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Note

Probe drug T-1032 *N*-oxygenation mediated by cytochrome P450 3A5 in human hepatocytes *in vitro* and in humanized-liver mice *in vivo*

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Running title: T-1032 *N*-oxygenation in humanized-liver mice

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Abstract

Polymorphic cytochrome P450 3A5 (CYP3A5) expression contributes to individual differences in the pharmacokinetics of probe drugs. The identification of suitable *in vivo* CYP3A5 probes would benefit drug metabolism and drug interaction studies using chimeric mice with humanized liver. In this study, we investigated the pharmacokinetic profiles of T-1032, which is known as an *in vitro* CYP3A5 probe substrate, using humanized-liver mice. Substantial *N*-oxygenation of T-1032 was observed in hepatocytes from humans and from humanized-liver mice. Hepatocytes from the human donor genotyped as *CYP3A5**3/*3 (poor expressers) showed significantly lower T-1032 *N*-oxidation rates than those from donors harboring *CYP3A5**1. After a single oral dose of T-1032 (10 mg/kg) in humanized-liver mice, the plasma levels of T-1032 *N*-oxide were higher in five mice with *CYP3A5**1/*7 hepatocytes than in four mice with *CYP3A5**3/*3 hepatocytes. The maximum concentration of T-1032 *N*-oxide after oral administration of T-1032 in humanized-liver mice with *CYP3A5**1/*7 hepatocytes were twice (a significant difference) those from humanized-liver mice with *CYP3A5**3/*3 hepatocytes. These results suggest that polymorphic CYP3A5-dependent T-1032 *N*-oxidation was observed in humanized liver mice *in vitro* and *in vivo*. However, the contribution of *CYP3A5* genotypes may have little or only limited effects on the overall pharmacokinetic profiles of T-1032 *in vivo*.

Keywords: CYP3A5.1; *CYP3A5**1; hepatocytes; *N*-oxide; T-5

1. Introduction

Approximately half of new medicines recently approved by the US Food and Drug Administration are reportedly metabolized by cytochrome P450 3A4 (CYP3A4) and 3A5 [1]. CYP3A4 and 3A5 have significant substrate overlap [2, 3], but there is currently no way to selectively monitor the activities of these two CYP3A enzymes; instead, the cumulative activity is often attributed to CYP3A4. Whereas CYP3A4 expression is ubiquitous, CYP3A5 is expressed variably in different populations, resulting in large individual differences in CYP3A5 expression levels [4].

T-1032 [methyl (2-(4-aminophenyl)-1,2-dihydro-1-oxo-7-(2-pyridinylmethoxy)-4-(3,4,5-trimethoxyphenyl)-3-isoquinoline carboxylate sulfate] was synthesized as a selective phosphodiesterase 5 inhibitor [5]. It has been suggested that the oxidation of T-1032 (also known as T-5) by polymorphic CYP3A5 could yield an *N*-oxide metabolite that may be more than 100-fold selective compared with P450 3A4 in human liver microsomal P450 forms in *in vitro* systems [6]. Against this background, we set out to investigate whether T-1032 is a suitable CYP3A5 probe in human hepatocytes *in vitro* and in humanized-liver mice *in vivo*. The identification of suitable CYP3A5 probes could contribute to drug metabolism and drug interaction studies. We report herein the significant but limited contributions of polymorphic CYP3A5 to the oxidation of T-1032 in *in vivo* systems.

2. Materials and methods

2.1 *In vitro* analyses

T-1032 (CAS no. 212500-03-3), its *N*-oxide, T-1032-*d*₃, and T-1032 *N*-oxide-*d*₃ were obtained from Biopredic International, Saint Grégoire, France. Cryopreserved hepatocytes from four human donors (a 2-year-old Caucasian boy [donor A], a 35-year-old Asian man [donor B], a 30-year-old African American woman [donor C], and a 12-year-old Caucasian girl [donor D], genotyped as *CYP3A5**1/*3, *3A5**1/*3, *3A5**1/*7, and *3A5**3/*3, respectively) were obtained from Biopredic International or Lonza Walkersville, Walkersville, MD, USA, and were transplanted into NOG-TKm30 mice [7] to create humanized-liver mice. Cryopreserved hepatocytes from 12 humanized-liver mice transplanted with human hepatocytes from the above four donors (Hu-Liver cells) were prepared using the collagenase perfusion method described previously [8, 9]. The percentages of human leukocyte antigen-positive cells in hepatocytes from these humanized liver mice were all >95%, as determined by fluorescence-activated cell sorting using a monoclonal anti-human leukocyte antigen antibody (clone G46-2.6; BD Biosciences, Franklin Lakes, NJ, USA) on a BD FACSAria cell sorter (BD Biosciences). This study was reviewed and approved by the Institutional Animal Care and Use Committees of the Central Institute for Experimental Animals (Permit Number: 20060 A).

