytoin</td>
<td>233.0 → 190.0</td>
<td>55</td>
<td>17</td>
<td>ESI -</td>
</tr>
<tr>
<td>1’-hydroxybufuralol</td>
<td>278.0 → 186.0</td>
<td>100</td>
<td>26</td>
<td>ESI +</td>
</tr>
<tr>
<td>Dextrophan</td>
<td>258.5 → 157.0</td>
<td>180</td>
<td>50</td>
<td>ESI +</td>
</tr>
<tr>
<td>6-hydroxychlorzoxazone</td>
<td>184.0 → 120.0</td>
<td>55</td>
<td>25</td>
<td>ESI -</td>
</tr>
<tr>
<td>1’-hydroxymidazolam</td>
<td>342.0 → 203.0</td>
<td>130</td>
<td>37</td>
<td>ESI +</td>
</tr>
<tr>
<td>4’-hydroxymidazolam</td>
<td>342.0 → 234.0</td>
<td>130</td>
<td>32</td>
<td>ESI +</td>
</tr>
<tr>
<td>6β-hydroxytestosterone</td>
<td>305.0 → 269.0</td>
<td>60</td>
<td>21</td>
<td>ESI +</td>
</tr>
<tr>
<td>6-hydroxychlorzoxazone</td>
<td>184.0 → 120.0</td>
<td>55</td>
<td>25</td>
<td>ESI -</td>
</tr>
<tr>
<td>Mephenamine (IS)</td>
<td>270.0 → 181.0</td>
<td>60</td>
<td>13</td>
<td>ESI +</td>
</tr>
</tbody>
</table>
Table 3. Parameters used in PBPK prediction

<table>
<thead>
<tr>
<th>Drug</th>
<th>Physical/Chemical Properties</th>
<th>Distribution Parameters</th>
<th>Elimination Parameters</th>
<th>Interaction Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td>MW:271.4 log Po:w: 3.8 Type:MB pKa 1:8.3 B:P: 1.32 Fraction unbound in plasma:0.5</td>
<td>Model: Minimal PBPK Vss: user input 14.3</td>
<td>Enzyme kinetics:HLM CLint: CYP2D6:253 CYP3A4:4.3, CYP2B6:4.7 μL/min/mg protein</td>
<td>Ki(μM)CYP2D6: 1.20</td>
</tr>
<tr>
<td>DXO</td>
<td>MW:257.37 log Po:w: 3.53 Type:MB pKa 1:9.66 B:P: 0.55 Fraction unbound in plasma:0.5</td>
<td>Full PBPK Model Vss:Predicted</td>
<td>Enzyme kinetics:HLM CLint: CYP3A4:60 μL/min/mg microsomal protein</td>
<td>/</td>
</tr>
<tr>
<td>GFA</td>
<td>MW:429.51 log Po:w:-0.49 Type:DB pKa 1:4.2 B:P: 8.8 Fraction unbound in plasma:0.88</td>
<td>Minimal PBPK Vss:1.67 L/kg CV(%) 25%</td>
<td>In vivo clearance CLiv:10.72 L/h CV(%) 28.7</td>
<td>/</td>
</tr>
</tbody>
</table>
Table 4. Inhibition of CYP2D by GFA and quinidine. The inhibition constants (Ki values) were calculated from the appropriate nonlinear regression enzyme inhibition model. The mechanism of inhibition was determined graphically.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Ki(µM)</th>
<th>Mechanism of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFA</td>
<td>1.20±0.33</td>
<td>non-competitive</td>
</tr>
<tr>
<td>HLM</td>
<td>0.37±0.16</td>
<td>non-competitive</td>
</tr>
<tr>
<td>rCYP2D6</td>
<td>0.38±0.12</td>
<td>competitive</td>
</tr>
<tr>
<td>MKLM</td>
<td>2.4±1.3</td>
<td>competitive</td>
</tr>
<tr>
<td>DLM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quinidine</td>
<td>0.19±0.079</td>
<td>competitive[r]</td>
</tr>
<tr>
<td>HLM</td>
<td>0.027</td>
<td>competitive</td>
</tr>
<tr>
<td>rCYP2D6</td>
<td>3.64±1.63</td>
<td>competitive</td>
</tr>
<tr>
<td>MKLM</td>
<td>2.5±1.29</td>
<td>non-competitive</td>
</tr>
<tr>
<td>DLM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Noncompartmental pharmacokinetic parameters for DM and DXO in beagle dog plasma after a single i.v. dose of DM (n = 6), pretreated with GFA or sodium saline. Data are expressed as means ± SD.

