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***In vivo* Formation of Dihydroxylated and Glutathione Conjugate Metabolites derived from Thalidomide and 5-Hydroxythalidomide in Humanized TK-NOG mice**

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Abstract

The formation of dihydroxythalidomide and glutathione (GSH) conjugate(s) of 5-hydroxythalidomide were investigated in chimeric mice modified with “humanized” liver: novel humanized TK-NOG mice were prepared by introduction of thymidine kinase, followed by induction with ganciclovir, and human liver cells were transplanted. Following oral administration of racemic thalidomide (100 mg/kg), plasma concentrations of 5-hydroxy- and dihydroxythalidomide were higher in humanized mice than in controls. After administration of 5-hydroxythalidomide (10 mg/kg), higher concentrations of dihydroxythalidomide were detected. These results indicate that livers of humanized mice mediate thalidomide oxidation, leading to catechol and/or the GSH conjugate *in vivo* and suggest that thalidomide activation occurs.

Thalidomide was previously withdrawn because of its teratogenic effects in humans but has been approved for the treatment of refractory multiple myeloma.^{1,2} It has been shown that rabbits are thalidomide-sensitive and rodents are thalidomide-resistant.^{1,2} Human P450 3A enzymes in liver mediate thalidomide 5-hydroxylation and further oxidation, leading to non-enzymatic GSH conjugation,^{3,4} which may be relevant to the pharmacological and toxicological actions. However, it is not known if these two-step aromatic oxidations of thalidomide and the trapping of reactive metabolites by GSH occur in human *in vivo* situations, especially secondary catechol formation and other oxidation. “Humanized” mice, in which various kinds of human cells can be engrafted and retain the same functions as in humans, are extremely useful.^{5,6} Recently developed TK-NOG mice⁶ were treated to express a herpes simplex virus type 1 thymidine kinase (HSVtk) within the livers of severely immunodeficient NOG mice and induced by a non-toxic dose of ganciclovir, and human

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Supporting Information. Experimental Procedures, a representative ESI-LC-MS/MS chromatogram of the dihydroxythalidomide, and a representative ESI-LC-MS/MS chromatogram of dihydroxythalidomide formed *in vivo*. This material is available free of charge via the Internet at <http://pubs.acs.org>.

liver cells were transplanted in the absence of ongoing drug treatment. Compared to other “humanized” system approaches, TK-NOG mice did not develop systemic morbidity (e.g., liver disease, renal disease, bleeding diathesis), nor were drug treatments required to suppress liver tumor development. Since such success has not previously been achieved in other liver reconstruction models,^{5,6} it is now possible to expand applications by human hepatocyte transplantation. The purpose of this study was to investigate the oxidative metabolism of thalidomide by human liver *in vivo* and to better understand the activation of thalidomide in TK-NOG mice containing human liver cells. Secondary oxidized thalidomide metabolites and conjugation of 5-hydroxythalidomide with GSH *in vivo* are reported here, indicating that activation of thalidomide occurs to produce an electrophilic product in humanized mice.

The metabolism of 5-hydroxythalidomide was first investigated *in vitro*, based on earlier work² (Figure S1, Supporting Information). 5-Hydroxythalidomide was oxidized to dihydroxythalidomide, possibly a mixture of catechol and other dihydroxylated metabolites, by recombinant P450 3A4 as judged from LC-MS analysis (m/z 289, $[M+H]^+$ 16). Consequently, the molecular ion peak (t_R 6.3 min) was also detected in plasma samples from TK-NOG mice following an oral administration of thalidomide (Figure 1 and Figure S2).

Thalidomide and its primary metabolite, 5'- or 5-hydroxythalidomide, and its secondary metabolite dihydroxythalidomide were detected by LC-MS/MS analysis in plasma samples obtained from mice following oral administration (100 mg/kg) (the t_R of thalidomide and 5'- or 5-hydroxythalidomide was 6.6 and 6.9 min, respectively, under these conditions^{3,4}). Thalidomide was detected 30 min after oral administration (Figure 2A). Plasma concentrations of 5'-thalidomide were significantly higher in control TK-NOG mice than in humanized TK-NOG mice (Figure 2B). In contrast, 5-hydroxythalidomide—a thalidomide metabolite known to be activated to reactive epoxide products²—was preferentially produced in humanized TK-NOG mice (Figure 2C). Dihydroxythalidomide (Figure 2D) and 5-hydroxythalidomide-GSH conjugate(s)³ (Figure 2E) formed *in vivo* were measured using LC-MS/MS (m/z 289→177 transition (Figure 1D) and m/z 580→451 transition,² respectively). Similarly, dihydroxythalidomide was preferentially produced in humanized TK-NOG mice (Figure 2D and Figure S2). Low levels of 5-hydroxythalidomide-GSH conjugates were detected in plasma samples from thalidomide administered to either control NOG mice or humanized NOG-NOG mice (Figure 2E).

