Targeted Proteomic Analysis of Drug Metabolizing Enzymes in Exosomes Secreted by HepG2 Cells
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Purpose
Exosomes are extracellular vesicles originated from inward budding of multi-vesicular bodies within cells. They can be found in bodily fluids (e.g. blood and urine) and cell culture medium, with a typical size ranging between 30 – 150 nm in diameter. The cargo (e.g. proteins, nucleic acids and lipids) in exosomes reflect the cells of origin and hence have the potential to serve as biomarkers for diseases (e.g. cancer) and monitoring a patient’s response to treatment. The purpose of this study is to determine if drug-metabolizing enzymes (DMEs) can be detected in exosomes secreted by HepG2 cells (a human hepatocellular cell line). If true, exosomal DMEs may serve as biomarkers for the liver DME activity.

Methods
HepG2 exosomes were isolated from the cell culture media using differential ultracentrifugation. The exosomes were then characterized by nanoparticle tracking analysis (NTA), and the exosomal and total cell lysate protein content were quantified by the BCA assay. Liquid chromatography-multiple reaction monitoring mass spectrometry (LC-MRM-MS)-based targeted proteomic analysis was performed to determine the exosomal expression of cytochrome P450s (CYPs) and flavin-containing monooxygenases (FMOs) using previously developed methods. Briefly, exosomal (or total cellular) proteins (30 μg) were reduced, alkylated, and trypsin-digested prior to separation on an analytical column (ACQUITY UPLC, C18, 1.7 μm, 2.1x100mm). Signature peptides for CYPs 1A1, 1A2, 1B1, 2C9, 2C19, 3A4, 3A5, 3A7, 4F2, 4F3B, 4F11, and FMOs 1, 3, and 5 were detected using specific MRM transitions and a triple-quadrupole mass spectrometer (Waters Xevo TQ-S). In addition, an untargeted global proteomic analysis of the HepG2 exosomal and total cellular proteins was performed using data-dependent acquisition (DDA) and a fourier-transform mass spectrometer (FTMS; Thermo LTQ-FT) to identify other proteins, such as exosomal marker proteins.

Results
The isolated HepG2 exosomes were first characterized by NTA and found to have an average (mean +/- SD) size of 138 +/- 3 nm, which is within the expected size range of exosomes. The exosomal and total cellular protein concentrations were 434 μg/mL and 33.4 mg/mL, respectively. Targeted proteomic analysis of HepG2 exosomes detected the expression of CYP3A4, CYP3A7, CYP4F11, CYP4F12 and FMO3. With the exception of CYP4F12, which was nearly 2 fold higher in abundance in the exosomes than in the cell lysate, the detected enzymes were found to be more abundant (3-20 fold) in the cell lysate than the exosomes. For example, CYP3A7 and FMO3 were about 3.5 and 5 fold higher in abundance in the cell lysate than in the exosomes, respectively. CYP3A4, however, was found to be 20 fold higher in the cell lysate than in the exosomes. Enzymes in the CYP2C and CYP1A family, such as 2C9, 2C19, 1A1, and 1A2, were not detected in the lysate or exosomes. To compare exosomal and cellular protein expression profiles, global proteomics was performed on the samples using DDA-based FTMS. This analysis identified 348 proteins and 228 proteins for the HepG2 cell lysate and exosomes, respectively. During this analysis, exosomal marker proteins HSP70 and HSP90 were identified in both the cell lysate and exosomal digestions. The cell lysate also contained ASGR1, which has been suggested to be a marker for liver derived exosomes, but it was not identified in the HepG2 exosomes. Additionally, both the cell lysate and exosomes were found to contain AFP and GPC3, which are proteins thought to act as biomarkers for hepatic carcinoma. However, neither CYPs/FMOs nor common exosomal marker protein, such as CD9, CD81, and CD63 were identified in the HepG2 exosomes by the DDA FTMS global proteomic analysis.

Conclusion
Using LC-MRM-based targeted proteomics, we demonstrated, for the first time, the detection of CYPs and FMOs in the exosomes of HepG2 cells. In most cases, exosomal abundances of these proteins were markedly lower than their cellular abundances, presenting a major challenge in detecting and quantifying exosomal DMEs. Indeed, DDA FTMS-based global proteomic analysis failed to identify these CYPs and FMOs, as well as common exosomal marker proteins, likely due to a lower dynamic range and sensitivity of the DDA method employed. In the future, data-independent acquisition method (e.g., LC-MS5), which offers an improved dynamic range and sensitivity, will be employed for global proteomic analysis of exosomes. In addition, LC-MRM-based targeted proteomic methods for common exosomal marker proteins (e.g., CD9, CD81, and CD63) will be developed to characterize exosomes isolated from hepatic cell cultures.