Construction of humanized CYP1A2 rats using CRISPR/Cas9 to promote drug metabolism and pharmacokinetic research

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Abbreviation:  BAC, bacterial artificial chromosome; CRISPR/Cas9, Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9; CYP, cytochrome P450; DMPK, drug metabolism and pharmacokinetics; FA, formic acid; H&E, hematoxylin and eosin; HLM, human liver microsomes; IS, internal standard; KI, knockin; KO, knockout; LHA, left homology arms; Mb, Megabase; RHA, right homology arms; RLM, rat liver microsomes; SD, Sprague-Dawley; ssODN, single-stranded oligodeoxyxynucleotide; WT, wild-type.
Abstract

Cytochrome P450 family 1 subfamily A member 2 (CYP1A2), performs an indispensable role in metabolism of both exogenous and endogenous substances. What is more, CYP1A2 functions in human diseases by regulating homeostasis of cholesterol. Despite the emergence of gene-editing animal models, genetically humanized animals that overcome species differences for further exploring the role of CYP1A2 in drug metabolism and human diseases have not yet been constructed. In this study, we inserted human CYP1A2 cDNA into the rat Cyp1a2 gene by using CRISPR/Cas9 technology. Results showed that human CYP1A2 was successfully expressed in humanized rat liver and there were no statistically significant differences of physiological symptoms compared with wild-type (WT) rats. In vitro incubation results indicated the different inhibition of furafylline on CYP1A2 activity in human liver microsomes, humanized CYP1A2 (hCYP1A2) rat liver microsomes, and WT rat liver microsomes, with IC₅₀ values of 7.1 μM, 36.5 μM, and 285.8 μM, respectively. Meanwhile, pharmacokinetic characteristics of clozapine were conducted, and the results suggested that in hCYP1A2 rats, clozapine tended to be metabolized into norclozapine. Both the in vitro and in vivo results demonstrated the different metabolic functions of CYP1A2 in humanized and WT rats. We successfully constructed a novel humanized CYP1A2 rat model using the CRISPR/Cas9 system, providing a powerful tool for better predicting CYP1A2-mediated drug metabolism and pharmacokinetics.

**Key words:** CYP1A2; CRISPR/Cas9; Humanized rat model; Drug metabolism and pharmacokinetics; Gene editing
Significance Statement

Human CYP1A2 takes active part both in the biotransformation of exogenous substances (such as drugs, environmental chemicals) and endogenous substances (such as fatty acids, hormones, and prostaglandins). Meanwhile, it plays a regulatory role in human diseases, including hypercholesterolemia, hypertension as well as various malignant tumors. However, due to natural differences with humans in various aspects, the results obtained from animal models cannot be directly applied to humans. This study successfully constructed humanized CYP1A2 rat model by CRISPR/Cas9 technology, providing a powerful model for promoting drug development and safety evaluation, as well as further exploring the role of CYP1A2 in human diseases.
Introduction

Due to natural differences with humans in various aspects, the results obtained from animal models cannot be directly applied to humans. One strategy to address this predicament is to construct humanized animal models. Currently, the transplantations of human immune system components, hepatocytes, skin, and iPSC are the main methods for constructing humanized mouse models (Fujiwara, 2018). Nowadays, megabase (Mb)-sized humanized rat models have not been established due to the instability of chromosome during in vitro culture, which results in the complex manipulation in embryonic stem cells (Tong et al., 2010). With the prosperous development of gene-editing technologies, the clustered regularly interspaced short palindromic repeats (CRISPR) / CRISPR-associated protein 9 (Cas9) has been widely applied to cause mutations or significant genomic deletions in orthologous gene cluster animals. Due to the more frequent use of rats for studying human diseases and evaluating the efficacy and toxicity of drugs, the humanization of rats will be more suitable for various applications compared to mice.

