SUPPORTING INFORMATION

In Vivo Formation of a Glutathione Conjugate Derived from Thalidomide in Humanized uPA-NOG Mice

Hiroshi Yamazaki, Hiroshi Suemizu, Sho Igaya, Makiko Shimizu, Norio Shibata, Masato Nakamura, Goutam Chowdhury, and F. Peter Guengerich

Chem. Res. Toxicol. 24, xx-xxx (2011)

TABLE OF CONTENTS

.

Experimental Procedures

Chemicals used were from previously reported sources *(1, 2)*. Male control uPA-NOG and humanized uPA-NOG mice (~20-30 g body weight) *(3)* were used in this study. In these chimeric mice, >70% of liver cells were estimated to be replaced with human hepatocytes, as judged by measurements of human albumin concentrations in plasma *(3)*. Blood samples were collected at 0.5, 1, 2, 4 and 7 h after single oral doses of racemic thalidomide (270 mg/kg, Wako Pure Chemicals, Tokyo, Japan) 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% (v/v) $HClO_4$, the aqueous supernatant was centrifuged at 2×10^3 *g* for 10 min at 4 °C and analyzed using LC-MS. Pooled liver microsomes from male uPA-NOG mice *(3)* were prepared in 10 mM Tris-HCl buffer (pH 7.4) containing 0.10 mM EDTA and 20% (v/v) glycerol. The use of animal livers 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)*. Briefly, a typical incubation mixture (total volume of 0.20 mL) contained microsomal protein (1.0 mg mL^{-1}) or recombinant P450 (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, and the 5-hydroxythalidomide glutathione conjugate were performed as described previously *(1)* with slight modifications. 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. The mass spectrometer was tuned using thalidomide and 5'- and 5-hydroxythalidomide. Thalidomide and 5'- and 5-hydroxythalidomide 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, 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).

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) Suemizu, H., Hasegawa, M., Kawai, K., Taniguchi, K., Monnai, M., Wakui, M., Suematsu, M., Ito, M., Peltz, G., and Nakamura, M. (2008) Establishment of a humanized model of liver using NOD/Shi-scid IL2Rgnull mice. *Biochem. Biophys. Res. Commun. 377*, 248-252.

Figure S1. Representative ESI-LC-MS/MS chromatogram of the GSH conjugate of oxidized 5-hydroxythalidomide. Extracted ion chromatogram of the product ion with *m/z* 451 of the 5-hydroxythalidomide-GSH conjugate in the plasma from chimeric mouse with humanized liver.