Cell suspension metabolic assays using cryopreserved hepatocytes from one human donor and fresh hepatocytes (1×10^5 cells/tube) from the 12 humanized-liver mice were incubated for 60 min at 37°C in an incubator on an orbital shaker. The reaction mixture (0.2 mL) contained T-1032 (10 µM) and Williams' Medium E without phenol red (Gibco, Grand Island, NY, USA) [10]. Reactions were started by adding the substrate and stopped by adding an equal volume of ice-cold acetonitrile containing the deuterium-labelled internal standards. Precipitates were then removed by centrifugation (10 min at $20,000 \times g$, 4°C). Supernatants (2.0 µL) were analysed using liquid chromatography–tandem mass spectrometry.

2.2 *In vivo* animal experiments

Humanized-liver NOG-TKm30 mice (4–5 males per group, weight ~20–30 g, Central Institute for Experimental Animals, Kawasaki, Japan [7]) were used in this *in vivo* study. The human hepatocyte replacement indices of the host livers were all >80%, as estimated using a human albumin ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX, USA). Plasma samples were collected 0.25–24 h after oral doses of T-1032 (1.0 mg/kg). The pharmacokinetic profiles of T-1032 and its *N*-oxide were determined between 0.25 and 24 h using liquid chromatography and tandem mass spectrometry.

A Nexera ultra-high-performance liquid chromatography system (Shimadzu, Kyoto, Japan) equipped with an API QTRAP 5500 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA, USA) and a YMC-Triart C18 column (100 mm × 3.0 mm i.d., particle size 3.0 μm; YMC, Kyoto, Japan) was used. For internal standards, T-1032-*d*₃ and T-1032 *N*-oxide-*d*₃ were used. Levels of T-1032 and its *N*-oxide in mouse plasma along with levels of T-1032-*d*₃ and T-1032 *N*-oxide-*d*₃ were quantified using a gradient elution program followed by a tandem mass spectrometry system with multiple reaction monitoring and positive ion mode electrospray ionization using the following transitions: *m/z* 568→476, *m/z* 584→476, *m/z* 571→479, and *m/z* 587→479, respectively. The mobile phase had a flow rate of 0.3 mL/min with a multistep gradient elution program using 0.1% formic acid in water (A) and methanol (B) as follows: 0–12 min, 20%–80% B; 12.1–15 min, 100% B; and 15.1–18 min, 20% B. The column temperature was maintained at 40°C throughout the run. The retention times for authentic T-1032 and its *N*-oxide were 10.3 and 9.1 min, respectively, under the present conditions. The pharmacokinetic parameters for T-1032 and its *N*-oxide were derived from the respective plasma concentration–time curves (0.25–24 h after oral doses) using trapezoidal

linear interpolation methods with uniform weighting and non-compartmental analysis in Phoenix 8.2 (Pharsight, Mountain View, CA, USA).

Statistical analyses of the pharmacokinetic parameters of T-1032 and its *N*-oxide in humanized-liver mice were performed using two-way analysis of variance and unpaired *t*-tests (Prism, GraphPad Prism Software, La Jolla, CA, USA).

3. Results and discussion

To evaluate *in vitro* the roles of polymorphic CYP3A5 in the hepatic *N*-oxidation of T-1032 in humans and humanized-liver mice, T-1032 *N*-oxide formation rates were investigated after incubation with human hepatocytes. T-1032 *N*-oxide was produced by all the hepatocytes tested (**Fig. 1**). The hepatocytes from one human donor genotyped as *CYP3A5**3/*3 (poor expressers) showed significantly lower T-1032 *N*-oxidation rates than those from donors harboring the *CYP3A5**1 allele (extensive expressers, $p < 0.01$).