The CYP1A subfamily has aroused interest due to its significant role in the biotransformation of drugs and pollutants, especially its close relationship with human diseases (Lu et al., 2020b). CYP1A can metabolize a variety of environmental procarcinogens into carcinogenic species, thereby increasing the risk of malignancies (Tamasi et al., 2011; He and Feng, 2015). CYP1A consists of two functional genes,
CYP1A1 (distributed in the urinary bladder, liver, and small intestine) and CYP1A2 (exclusively expressed in the liver). CYP1A2 is primarily involved in drug metabolism, carcinogen activation, and some drug-drug interactions, accounting for about 20% of clinically used drugs, including antidepressants, antipsychotics, and analgesics (Wang and Zhou, 2009). Additionally, it takes part in the metabolic activation and inactivation of environmental toxins, including aristolochic, benzopyrene, and ellipticine, thus resulting in the formation of matching reactive intermediates that cause DNA adducts and mutagenesis later (Dai et al., 2023). It also takes part in the biotransformation of various endogenous substances, such as retinol and linoleic acid, as well as the biosynthesis of steroid hormones (Marill et al., 2000; Rendic and Guengerich, 2012; Sun et al., 2021). In addition, CYP1A2 is widely involved in cholesterol metabolic network, and we have demonstrated that the deletion of CYP1A1/2 in rats activates the lipogenesis pathway (LXRα-SREBP1-SCD1) and inhibits the key protein of cholesterol ester hydrolysis (Lu et al., 2023). Interestingly, lansoprazole, the inducer of CYP1A, could significantly alleviate hepatic lipid deposition in rats induced by a high-cholesterol diet, indicating that CYP1A is a potential regulator for cholesterol homeostasis (Lu et al., 2023).

So far, we have constructed CYP1A1, CYP1A2, and CYP1A1/2 knockout rats by using CRISPR/Cas9 technology in 2021 and 2023, respectively (Sun et al., 2021; Lu et al., 2023). Although they have made significant contributions to exploring the function of CYP1A, in order to overcome differences in substrate specificity and
expression regulation among species, there is an urgent need to construct humanized animal models to better predict the human CYP1A pathway in drug metabolism and regulation in human diseases. Since 2005, efforts have been devoted in humanization CYP1A, such as microinjection cloning of bacterial artificial chromosomes (BACs) containing human CYP1A1 and CYP1A2 genes into fertilized FVB/N mouse eggs, and further mating with CYP1A1 null or CYP1A2 null mice to generate homozygous mice (Cheung et al., 2005; Dragin et al., 2007; Jiang et al., 2005). The fast development of CRISPR/Cas9 in recent years has increased our ability to genomically humanize rodent genomes. The use of a single-stranded oligodeoxynucleotide (ssODN) as a repair template in CRISPR/Cas9-assisted HR in zygotes is adequate for effective editing in zygotes without selection, resulting in no genomic scarring formation (Zhu et al., 2019).

In this report, in order to develop a humanized animal model for better exploring the function of CYP1A2 in humans, we deleted the murine Cyp1a2 gene and inserted human CYP1A2 into rats by using CRISPR/Cas9 technology. We confirmed that humanized CYP1A2 rats successfully expressed human genes and simulated the metabolism of probe substrates in humans. Therefore, this model can not only be used to predict human DMPK, promoting drug development and safety evaluation, but also to explore the role of CYP1A2 in human diseases.

Materials and methods

Chemicals and reagents
The SYBR Green real-time fluorescence quantitative PCR master mix and reverse transcription kit were provided by Yeasen (Shanghai, China). The anti-AHR (67785-1-lg) and anti-His rabbit primary antibody (66005-1-lg) was obtained from Proteintech (Wuhan, China). The anti-PXR rabbit primary antibody (DF6478) and anti-FXR rabbit primary antibody (DF12511) were achieved from Affinity Biosciences (Ohio, USA). The anti-CAR primary antibody (A1970) was bought from Abclonal Technology (Wuhan, China). The anti-LXRα rabbit primary antibody (ab176323) and anti-GAPDH rabbit primary antibody (ab8245) was bought from Abcam (Cambridge, UK). Clozapine, norclozapine and furafylline were purchased from Meilunbio (Dalian, China). Phenacetin and 4-acetamidophenol were purchased from Aldrich, and verapamil was achieved from MP Biochemicals (California, USA). Caffeine, paraxanthine, and 3-acetamidophenol were bought from Sigma (St. Louis, USA).

