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In vivo Drug Interactions of the Teratogen Thalidomide with Midazolam: Heterotropic Cooperativity of Human Cytochrome P450 in Humanized TK-NOG Mice

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Abstract

In vivo drug interactions of the teratogen thalidomide with the model cytochrome P450 (P450) 3A substrate midazolam were investigated in mice with humanized livers. The clearance of midazolam (administered intravenously, 10 mg kg^{-1}) in chimeric mice was enhanced by orally co-administered thalidomide (100 mg kg^{-1}). A higher area-under-the-curve of the major metabolite 1′-hydroxymidazolam (1.7-fold) was obtained with thalidomide due to heterotropic cooperativity of human P450 3A enzymes. A higher area-under-the-curve of the minor metabolite 4-hydroxymidazolam (3.5-fold) was seen by pre-treatment with thalidomide daily for 3 days, presumably because of human P450 3A induction. These results demonstrate that livers of humanized mice mediate drug interactions of thalidomide and suggest interactions of therapeutic agents during therapies with thalidomide.

Introduction

Many drug interactions are due to alterations in drug metabolism in the body, generally through enzyme inhibition or enzyme induction.[1] There is growing clinical interest in thalidomide because of its immunomodulatory and anti-angiogenic properties, despite its notorious teratogenicity in humans[2] (but its absence in rodents) [3]. Little information about thalidomide is available regarding its specific effects on human drug-metabolizing enzymes

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or drug interactions.[4] We previously reported, using *in vitro* systems, that midazolam metabolism or cyclosporine A clearance mediated by human liver microsomal cytochrome P450 3A4/5 may be increased by thalidomide in a dose-dependent manner, showing ligand cooperativity with P450 3A enzymes[5]

We recently reported thalidomide 5-hydroxylation by human liver microsomal P450 3A4/5 and further aromatic ring oxidation leading to GSH conjugation—which may be relevant to the pharmacological and toxicological actions—in *in vitro* human situations[6] and *in vivo* chimeric mice with humanized liver cells.[7,8] *In vivo* ligand cooperativity with human P450 3A enzymes with humanized non-obese diabetes-severe combined immunodeficiency-interleukin-2 receptor gamma chain-deficient mice (NOG mice) has not previously been reported. The purpose of this study was to investigate *in vivo* drug interactions of thalidomide with midazolam in NOG mice[9] containing human liver cells.[8] High midazolam clearance and a higher area-under-plasma-concentration time curve (AUC) of the metabolite 1′-hydroxymidazolam were obtained following co-administration of thalidomide, indicating that heterotropic cooperativity occurs *in vivo.* Thalidomide also has the potential for human P450 3A induction in chimeric mice with humanized liver, as judged by the rapid clearance of midazolam.

Experimental Procedures

Chimeric Mice

Chemicals used were from sources reported previously.[5,6,8] Male control TK-NOG and humanized TK-NOG mice (20–30 g body weight)[9] were used in this study. All mouse studies were performed in accordance with the guidelines of and were approved by the Animal Care Committee of the Japan Central Institute for Experimental Animals. All studies involving mouse tissue with transplanted human cells were approved by the Ethical and Biosafety Committee of the Japan Central Institute for Experimental Animals. Eight weeks old male TK-NOG mice received intraperitoneal injections of sodium ganciclovir (Denosine-IV; Mitsubishi Tanabe Pharma, Osaka, Japan) on days -7 and -5 prior to transplantation. One week after ganciclovir treatment, the degree of liver damage was examined by determining serum aspartate aminotransferase and alanine aminotransferase values (FUJI DRI-CHEM 7000; Fujifilm, Tokyo, Japan). For transplantation, $^{1} \times 10^{6}$ commercially available cryopreserved human hepatocytes (4-year-old, female; Lonza Walkersville, MD) were transplanted by intra-splenic injection as described.[9] Twelve weeks after transplantation, small amounts of blood were collected and human albumin concentrations were measured with the Human Albumin ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, TX) according to the manufacturer's protocol. In these chimeric mice, >80% of liver cells were estimated to be replaced with human hepatocytes, as judged by measurements of human albumin concentrations in plasma.[9]

