SUPPORTING INFORMATION

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

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TABLE OF CONTENTS

Experimental Procedures

Chemicals used were from sources reported previously.[1-3] Male control TK-NOG and humanizedTK-NOG mice (~20-30 g body weight)[4] were used in this study. 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.[4] Blood samples were collected 0.5, 1, 2, 4, and 7 h after single oral doses of racemic thalidomide (100 mg/kg, Wako Pure Chemicals, Tokyo, Japan) and 5-hydroxythalidomide[2] (10 mg/kg) administered to four animals (this dose was chosen because of the lack of apparent toxicity of thalidomide). After treatment of the plasma with one-tenth volume of ice-cold 60% (w/v) $HClO_4$, 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. Thalidomide hydroxylation activities were determined using LC-MS.[1,2] Briefly, a typical incubation mixture (total volume 0.20 mL) contained recombinant P450 3A4 (0.10 μM), an NADPH-generating system (0.25 mM $NADP^+$, 2.5 mM glucose 6-phosphate, and 0.25 unit mL^{-1} yeast glucose 6-phosphate dehydrogenase), and thalidomide (1.0 mM) in 0.10 M potassium phosphate buffer (pH 7.4), unless otherwise specified. Incubations were carried out at 37 °C for 60 min. In order to trap an intermediate metabolite(s), GSH (5.0 mM) was added to the mixtures. Incubations were terminated by adding 0.20 mL of ice-cold Cl_3CCO_2H. Following centrifugation at 2×10^3 *g* for 10 min, the aqueous supernatant was analyzed using an LC-MS system.

LC-MS/MS analyses of thalidomide, 5- or 5'-hydroxythalidomide, dihydroxythalidomide, and the 5-hydroxythalidomide glutathione conjugate were performed as described previously[1,3] with slight modification. An LCQ Duo mass analyzer (ThermoFisher Scientific, Yokohama, Japan) equipped with Xcalibur software was operated in the electrospray negative and positive ionization modes and was directly coupled to an Agilent 1100 system (Agilent Technology, Tokyo, Japan) equipped with an octadecylsilane (C_{18}) column (XBridge, 3.5 μm, 2.1 mm × 150 mm, Waters, Tokyo, Japan). LC conditions were as follows: Buffer A contained 0.1% CH_3CO_2H in CH_3CN and Buffer B contained 0.1% CH_3CO_2H in H_2O (v/v). The following gradient program was used, with a flow rate of 0.25 mL min^{-1}: 0-3 min, linear gradient from 0% A to 95% A (v/v); 3-8 min, hold at 95% A; 8-10.5 min, linear gradient to 0% A; 10.5-14 min, hold at 0% A. The temperature of the column was maintained at 35 °C. Samples (5 μL) were infused with an auto-sampler. MS analyses were performed in the negative ion mode for thalidomide and its hydroxylated metabolites and in the positive mode for the GSH conjugate.[1] The mass spectrometer was tuned using thalidomide, 5'- and 5-hydroxythalidomide, and dihydroxythalidomide. Thalidomide, 5'- and 5-hydroxythalidomide, and dihydroxythalidomide were quantified using the *m/z* 257→146 transition of thalidomide, the *m/z* 273→146 transition of 5'-hydroxythalidomide, and the *m/z* 273→161 transition of 5-hydroxythalidomide, and the *m/z* 289→177 transition of dihyroxythalidomide, respectively. The 5-hydroxythalidomide-GSH conjugate was measured using the *m/z* 580→451 transition, on the basis of a standard curve of 5-hydroxythalidomide. Statistical analysis for the plasma concentrations of thalidomde and

its metabolites in control and humanized mice was done using two-way analysis of valiance (ANOVA) with Bonferroni post tests (Prism, GraphPad Software, La Jolla, CA). Area under the curve (AUC) values were derived from plots of thalidomide and its metabolites vs time and were calculated with the trapezoidal rule with the program WinNonlin (Pharsight, Sunnyvale, CA) .

References

(1) Chowdhury, G., Murayama, N., Okada, Y., Uno, Y., Shimizu, M., Shibata, N., Guengerich, F. P., and Yamazaki, H. (2010) Human liver microsomal cytochrome P450 3A enzymes involved in thalidomide 5-hydroxylation and formation of a glutathione conjugate. *Chem. Res. Toxicol. 23*, 1018-1024.

(2) Yamamoto, T., Shibata, N., Sukeguchi, D., Takashima, M., Nakamura, S., Toru, T., Matsunaga, N., Hara, H., Tanaka, M., Obata, T., and Sasaki, T. (2009) Synthesis, configurational stability and stereochemical biological evaluations of (*S*)- and (*R*)-5-hydroxythalidomides. *Bioorg. Med. Chem. Lett. 19*, 3973-3976.

(3) Yamazaki, H., Suemizu, H., Igaya, S., Shimizu, M., Shibata, M., Nakamura, M., Chowdhury, G., and Guengerich, F. P. (2011) *In vivo* formation of a glutathione conjugate derived from thalidomide in humanized uPA-NOG mice. *Chem. Res. Toxicol. 24*, 287-289.

(4) Hasegawa, M., Kawai, K., Mitsui, T., Taniguchi, K., Monnai, M., Wakui, M., Ito, M., Suematsu, M., Peltz, G., Nakamura, M., and Suemizu, H. (2011) The reconstituted 'humanized liver' in TK-NOG mice is mature and functional. *Biochem. Biophys. Res. Commun. 405*, 405-410.

Figure S1. Representative ESI-LC-MS chromatogram (A) and mass spectra (B) of dihydroxythalidomide formed *in vitro.*
The extracted ion chromatogram of the product ion with *m/z* 289 (of dihydroxythalidomide formed from 5-hydoroxythalidomide) is shown for *in vitro* P450 3A4 reaction mixtures.

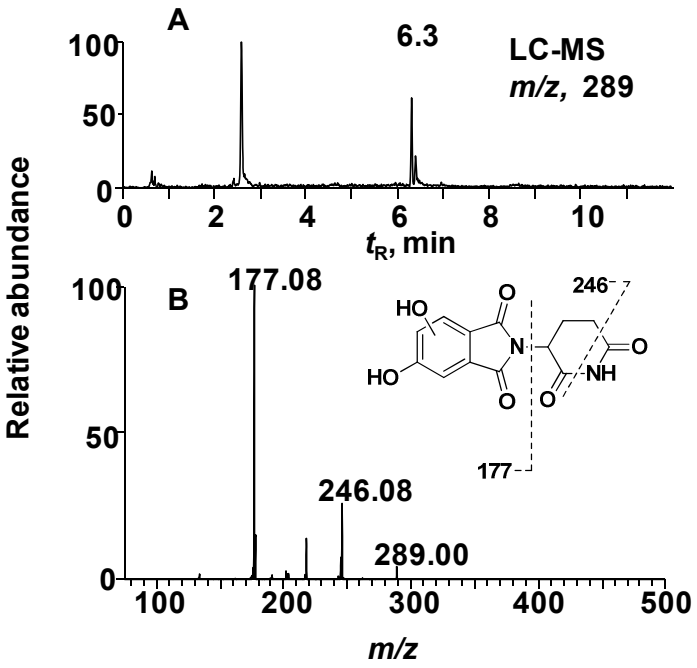

Figure S2. Representative ESI-LC-MS/MS chromatogram of dihydroxythalidomide. An extracted ion chromatogram of the product ion with *m/z* 289 of dihydroxythalidomide is shown for plasma from chimeric mice containing humanized liver treated with thalidomide.