**Animals and ethical issues**

All Sprague-Dawley (SD) rats were bought from East China Normal University (ECNU) Multifunctional Platform for Innovation (Shanghai, China). CYP1A2 knockout rats were generated in our previous studies (Sun et al., 2021). All animal experiments comply with relevant ethical regulations and the animal procedures were approved by the Ethics Committee on Animal Experimentation of ECNU (r20160501).

**Humanization of CYP1A2 rats**

To increase the probability of cleavage, four target sites characterized by a 21 bp
base sequence ending in NGG (N for any base) were chosen at the first exon of the rat CYP1A2 gene using the online tool (https://benchling.com). Based on the above requirements, the target selection includes:

sgRNA-1: CGCCATCTGTACGACTGCAGG;
sgRNA-2: CTCCTGCAGTCGTACAGATGG;
sgRNA-3: TGCCACCAGAGAACTCCCAGG;
sgRNA-4: GAGTACCTGGGAGTTCTCTGG.

As shown in Fig. 1A, the length of the left and right homology arms (LHA, RHA) selected in this study were all about 800 bp, and the Flag and His sequences were fused after the human CYP1A2 sequence to facilitate the detection of the human CYP1A2 protein level. After successful construction, amplification, and extraction, the plasmid was delivered to Bio-sune Biotech Co., Ltd (Shanghai, China) for first-generation sequencing to ensure the integrity and accuracy of sequence. The microinjection system containing sgRNA (50 ng/μL), homologous recombination plasmid (200 ng/μL) and Cas9 mRNA (50 ng/μL).

Genotype identification

The genomic DNA was extracted from the toes of newborn and consequently amplified with DNA polymerase and primers (Table 1), and the PCR products (3260 bp) was electrophoresed on the 1.5% agarose gel. PCR products of the F0 generation (the newborn rats after microinjection) with correct band position were selected and sent to GENEWIZ (Suzhou, China) for precise sequence. The identified F0 offspring were crossed with WT rats to obtain heterozygous F1 generation. Then after
electrophoresing and sequencing, the healthy adult F1 rats were chosen and crossed to
breed homozygous F2 generation. The F2 rats also need to be genotyped before
proceeding to the further experiment.

**qPCR analysis**

Total mRNA in liver was extracted and reverse-transcribed, and the main
subtypes of CYP were detected as described previously (Sun et al., 2021). The primer
information is shown in Table 1. The β-actin was used as an internal reference.

**Western blot analysis**

The liver tissue was homogenized in RIPA buffer, and total protein (40 μg)
detected by BCA kit was electrophoresed in 10% SDS-PAGE and transferred to
PVDF membrane. The blot was blocked in 5% BSA for 1h, followed by incubating
with primary antibodies overnight at 4°C. Rabbit anti-His (1:10000), rabbit anti-PXR
(1:1000), rabbit anti-CAR (1:1000), rabbit anti-FXR (1:1000), rabbit anti-LXRα
(1:2000), rabbit anti-AHR (1:2000) and anti-GAPDH (1:10000) were used as the
primary antibody. The secondary antibody was anti-rabbit (1:10000) and the
incubations were performed at room temperature for 1h and then scanned by Odyssey
imager system (LI-COR, MA, USA).

**Immunohistochemistry analysis**

Liver tissue was fixed with 3% PFA for 15 min, and then the following primary
antibody: anti-His (Proteintech, 66005-1-lg) was incubated at 4°C overnight. The
same buffer was used to incubate the secondary antibody at room temperature for 60
min.
Serum biochemical indexed detection

Blood samples were collected from 8-week-old rats fasting for 12 h, and the serum was sent to Shanghai ADICON Clinical Laboratories (Shanghai, China) to detect the biochemical indexes.

Liver staining

Male 8-week-old WT and hCYP1A2 rats were euthanized. According to the standard procedure, the liver tissue was carefully separated, diced into small pieces, fixed with the 4% paraformaldehyde PBS buffer, embedded in paraffin for cutting into sections, and then stained with Hematoxylin and Eosin (H&E).