In vivo Analysis

The United States Food and Drug Administration recommends the choice of midazolam for initial *in vivo* drug interaction studies for human P450 3A enzymes (http://www.fda.gov/). Blood samples were collected prior to and 5, 35, 95, 215, and 395 min after single oral doses

of midazolam (10 mg kg^{-1}) administered to four animals. Racemic thalidomide (100 mg kg^{-1}, Wako Pure Chemicals, Osaka, Japan) was orally administered 30 min before the midazolam treatment for the ligand cooperativity study. This dose was chosen because of the known pharmacokinetic profile[8] and the lack of apparent toxicity of thalidomide. In separated experiments, thalidomide (100 mg kg^{-1}) was administered to mice daily for 3 days for the induction study and followed by intravenous injection of midazolam on the fourth day. After treatment of the plasma samples with β-glucuronidase (from *Ampullaria*, 100,000 units, Wako Pure Chemicals, Osaka, Japan), a 4-fold volume of CH_3OH containing 0.0625 μM caffeine (as an internal standard) was added. The aqueous supernatant was centrifuged at 2×10^3 *g* for 10 min at 4 °C and analyzed using LC-MS. The use of animals for this study was approved by the Ethics Committees of the Japan Central Institute for Experimental Animals and Showa Pharmaceutical University.

LC-MS/MS analyses

LC-MS/MS analyses of midazolam and 1′- and 4-hydroxymidazolam, were performed as described previously[5] with slight modification. A Quattro micro API mass analyzer was used for the metabolite analysis (Waters, Tokyo, Japan). The instrument was operated in the electrospray negative ionization mode and was directly coupled to a Waters LC 2695 system with an octadecylsilane C_{18} column (Atlantis, 3 μm, 2.1 mm × 50 mm) and MassLynx NT4.1 software for data acquisition (Waters). To tune the mass spectrometer, the cone voltage was optimized to maximize the intensity of the precursor ions for midazolam at *m/z* 326.15. The collision energy was then adjusted to optimize the signal. Typical tuning conditions were as follow: electrospray capillary voltage, 3.0 kV; sample cone voltage, 36 V; and collision energy, 21.0 eV at a collision gas (Ar) pressure of 1.6×10^{-4} kPa. The gradient mobile phase consisted of 0.1% CH_3CO_2H and CH_3CN in 0.1% CH_3CO_2H (v/v): 0–2.5 min with 0 to 95% CH_3CN (v/v) with 0.1% CH_3CO_2H (v/v); 3–6.5 min with 95% CH_3CN (v/v); 6.5–7.0 min with 95 to 0% CH_3CN with 0.1% CH_3CO_2H (v/v); 7.0–13 min with 0.1% CH_3CO_2H (v/v), all at a flow rate of 0.25 mL min^{-1}. Midazolam, its metabolites 1′- and 4-hydroxytmidazolam, and the internal standard caffeine were quantified using the *m/z* 326→291 transition of midazolam, the *m/z* 342→325 transition of 1′- and 4-hydroxymidazolam, and the *m/z* 195→138 transition of caffeine respectively.

Concentrations of midazolam and its metabolites were kinetically analyzed by WinNonlin software (Pharsight, Sunnyvale, CA) and statically evaluated by paired *t*-test using Prism (Graphpad, San Diego, CA).

Results

Because thalidomide was detected in mouse plasma 30 min after an oral single administration, as described previously,[7,8] intravenous midazolam administration (10 mg/kg) followed 30 min after thalidomide treatment (100 mg/kg) of male TK-NOG mice.[9] Thalidomide was previously administered orally to NOG mice daily for 3 days (100 mg/kg) and followed by intravenous injection of midazolam on the fourth day. Midazolam and 1′- and 4-hydroxymidazolam were detected by LC-MS/MS analysis in mouse plasma samples prior to or 5 to 395 min after midazolam treatment. The time-dependent profiles of