The *in vivo* pharmacokinetics of T-1032 and its *N*-oxygenated metabolite were investigated after administrations in humanized-liver mice. Orally administered T-1032 (1.0 mg/kg) was oxidized to its *N*-oxide in humanized-liver mice and was detected in plasma (**Fig. 2A and 2B**). The area under the concentration–time curve of T-1032 *N*-oxide was one-tenth that of T-1032 in humanized-liver mice. Concentrations of T-1032 *N*-oxide in plasma from humanized-liver mice with *CYP3A5**1/*7 human hepatocytes were significantly higher than those in plasma from humanized-liver mice with *CYP3A5**3/*3 hepatocytes by two-way analysis of variance ($p < 0.01$, **Fig. 2B**). The nonparametric pharmacokinetic parameters for T-1032 and its *N*-

oxygenated metabolite in individual humanized-liver mice *in vivo* are summarized in **Fig. 2C and 2D**. The mean maximum concentrations (C_{\max}) and standard deviations in plasma from humanized-liver mice with *CYP3A5**3/*3 and *CYP3A5**1/*7 hepatocytes were calculated to be 0.069 ± 0.024 nmol T-1032/mL and 0.005 ± 0.001 nmol T-1032 *N*-oxide/mL and 0.092 ± 0.053 nmol T-1032/mL and 0.011 ± 0.005 nmol T-1032 *N*-oxide/mL, respectively. The mean areas under the concentration–time curves (AUC) in plasma from humanized-liver mice with *CYP3A5**3/*3 and *CYP3A5**1/*7 hepatocytes, respectively, were calculated to be 0.41 ± 0.13 nmol T-1032 h/mL and 0.042 ± 0.018 nmol T-1032 *N*-oxide h/mL and 0.45 ± 0.11 nmol T-1032 h/mL and 0.066 ± 0.023 nmol T-1032 *N*-oxide h/mL. After oral administration of T-1032, the C_{\max} values of T-1032 *N*-oxide in plasma from humanized-liver mice with *CYP3A5**1/*7 hepatocytes were twice ($p < 0.05$) those from humanized-liver mice with *CYP3A5**3/*3 hepatocytes (**Fig. 2D**).

In this study, the pharmacokinetic profiles of T-1032 and its *N*-oxide were determined up to 24 h post-administration in humanized-liver mice. The ratio of the AUC of T-1032 *N*-oxide to the sum of the AUCs of T-1032 and its *N*-oxide were 13% (0.066 nmol T-1032 *N*-oxide h/mL compared with a total of 0.519 ± 0.133 nmol h/mL) in plasma from humanized-liver mice with *CYP3A5**1/*7 hepatocytes. In contrast, the ratio of the AUC of T-1032 *N*-oxide to the sum of the AUCs of T-1032 and its *N*-oxide were 9.2% (0.042 nmol T-1032 *N*-oxide h/mL compared with a total of 0.452 ± 0.144 nmol h/mL) in plasma from humanized-liver mice with *CYP3A5**3/*3 hepatocytes (poor expressers). The data shown in **Figs. 2B and 2D** imply significant but little effects of wild-type CYP3A5.1 on the total CYP3A activities in *in vivo* systems. *In vitro* T-1032 deletion mediated mainly by recombinant CYP3A5 and minorly by CYP2C8, 2C9, and 3A4 has been reported, probably forming the *O*-dealkylated metabolite

[6]. Possible contributions of flavin-containing monooxygenase could be ruled out on the *N*-oxide formation from T-1032 (with a *in silico* pKa value of 4.3 [11]), because of the previous findings that *in silico* pKa (base) values greater than 8.4 for the main metabolic sites for *N*-oxidation suggested a substrate of flavin-containing monooxygenases [12]. The present results collectively suggest that polymorphic CYP3A5-dependent T-1032 *N*-oxidation was observed in humanized liver mice *in vitro* and *in vivo* and that T-1032 was one of useful CYP3A5 probes. However, the contribution of *CYP3A5* genotypes (extensive or poor expressers) may have little or only limited effects on the overall pharmacokinetic profiles of T-1032 *in vivo*. Further detailed investigations for some limited drug interactions mediated by CYP3A5.1 are required.