In vitro determination of CYP1A2 activity

We used phenacetin as the probe substrate and furafylline as an inhibitor to detect CYP1A2 metabolic activity. The incubation system included liver microsomes protein (0.1 mg/mL), phenacetin (5 μM) and furafylline (1 - 1000 μM). After 20 min of incubation, ice-cold ACN containing verapamil (the internal standard) was added to terminate the reaction. Meanwhile, caffeine was also used as the probe substrate to detect CYP1A2 metabolic activity in vitro. The incubation system was followed our previous studies (Sun et al., 2021).

In vivo determination of CYP1A2 activity

Male rats (8-week-old, humanized or WT) were treated with clozapine at a single dose of 5 mg/kg (volume 5 mL/kg, dissolved in 0.5% CMC-Na) by gavage. Blood samples were collected from the tail vein at 0.08, 0.25, 0.5, 0.75, 1, 2, 4, and 8 h after administration. The blood samples were centrifuged at 10,000 g for 15 min at 4°C,
and the supernatant was stored at -20°C for analytical experiments.

**Mass spectrometry analysis**

The LC-MS/MS system was described in our previous studies (Sun et al., 2021). Metabolites from phenacetin and mebendazole (IS) were separated by using a Kinetex XB-C18 column (3 × 100 mm, 2.6 μm). The flow rate was 0.3 mL/min, with a gradient ranging from 10 to 90% methanol containing 0.1% formic acid (FA) in a 12.5 min run. MS was operated in positive mode with the ion transitions of 152.1→110.1, 296.4→264.1 for 4-acetamidophenol and mebendazole, respectively. Metabolites from caffeine, paraxantine, and 3-acetamidophenol (IS) were separated and detected according to our previous studies (Sun et al., 2021).

Metabolites of clozapine and verapamil (IS) were separated on an Omega C18 column (3 × 100 mm, 2.6 μm). The mobile phase consisted of H₂O containing 1 mM ammonium formate (A) and methanol containing 0.1% FA (B). The gradient of HPLC was: 0–3 min, 40%–90% B; 3–4 min, 90% B; 4–5 min, 90%–40% B. The flow rate was 0.2 mL/min, and the injection volume was 5 μL. Clozapine, norclozapine, clozapine-N-oxide and IS were monitored in the positive electrospray ionization mode, with the ion transitions of 327.1→192.0, 313.0→192.0, 343.0→192.0, and 455.2→165.1, respectively.

**Statistical data analysis**

All images were drawn using GraphPad Prism 8.0.1 (GraphPad Software Inc., San Diego, USA) and presented as mean ± SEM. The statistical analysis was performed using a two-tailed t-test, while the statistical analysis among multiple
groups was analyzed by using one-way analysis of variance (ANOVA). A significant difference was considered when the P value is less than 0.05.

Results

Generation of humanized CYP1A2 rats

In order to block murine CYP1A2 metabolism and insert human CYP1A2 in rats, we achieved rat Cyp1a2 knockout (KO) and human CYP1A2 knockin (KI) by using CRISPR/Cas9 technology. Agarose gel electrophoresis revealed that in 10 F0 generation rats, the target site of 3# integrated the human CYP1A2 sequence (Fig. 1B), and further sequencing confirmed the accuracy of the insertion position and the correctness of the inserted sequence (Fig. 1E). After caging with WT rats, 20 F1 generation rats were obtained. Further agarose gel electrophoresis and sequencing results showed that 1#, 2#, 7#, 8#, 17#, and 20# were positive offspring (Fig. 1C and E). All rats with the same mutation were chosen for crossing to obtain F2 generation (Fig. 1D).

All WT rats in this work were littersmates of humanized CYP1A2 rats, and the KO rats following F2 generation were employed for additional research. Serum physiological indicators showed that there was no statistically significant difference in lipid and liver function between humanized CYP1A2 and WT rats (Fig. 2A). Meanwhile, humanized CYP1A2 rat liver showed no significant difference compared with WT rat liver, without infiltration and necrosis (Fig. 2B).

Characterization of humanized CYP1A2 rats
The expression of the murine CYP at mRNA level was detected and analyzed. The results showed that there were no statistically significant changes between WT and humanized CYP1A2 rats (Fig. 3A). At the same time, the protein expression of main nuclear receptors was also detected. Compared with WT rats, protein expression of AHR, PXR, LXRα, CAR and FXR of humanized CYP1A2 rats showed no significant difference (Fig. 3B). Western blot of His results revealed the efficient insertion of human CYP1A2 (Fig. 3C), and immunostaining for His (Fig. 3D) confirmed this finding.