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>DM Saline group</th>
<th>DM GFA group</th>
<th>DXO Saline group</th>
<th>DXO GFA group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax (ng/ml)</td>
<td>291.2±66.4</td>
<td>302.0±126.4</td>
<td>2500.6±1585.5</td>
<td>862.5±569.5*</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>/</td>
<td>/</td>
<td>0.9±0.7</td>
<td>1.1±0.7</td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>2.08±0.20</td>
<td>2.16±0.27</td>
<td>7.67±2.66</td>
<td>8.36±2.78</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>2.27±0.24</td>
<td>2.50±0.49</td>
<td>7.31±2.58</td>
<td>9.47±1.77</td>
</tr>
<tr>
<td>AUC 0-τ (ng⋅h/ml)</td>
<td>548.7±128.8</td>
<td>541.7±64.7</td>
<td>9093.5±3091.3</td>
<td>4609.4±2388.3*</td>
</tr>
<tr>
<td>AUC 0-∞ (ng⋅h/ml)</td>
<td>556.8±132.5</td>
<td>551.4±66.7</td>
<td>9433.8±3149.1</td>
<td>4885.6±2730.0*</td>
</tr>
</tbody>
</table>

*p < 0.05
Table 6. *In vivo* pharmacokinetic profile and AUCi/AUC predictions using GFA as the inhibitor in human\(^a\)

<table>
<thead>
<tr>
<th>GFA (mg/kg)</th>
<th>Experimental data</th>
<th>SimCYP prediction</th>
<th>Eq 5 prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DM*</td>
<td>DXO*</td>
<td>DM</td>
</tr>
<tr>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>1.063 (1.028-1.096)</td>
</tr>
<tr>
<td>10</td>
<td>0.99</td>
<td>0.518</td>
<td>1.061 (1.031-1.090)</td>
</tr>
</tbody>
</table>

*: experimental data from beagle dogs
ND: Not determined
NA: Not available

\(a\): The SimCYP and equation 5 estimated ratio of AUC in the absence (AUC) or present of GFA inhibitor (AUCi) at indicated GFA dose (mg/kg) were presented in comparison with the experimental data collected in beagle dogs.
Figure 1
Figure 2

A

Enzyme Remaining activity (%)

B

GFA concentration (µM)

- CYP1A2 (PN-ACE)
- CYP2C9 (TB)
- CYP2C19 (OPZ)
- CYP2D6 (DM)
- CYP3A4 (TS)
- CYP3A4 (1-OH-MDZ)
- CYP2E1 (CLZ)
- rCYP2D6
Figure 5

Graph showing the enzyme remaining activity (%) against concentration (μM) for different inhibitors.

Legend:
- rCYP Inhibitor
- 2D6 GFA
- 2D6 QND
- 2D1 GFA
- 2D1 QND
- 2D2 GFA
- 2D2 QND
Figure 6

(a) $K_i = 1.2 \mu M$

(b) $K_i' = 1.2 \mu M$

Non-competitive
Figure 8

A

Enzyme remaining activity (%)

Time (min)

No time dependent inhibition

B

Enzyme remaining activity (%)

Time (min)

No time dependent inhibition
Fig 9

Graph showing the concentration of dextrophan (ng/ml) over time (h) for Control (+Saline) and Test (+GFA, 10 mg/kg) groups.