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# Detection of Efflux of Reactive Metabolites of Troglitazone from Primary Human Hepatocytes Using Stable Isotope Labeled Glutathione

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## PURPOSE

Drug-induced liver toxicity is one of the most common adverse drug effects, and formation of reactive metabolites (RM) could be a contributing factor. Troglitazone (TGZ), the model compound used in this study, was withdrawn from the market due to a high incidence of liver failure. The metabolism of TGZ is known to generate RM, which were detected as stable glutathione (GSH) conjugates in vivo and in vitro. We previously reported application of stable isotope-labeled (SIL) GSH methodology to trap and detect RM of TGZ generated in cryopreserved human hepatocytes in suspension. A major finding was different degrees of incorporation of exogenous GSH/SIL-GSH mixture into trapped RM. More recently, we have also reported evidence, obtained using oil-filtration methodology, in support of one of the hypothesized mechanisms: differential efflux of RM from cryopreserved human hepatocyte suspensions, followed in some cases by extracellular trapping as GSH conjugates. The aim of this work was to investigate if efflux of RM is also intrinsic to primary cultures of human hepatocytes.

## METHOD

Materials: TGZ and ($^{13}C_2$,$^{15}$N-Gly)-GSH (isotopic purity>90%) were purchased from Toronto Research Chemicals (Toronto, ON, Canada) and Cambridge Isotope Labs (Cambridge, MA, USA), respectively. All other reagents were of analytical or HPLC grade (Sigma, St. Louis, MO, USA).

Biology: Primary cultures of hepatocytes were purchased from Life Technologies (Grand Island, NY, USA), TRL (Research Triangle Park, NC, USA), and Bioreclamation IVT (Baltimore, MD, USA). Incubations with TGZ (5 and 50 µM), with and without addition of a mixture of GSH/SIL-GSH (ratio 1:1, total concentration 5 mM), were performed in duplicate. The medium was removed for analysis and replaced with fresh medium after each 24-hour interval. After 72 hours, cells were extracted for metabolite profiling using LC-HRAMS analysis. The concentrated medium (5-fold) and cell extracts were analyzed by reverse phase HPLC-HRAMS implemented on an LTQ Orbitrap XL equipped with HESI-II probe. The Orbitrap was operated at resolution 60,000 in survey scans (range 250-900 Th) and at resolution 15,000 in MSn scans The acquired data files were processed manually for the presence of known GSH conjugates using Xcalibur software (Thermo). In addition, a comprehensive search for GSH conjugates was performed using MetWorks software (Thermo) via automatic detection of the monoisotopic pattern characteristic for the GSH/SIL-GSH mixture (split 3.0037 Da); a range of peak intensity ratios from 1:0.1 to 1:0.9 was evaluated.

## RESULTS

At 5 µM TGZ, no trapped RM were detected without GSH/SIL-GSH addition and limited metabolite coverage was observed in "+GSH/SIL-GSH" samples. Data processing has revealed the presence of a number of known metabolites, including GSH conjugates, in the culture medium from cells treated with 50 uM TGZ (Figure 1). The levels of GSH conjugates were both donor-dependent and greatly increased by GSH/SIL-GSH addition; moreover, in some donors a few GSH conjugates were not detected at all in the absence of exogenous GSH/SIL-GSH. The observed ratio of GSH/SIL-GSH (Figure 2) in the medium allows for grouping of GSH conjugates into those showing: i) no incorporation of SIL-GSH (e.g., M5); ii) significant incorporation (e.g., M2i), and iii) intermediate incorporation (e.g., M3). Similar results – different levels of incorporation of GSH/SIL-GSH in different conjugates – were obtained for trapped RM detected in cell extract samples (Figure 3). Besides the trapped RM already described, a novel conjugate (with a high level of GSH/SIL-GSH incorporation) was detected and its structure was tentatively assigned based on HRAMS$^n$ data (Figure 4) as the net result of bis-oxygenation, thiazolidinedione ring scission, GSH conjugation (plus hydrolysis). The different levels of incorporation of exogenous SIL-GSH in trapped RM in the medium are most likely due to differences in the rate and/or extent of efflux of different RM, which are then trapped extracellularly by exogenous GSH and SIL-GSH. The efflux of reactive metabolites from hepatocytes was previously observed only for small lipophilic metabolites such as naphthalene oxide. While such efflux could be due to passive diffusion, the observed differences in the rate of efflux of RM of TGZ imply the existence of a specific, transport-mediated mechanism. It follows that inactivation of efflux transporters by RM (substrates that are also chemically reactive) could be a contributing factor in hepatotoxicity.