**Xenobiotic metabolism of humanized CYP1A2 rats**

To validate the effect of humanized CYP1A2 rats on drug metabolism, we have studied the inhibition of furafylline in vitro and the metabolism of clozapine in vivo. Furafylline presented selective inhibition on CYP1A2 activity in human liver microsomes (HLM), humanized CYP1A2 rat liver microsomes (hCYP1A2) and WT rat liver microsomes (RLM), with the IC50 value at 7.1 μM, 36.5 μM, and 285.8 μM, respectively (Fig. 4A and B). Meanwhile, we also selected caffeine as the probe substrate to assess the metabolic activity of CYP1A2. Results showed that at the same time, more paraxanthine were generated in HLM group compared with RLM and hCYP1A2 group (Fig. 4C). To confirm the activity of CYP1A2 in vivo, the metabolites of CYP1A2 substrate clozapine, norclozapine and clozapine-N-oxide were detected. The ratio of areas under the curve (AUC) for norclozapine and clozapine (Nor/Clo) in humanized CYP1A2 rats was 2.21-fold higher than that in Cyp1a2 KO rats (Fig. 5A), while the AUC ratio of norclozapine and
clozapine-N-oxide (Nor/Clo-o) in humanized CYP1A2 rats was two-fold higher than that in WT rats (Fig. 5B).

Discussion

Animal models play an indispensable role in experimental physiology, experimental pathology, and experimental therapeutics, especially in drug research, to predict human pharmacodynamics, pharmacokinetics and toxicology (Guengerich and MacDonald, 2007). As one of the most suitable animal models for human diseases, rats are experimental animal models that can combine genome and function. At the same time, rats are also the first choice for pharmacological research, and are widely used in the research fields of cardiovascular diseases and sports diseases (Kazuki et al., 2019; Lucroy and Suckow, 2020). Rats can be used to simulate almost all known human diseases (Ma et al., 2020). Understanding the genetic structure of rats can help to find disease-related genes, and further better understand the impact of genes and environment on human health. In addition, rats are widely used in drug efficacy and safety testing (Zonzini et al., 2010). The understanding of rat genome sequence will provide new targets for drug discovery, thus greatly promoting the development of new drugs (Lu et al., 2021; Lai et al., 2022). The traditional animal model constructed by homologous recombination of embryonic stem cells has achieved knockout of foreign genes, but the recombination efficiency is low and the recombination sites have a certain degree of randomness (Li et al., 2019). Fortunately, with the development of gene editing technology in recent years, rat gene editing
animal models can be rapidly constructed, thus providing new models for drug research & development and disease treatment (Liang et al., 2019; Lu et al., 2020a; Liu et al., 2021; Zhang et al., 2022).

Drug metabolism is of great significance for evaluating the pharmacokinetics and toxicological properties of drugs, which affects drug efficacy and toxicity. Therefore, it is essential to identify metabolites to determine whether new medications are safe (Baillie, 2006; Guengerich and MacDonald, 2007). Assessment of drug metabolism is normally operated by using in vitro systems, such as liver microsomes, liver sectioning, hepatocytes, and recombinant enzymes, as well as in vivo animal models (Rodrigues, 1999; LeCluyse, 2001; Ozaki et al., 2013). In vitro systems have the advantages of fast metabolic process, good reproducibility, simple preparation, easy collection and so on, but they are limited to simulate complex environments in vivo to predict more complicated biotransformation (Pelkonen et al., 2005; Dalvie et al., 2009). Due to the fact that rats are commonly employed for studying human diseases and drug screening, humanized rat models are more popular in some applications than mice (Kazuki et al., 2019).

CYP1A2, as the main drug metabolizing enzyme, is mainly distributed in the human liver. Due to its high abundance, various clinical drugs including caffeine, phenacetin, tizanidine and antipsychotics, antidepressants drugs like clozapine, olanzapine, undergo significant metabolism by CYP1A2 (Aitchison et al., 2000; Klomp et al., 2020; Zubiaur et al., 2021). Meanwhile, CYP1A2 is actively involved in the deactivation of many anticancer drugs, leading to the drug resistance (Dai et al.,
More importantly, CYP1A2 actively participates in the biotransformation of many endogenous substances which are involved in the regulation of human diseases, such as hypercholesterolemia and hypertension (Dai et al., 2023).