midazolam and its metabolites in chimeric mice with humanized liver are presented in Figure 1. In the control mice, midazolam was cleared from the plasma with a clearance (CL) value of 0.058 L/kg min^{-1} (Table 1). The main metabolite was 1′-hydroxymidazolam under these conditions. Co-treatment with thalidomide caused weak inhibition of midazolam oxidation but did not affect the CL or AUC significantly (Table 1). In contrast, midazolam clearance was enhanced (1.6-fold) by co-treatment with thalidomide (100 mg kg^{-1}) in chimeric mice with humanized liver (Figure 1A, Table 1). Significantly higher AUC of the major metabolite 1′-hydroxymidazolam (1.7-fold, Figure 1B, Table 1) was observed when thalidomide was used, but not for the minor metabolite 4-hydroxymidazolam (Figure 1C), apparently due to heterotropic cooperativity of human P450 3A enzymes *in vivo*. Pretreatment with thalidomide induced midazolam clearance in humanized NOG mice (Figure 1A, Table 1), but the low AUC of the minor metabolite 4-hydroxymidazolam was increased 3.5-fold (Figure 1C, Table 1) under these conditions. The pharmacokinetics of thalidomide and its metabolites in the humanized mice were described previously,[8] and rapid disappearance from the plasma to undetectable levels had been confirmed to occur within 24 h after oral treatment (100 mg kg^{-1}).

Discussion

Limited information is available regarding thalidomide metabolism and drug interactions *in vivo*, especially in humans. With renewed interest in thalidomide, subsequent work has confirmed that (aromatic ring) 4- and 5-hydroxylated metabolites of thalidomide are recovered in the urine of rabbits but not from rats.[10,11] Because humanized mice may generate more of the epoxide metabolite of thalidomide than control mice,[7,8] humanized liver might generally be a better model for possible drug interactions of thalidomide with midazolam. These results were similar when evaluated using *in vitro* human liver microsomes or recombinant P450 3A4/5 enzymes[5] and hepatic cell culture systems (data not shown) with regard to ligand cooperativity for the P450 3A enzymes.

Because human P450s 3A4 and 3A5 would be activated by heterotropic cooperativity of thalidomide, transformation of thalidomide to its 1′-hydroxymetabolite were enhanced by co-administration in chimeric mice with humanized liver (Fig. 1 and Table 1). When the total mass balance was considered, an increase in the AUC of 1′-hydroxymidazolam after co-administration with thalidomide would be recognized, probably because of the slower elimination of 1′-hydroxymidazolam than the parent compound. In our preliminary experiments using human hepatocyte systems, thalidomide, but not the primary human metabolite 5-hydroxythalidomide, was a good activator for P450 3A enzymes responsible for midazolam 1′-hydroxylation. Regarding ligand cooperativity, unmetabolized thalidomide was responsible for activation of human P450 3A enzymes. Because the primary metabolites of thalidomide might not be involved the P450 3A activation, the secondary reactive epoxide metabolite(s) derived from 5-hydroxythalidome should also not be the human P450 3A activator This hypothesis was consistent with the high plasma concentrations of 1′-hydroxymidazolam in chimeric mice at the earliest time after the administration of thalidomide when parent compound concentrations would be sufficient. On the other hand, human P450 3A4 in chimeric livers might be more highly induced than P450 3A5 in the case of pretreatment with thalidomide, resulting in AUC of 4-hydroxymetabolite being

predominantly increased. Rapid elimination of 4-hydroxymidazolam might be accounted for possible secondary metabolism of the primary metabolite mediated by induced human P450 3A4. Anyway, differences in species susceptibility may result from differences in the *in vivo* disposition and biotransformation of thalidomide by drug-metabolizing enzymes, resulting in drug interactions.

In the present study, we analyzed the effects of thalidomide on midazolam metabolism *in vivo* using humanized NOG mice in which the liver was replaced with transplanted human liver cells. Thus, the present results address the issue of drug interactions of thalidomide *in vivo* (Figure 1). The possible metabolism of thalidomide by intestinal mouse P450s has not yet been considered in a setting in which the drug is administered orally to chimeric mice with humanized liver.