Following administration of the primary metabolite 5-hydroxythalidomide (10 mg/kg), significantly higher concentrations of dihydroxythalidomide were detected (Figure 3A). 5-Hydroxythalidomide-GSH conjugates were also detected in plasma samples after 5-hydroxythalidomide was administered to either control or humanized NOG mice (Figure 3B). Although humanized mice may generate more epoxide metabolite than control mice, mouse liver samples generally might have higher glutathione *S*-transferase activity than human liver samples. In separate experiments in which 5-hydroxythalidomide (27 mg/kg) was orally administered to a different kind of chimeric uPA-NOG mice, humanized mice showed a 3-fold higher plasma concentration of 5-hydroxythalidomide-GSH conjugate than control mice 2 h after administration (results not shown). These results were not altered appreciably when evaluated by another parameter, AUC₀₋₇, which had similar or less sensitive statistical power, than plasma concentrations.

Numerous hypotheses have been proposed to explain thalidomide-induced teratogenesis, including oxidative stress, growth control of angiogenesis, and gene expression control for adhesion receptors.⁷⁻⁹ With renewed interest in thalidomide, earlier work showing the presence of phenolic products produced in rabbits (a sensitive species) but not in rats^{10,11} has been developed again. Subsequent work has confirmed that the 4- and 5-hydroxylated

and 5'-hydroxylated products are formed in various species including in humans.^{10,12–15} Schumacher *et al.*¹¹ reported that (aromatic ring) 4- and 5-hydroxylated metabolites of thalidomide were recovered in the urine of rabbits but not from rats, and more thalidomide metabolites were bound to liver macromolecules in the rabbit than in the rat. Differences in species susceptibility may result from differences in biotransformation of the compound by drug metabolizing enzymes. Limited information is available regarding thalidomide metabolism *in vivo*, especially in humans.

In the present study, we analyzed thalidomide metabolism *in vivo* using humanized TK-NOG mice in which the liver was replaced with transplanted human liver cells. In spite of one of the long-held tenants of thalidomide metabolism, that it is rapidly and non-enzymatically hydrolyzed,^{1,2} preferentially higher levels of 5-hydroxy- and dihydroxythalidomide (that would be metabolized by human P450 3A enzymes³) were observed in humanized mouse plasma samples (Figure 2). The present results (administration of 5-hydroxythalidomide to humanized mice) address the issue of reactive metabolite formation *in vivo* (Figure 3). GSH was attached to the phenyl ring of 5-hydroxythalidomide.³ The presence of phenolic derivatives of thalidomide suggests that the drug might undergo oxidative metabolism via an arene oxide intermediate.¹⁶ Our *in vitro* work showed that the conjugate can best be explained by an epoxide intermediate.² Thus, congenital malformation of limbs in children caused by thalidomide may result from an arene oxide metabolite that covalently binds to critical structures in the developing fetus, as earlier proposed by Gordon et al.¹⁶ The possible production of reactive metabolites of thalidomide by intestinal mouse P450s has not yet been considered (when the drug is administered orally to chimeric mice with humanized liver). Taken together, evidence is presented that 5-hydroxythalidomide can be further oxidized by human liver and finally forms (multiple) phenyl ring-based 5-hydroxythalidomide-GSH conjugates via a highly reactive and unstable intermediate(s). It is possible that reactive intermediates produced from the bio-transformation of thalidomide are acting through the disruption of signaling pathways that are necessary for normal development and morphogenesis.

In conclusion, the present study demonstrates that human liver cells expressed in novel chimeric TK-NOG mice effectively mediate thalidomide 5-hydroxylation and further oxidation leading to dihydroxythalidomide and/or a GSH conjugate *in vivo*, which may be relevant to its pharmacological and toxicological actions via adduct formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AUC	area under plasma concentration–time curve
HSVtk	herpes simplex virus type 1 thymidine kinase

NOG mice non-obese diabetes- severe combined immunodeficiency- interleukin-2 receptor gamma chain-deficient mice

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Figure 1.

Representative ESI-LC-MS chromatograms (A) and mass spectra (B) of dihydroxythalidomide formed *in vivo*. The extracted ion chromatogram of the product ion with m/z 289 of dihydroxythalidomide is shown for the plasma from chimeric mouse with humanized liver treated with thalidomide. The mass spectrum in B was obtained from the peak of t_R 6.3 min shown in A.

Figure 2.

Plasma concentrations of thalidomide (A), 5'-hydroxythalidomide (B), 5-hydroxythalidomide (C), dihydroxythalidomide (D), and GSH conjugate (E) in control TK-NOG mice (○) and chimeric TK-NOG mice with humanized liver cells (●) after oral administration of thalidomide (100 mg/kg). Results are expressed as mean values ($\pm$ SD) obtained with four mice (* p < 0.05 and ** p < 0.01, two-way ANOVA with Bonferroni post tests).

Figure 3.

Plasma concentrations of dihydroxythalidomide (A) and GSH conjugate (B) in control TK-NOG mice (○) and chimeric TK-NOG mice with humanized liver cells (●) after oral administration of 5-hydroxythalidomide (10 mg/kg). Results are expressed as mean values ($\pm$ SD) obtained with four mice (* $p < 0.05$, two-way ANOVA with Bonferroni post tests). 5-Hydroxythalidomide concentrations were lower than the detection level.