In this study, we successfully deleted murine Cyp1a2 and inserted human CYPIA2 at the same time by using CRISPR/Cas9. Compared with the conventional methods, CRISPR/Cas9 technology can delete the murine genes and insert human gene simultaneously, providing a powerful tool to achieve reproducible human gene expression in a variety of different species. The liver expression of human CYP1A2 were observed in this model. Since there are no antibodies on the market to distinguish CYP1A2 from humans and rats, we indirectly demonstrated the expression of human CYP1A2 through the expression of His-tagged protein.

In the functional analysis of humanized CYPIA2 rats, we used phenacetin as the probe drug and furafylline as the inhibitor, respectively. Previous studies have reported that furafylline is a selective and noncompetitive inhibitor of CYP1A2, which inhibits rat CYP1A2 at a concentration 1000 times higher than that required to inhibit human CYP1A2, indicating a significant difference in the active site geometry between rat and human orthologues of CYP1A2 (Sesardic et al., 1990; Martignoni et al., 2006). Our data showed that the relative activity of CYP1A2 in HLM was significantly lower than that in RLM, and the relative activity of humanized rats is between the two, indicating that the inhibitory effect of furafylline on CYP1A2 in three liver microsomes is different, while the inhibitory effect of furafylline on humanized rat liver microsomes is between the two. Caffeine, as a probe substrate of
CYP1A2, was also used to detect CYP1A2 activity *in vitro*. It is oxidized at several points in its chemical structure, including 1-N-demethylation, 3-N-demethylation, 7-N-demethylation, and 8-hydroxylation (to theobromine, paraxanthine, theophylline, and 1,3,7-trimethyluric acid, respectively). In the rat liver, 3-N-demethylation accounts for about 13% when measured at a substrate concentration of 100 μM. In contrast, 3-N-demethylation was the main oxidation pathway of caffeine in human liver microsomes (~70%) (Kot and Daniel, 2008a; 2008b). Consequently, at the same time, more paraxanthine were generated in HLM group compared with RLM and hCYP1A2 group. Clozapine, as a dibenzodiazepine derivative with great risk of severe adverse effects, is extensively metabolized by CYP1A2 in the liver (Luft and Taylor, 2006). The major metabolites of clozapine are norclozapine and clozapine-N-oxide (Jaquenoud Sirot et al., 2009). In this study, we used clozapine as a probe drug to compare the performance of CYP1A2 enzyme in humanized and WT rats. In humanized CYP1A2 rats, norclozapine was preferentially formed rather than clozapine-N-oxide, indicating different metabolic characteristics from WT rats.

In conclusion, we have successfully constructed humanized CYP1A2 rats. This rat model will promote the research related to DMPK and has many key application values in basic research, drug development and safety evaluation.

**Data Availability Statement**

The authors declare that all the data supporting the findings of this study are contained within the paper.
Author contributions

Participated in research design: Wang.

Conducted experiments: Liu, Lu, Zhang, Huang, Chen, and Shen.

Performed data analysis: Liu, Lu, and Yao.

Wrote or contributed to the drafting of the manuscript: Liu, Lu, Yao, Zhang, and Wang.

Obtained the funding and supervised the whole study: Wang.

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Footnotes

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Table 1 Primers used in this study.

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Figure legends:

**Figure 1.** Genotyping of humanized *CYP1A2* rats generated by CRISPR/Cas9. (A) The construction of humanized *CYP1A2* plasmid. The length of the left and right homologous arms (LHA, RHA) is about 800 bp, and the Flag and His sequences are fused after the human *CYP1A2* sequence. The mutations in the (B) F0, (C) F1, and (D) F2 generation for humanized *CYP1A2* rats, which were detected by 1.5% agarose gel using PCR products amplified from F0 rat toes genomic DNA by primer. H2O, blank control; WT, negative control. (E) The sequencing results of F0 and F1 generation for humanized *CYP1A2* rats.