In conclusion, the present study demonstrates that human liver cells expressed in chimeric NOG mice effectively mediate enhancement of midazolam metabolism by thalidomide *in vivo* via ligand cooperativity. The possibilities of reactive metabolite formation and of drug interactions during thalidomide therapy in humans should be evaluated further.

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Abbreviations

AUC	area-under-plasma-concentration time curve
CL	clearance
NOG mice	non-obese diabetes-severe combined immunodeficiency-interleukin-2 receptor gamma chain-deficient mice

References

1. Rendic S, Guengerich FP. Update information on drug metabolism systems—2009, part II: summary of information on the effects of diseases and environmental factors on human cytochrome P450 (CYP) enzymes and transporters. Curr Drug Metab. 2010; 11:4–84. [PubMed: 20302566]
2. Calabrese L, Resztak K. Thalidomide revisited: pharmacology and clinical applications. Expert Opin Invest Drugs. 1998; 7:2043–2060.
3. Kim JH, Scialli AR. Thalidomide: the tragedy of birth defects and the effective treatment of disease. Toxicol Sci. 2011; 122:1–6. [PubMed: 21507989]
4. Trapnell CB, Donahue SR, Collins JM, Flockhart DA, Thacker D, Abernethy DR. Thalidomide does not alter the pharmacokinetics of ethinyl estradiol and norethindrone. Clin Pharmacol Ther. 1998; 64:597–602. [PubMed: 9871424]
5. Okada Y, Murayama N, Yanagida C, Shimizu M, Guengerich FP, Yamazaki H. Drug interactions of thalidomide with midazolam and cyclosporine A: heterotropic cooperativity of human cytochrome P450 3A5. Drug Metab Dispos. 2009; 37:18–23. [PubMed: 18948377]
6. Chowdhury G, Murayama N, Okada Y, Uno Y, Shimizu M, Shibata N, Guengerich FP, Yamazaki H. Human liver microsomal cytochrome P450 3A enzymes involved in thalidomide 5-hydroxylation

and formation of a glutathione conjugate. Chem Res Toxicol. 2010; 23:1018–1024. [PubMed: 20443640]

7. Yamazaki H, Suemizu H, Igaya S, Shimizu M, Shibata M, Nakamura M, Chowdhury G, Guengerich FP. *In vivo* formation of a glutathione conjugate derived from thalidomide in humanized uPA-NOG mice. Chem Res Toxicol. 2011; 24:287–289. [PubMed: 21299192]
8. Yamazaki H, Suemizu H, Shimizu M, Igaya S, Shibata N, Nakamura N, Chowdhury G, Guengerich FP. *In vivo* formation of dihydroxylated and glutathione conjugate metabolites derived from thalidomide and 5-hydroxythalidomide in humanized TK-NOG mice. Chem Res Toxicol. 2012; 25:274–276. [PubMed: 22268628]
9. Hasegawa M, Kawai K, Mitsui T, Taniguchi K, Monnai M, Wakui M, Ito M, Suematsu M, Peltz G, Nakamura M, Suemizu H. The reconstituted 'humanized liver' in TK-NOG mice is mature and functional. Biochem Biophys Res Commun. 2011; 405:405–410. [PubMed: 21238430]
10. Eriksson T, Bjorkman S, Roth B, Bjork H, Hoglund P. Hydroxylated metabolites of thalidomide: formation *in-vitro* and *in-vivo* in man. J Pharm Pharmacol. 1998; 50:1409–1416. [PubMed: 10052858]
11. Schumacher H, Smith RL, Williams RT. The metabolism of thalidomide: the fate of thalidomide and some of its hydrolysis products in various species. Br J Pharmacol Chemother. 1965; 25:338–351. [PubMed: 5866716]