Conflicts of interest

All authors declare that they have no conflicts of interest.

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Author contributions

H.S. and H.Y. designed the study; K.P. and S.R. optimized chemical synthesis of T-1032 and its *N*-oxide as well as the design and synthesis of all deuterated analogues; S.U., M.S., N.Y., Y.H., and H.S. performed the experiments; and M.S. and H.Y. modeled the pharmacokinetics. All co-authors performed data analysis. S.U. and H.Y. wrote the manuscript.

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Figure legends

Figure 1. T-1032 *N*-oxidation in hepatocytes from humans and humanized-liver mice.

Hepatocytes (5.0×10^5 cells/mL) were incubated with T-1032 (10 μ M) at 37°C for 60 min. Plots represent the mean values of triplicate determinations of Hu-Liver cells from one host mouse transplanted with human hepatocytes harboring the *CYP3A5*1* allele (closed symbols) and the *CYP3A5*3/*3* genotype (open symbols), and bars give the mean values of hepatocyte batches from three mice. **Significantly different, $p < 0.01$.

Figure 2. Plasma concentrations (A, B) and pharmacokinetic parameters (C, D) for T-1032 and its *N*-oxide after oral administration of T-1032 (1.0 mg/kg body weight) in humanized-liver mice transplanted with hepatocytes previously genotyped as *CYP3A5*3/*3* (open symbols, n=4) and as *CYP3A5*1/*7* (closed symbols, n=5). In panels A and B, mean values and SD bars are shown. In panels C and D, individual plots and mean bars are shown. Concentrations of T-1032 *N*-oxide in plasma from humanized-liver mice with *CYP3A5*1/*7* hepatocytes were significantly higher than those in plasma from humanized-liver mice with *CYP3A5*3/*3* hepatocytes by two-way analysis of variance ($p < 0.01$). *Significantly different, $p < 0.05$.

Figure 1

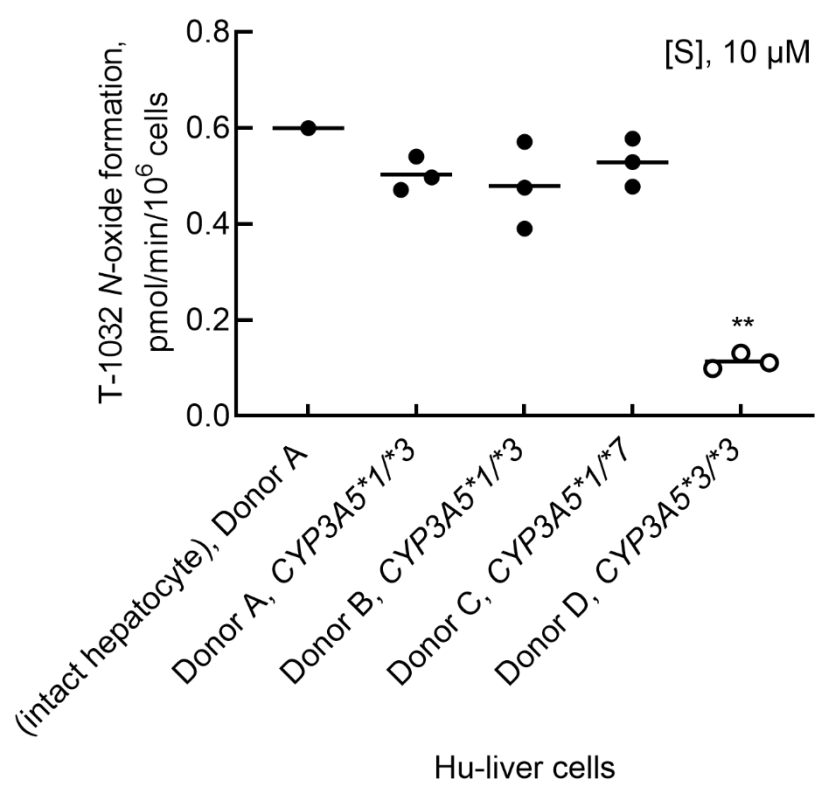


Figure 2