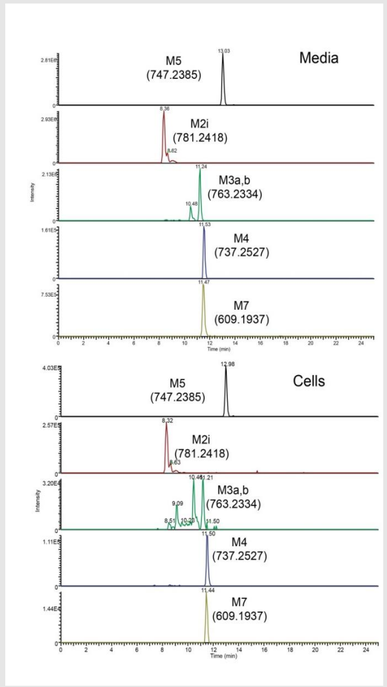


**Figure 1. XIC Traces of GSH Conjugates in Media and Cells Extracts**

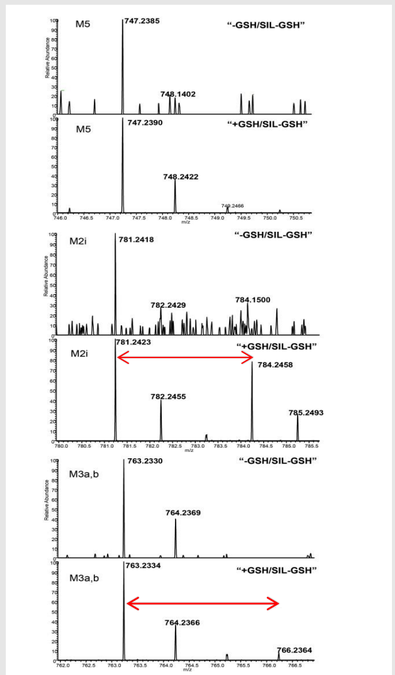


**Figure 2. HRAMS Survey Spectra of GSH Conjugates in Media**

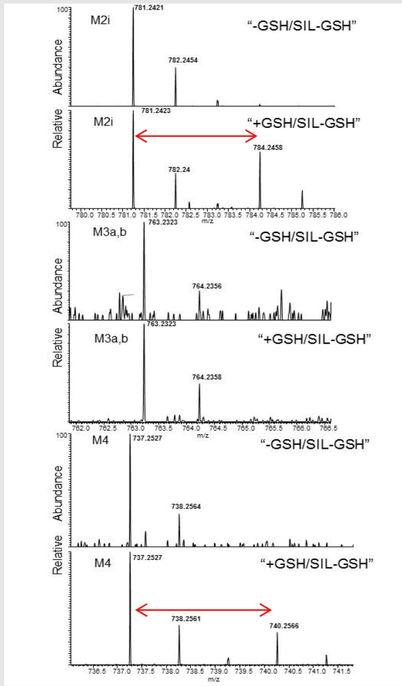


**Figure 3. HRAMS Survey Spectra of GSH Conjugates in Cells Extracts**

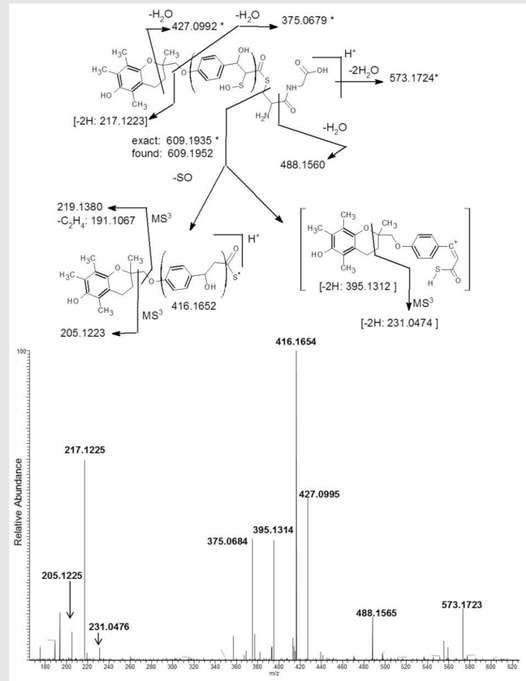


**Figure 4. HRAMS$^n$ Spectra of Novel GSH Conjugate and Tentative Structure Assignment**

## CONCLUSION

Addition of a mixture of GSH/SIL-GSH to primary cultures of human hepatocytes incubated with TGZ resulted in detection of a number of GSH conjugates of RM with monoisotopic patterns showing distinctly different degrees of incorporation of endogenous GSH/SIL-GSH. The observation is consistent with efflux of various RM (at different rate and/or extent) from primary human hepatocytes prior to extracellular GSH trapping, consistent with the involvement of efflux transporters. Efflux of RM may contribute to extracellular hepatotoxicity of TGZ via haptenization of outward-facing membrane proteins and/or intracellular hepatotoxicity via inactivation of the involved transporters. A novel GSH conjugate not observed in cryopreserved suspensions was detected, for which tentative structure assignment was proposed based on MS$^n$ data interpretation.