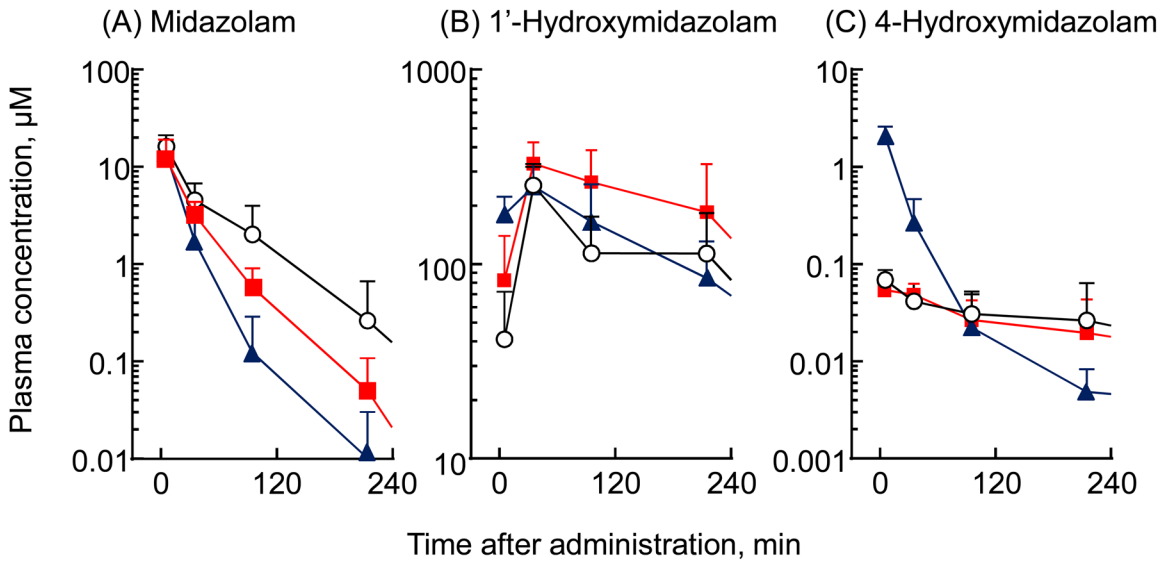

Figure 1. Plasma concentrations of midazolam (A), 1′-hydroxymidazolam (B), and 4-hydroxymidazolam (C) in chimeric NOG mice with humanized liver after single administrations intravenously (10 mg kg^{-1}) without (○) or with co-administration (■) or pretreatment (▲) with thalidomide (100 mg kg^{-1})

Thalidomide (100 mg kg^{-1}) was orally co-administered or administered daily for three days prior to intravenous midazolam treatment (10 mg/kg) in chimeric NOG mice with humanized liver. Results are expressed as mean values ± SD obtained with four mice in each set (*$p < 0.05$).

Table 1

Pharmacokinetic parameters for midazolam and its metabolites in control NOG mice and chimeric mice with humanized liver after single administration intravenously (10 mg kg^{-1}), with or without co-administration or pretreatment of thalidomide (100 mg kg^{-1}).

Experiments	CL (L/kg min^{-1})	$AUC_{0-\infty}$ (μM min)		
	Midazolam	Midazolam	1′-Hydroxy-midazolam	4-Hydroxy-midazolam
1) Control NOG mice				
Midazolam alone	0.058 ± 0.005	536 ± 46	29700 ± 6800	72.7 ± 5.3
Co-administered with thalidomide	0.051 ± 0.010	616 ±130	14300 ± 2500	51.2 ±15.3
2) Mice with humanized liver				
Midazolam alone	0.049 ± 0.022	759 ± 431	42600 ± 15000	15.0 ± 8.0
Co-administered with thalidomide	0.078 ± 0.034*	459 ± 202	72300 ± 31500*	14.0 ± 5.9
Pre-treated with thalidomide	0.087 ± 0.031*	383 ± 120	46800 ± 18000	52.1 ±16.7*

Thalidomide (100 mg kg^{-1}) was orally co-administered or administered daily for three days prior to intravenous midazolam treatment (10 mg/kg) in control NOG mice and chimeric NOG mice with humanized liver. Data are means ± SD with four mice in each set (*$p < 0.05$).