**Figure 2.** Physiologic phenotype of humanized *CYP1A2* rats. (A) Clinical chemistry and physiologic analysis of serum from male WT and humanized *CYP1A2* rats at 8 weeks old. The results are shown as mean ± SEM of six rats. (B) Hematoxylin and eosin (H&E) staining on liver sections from WT and humanized *CYP1A2* rats at 8 weeks (n = 3).

**Figure 3.** Gene expression profiling of humanized *CYP1A2* rats. (A) Compensatory expression of other main CYP enzymes in humanized *CYP1A2* rats at 8 weeks old. The results are expressed as mean ± SEM (n = 6). *P < 0.05, and **P < 0.01 compared with the WT group. (B) Protein expression of main nuclear receptors in rats at 8 weeks old (n = 3) (C) Western blotting for His protein expression in the liver of rats. (D) Immunohistochemistry for His protein expression in the liver of rats. Red arrows are used for indication.
**Figure 4.** Xenobiotic metabolism in humanized *CYPIA2* rats *in vitro*. (A) Selective inhibition of furafylline on phenacetin metabolism in human liver microsomes (HLM), humanized *CYPIA2* rat liver microsomes (hCYP1A2) and WT rat liver microsomes (RLM). (B) The inhibition (IC$_{50}$ value) of furafylline on CYP1A2 activity. (C) Caffeine metabolism in HLM, hCYP1A2, and WT rat liver microsomes.

**Figure 5.** Xenobiotic metabolism in humanized *CYPIA2* rats *in vivo*. (A) Ratio of AUC for Nor/Clo and Clo-o/Clo in WT, Cyp1a2 KO, and humanized *CYPIA2* rats. (B) Ratio of AUC for Nor/Clo-o in WT and humanized *CYPIA2* rats. Rats were gavage administered with clozapine (5 mg/kg). The results are expressed as mean ± SEM (n = 6). *P < 0.05, **P < 0.01, and ***P < 0.001.
**Fig. 1**

A  
| LHA | Human CYP1A2 | Flag | His | RHA |

B  

WT: 2288 bp  
KI: 3260 bp

C  

WT: 2288 bp  
KI: 3260 bp

D  

WT: 2288 bp  
KI: 3260 bp

E  

WT  
CATCTCCATTTCTCCTGCAGTCCCAAGGTACTCCCGG GTGC

F0  
CATCTCCATTTCTCCTGCAG.................TCCCAAGGTACTCCCGG GTGC
  3’ segment of the LHA  
  5’ segment of the RHA  
  KI: 1912bp

F1  
CATCTCCATTTCTCCTGCAG.................TCCCAAGGTACTCCCGG GTGC
  3’ segment of the LHA  
  5’ segment of the RHA  
  KI: 1912bp

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Fig. 2

A

B

WT

hCYP1A2

H&E

200 µm

200 µm
Fig. 3

A

B

C

D

WT and hCYP1A2

Relative mRNA expression

WT

hCYP1A2

Cyp1a1, Cyp2a1, Cyp2c11, Cyp2d1, Cyp2d2, Cyp2e1, Cyp3a1, Cyp3a2

0.0
0.5
1.0
1.5
2.0
2.5

0.0
0.5
1.0
1.5
2.0
2.5

AHR

PXR

LXRα

CAR

FXR

GAPDH

96 KDa

50 KDa

50 KDa

45 KDa

55 KDa

36 KDa

55 kDa

36 kDa

WT

hCYP1A2

His

GAPDH

96 KDa

50 KDa

45 KDa

36 KDa

WT

hCYP1A2

100 μm

100 μm
Fig. 4

A

![Graph showing relative activity as a function of log [Furafylline, μM].](image)

B

![Bar graph showing IC₅₀ values for different conditions.](image)

C

![Graph showing velocity as a function of concentration of caffeine.](image)
Fig. 5

A

![Bar chart showing relative ratio of AUC (fold over WT) for WT, Cyp1a2 KO, hCYP1A2, Nor/Clo, and Clo-o/Clo.](image)

B

![Bar chart showing relative ratio of AUC (fold over WT) for WT, hCYP1A2, and Nor/Clo-o.](image)