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Absolute Difference of Hepatobiliary Transporter MRP2/Mrp2 in Liver Tissues and Isolated Hepatocytes from Rat, Dog, Monkey and Human

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Abbreviations: MRP/Mrp: multidrug resistance-associated protein; AQUA: absolute quantification; LC-MS/MS: liquid chromatography tandem mass spectrometry; MRM: multiple reaction monitoring; XIC: extract ion chromatogram; SIL: stable isotope labeled; QC: quality control; LOQ: limit of quantification. qRT-PCR: quantitative real time polymerase chain reaction. GS-MF: GSH conjugates of 5-Chloromethylfluorescein diacetate; LTC4: leukotriene C4.

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ABSTRACT

Previously, we have reported that hepatobiliary transporter MRP2/Mrp2 is considered as the major cause of the interspecies differences detected by fluorescent substrates efflux in isolated hepatocytes. In the present study, the interspecies differences of MRP2/Mrp2 were firstly evaluated by qRT-PCR and western blotting. The mRNA levels were able to distinguish the difference among species with the rank order comparable to the corresponding activities observed, while the extents of the differences remained unwarranted. The cross reactions of MRP2/Mrp2 protein of different species with anti-human MRP2 polyclonal antibody were found by western blotting. However, due to the unknown binding affinity of antibody to MRP2/Mrp2 protein across species and lack of purified MRP2/Mrp2 proteins for calibration, the immunoblotting assay was excluded from the absolute quantification of MRP2/Mrp2 protein for multiple species. By using our newly developed LC-MS/MS quantification method, we were able to measure the absolute amount of MRP2/Mrp2 in liver tissues and isolated hepatocytes across species. Freshly isolated hepatocytes conserved the MRP2/Mrp2 protein levels that are comparable to the liver tissues. The amount of Mrp2 in rat liver was approximately 10 fold higher than that in other species. Moreover, a significant loss of Mrp2 protein in the membrane fraction of rat cryopreserved hepatocytes was observed. Taken together, the absolute differences of MRP2/Mrp2 level in various species were determined, for the first time, by direct quantification. The results could potentially fill the translational gaps of *in vitro/ in vivo* or preclinical species to human extrapolation of hepatobiliary elimination mediated by MRP2/Mrp2.

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Introduction

Xenobiotics and their metabolites are generally eliminated and detoxified by phase I and phase II enzymatic metabolism, phase III transporter mediated drug efflux to bile, or by both mechanisms. The excretion of drugs by hepatocytes into bile is one of the primary elimination routes for xenobiotics and the conjugate metabolites (Arias et al., 1993). In each stage of drug discovery, accurate prediction of human pharmacokinetics for a potential drug candidate is of great value (Mahmood, 1999). Even though the interspecies scaling methods based on physiologically allometric procedures have been successfully applied particularly into extrapolation of hepatic enzymatic metabolism and urinary excretion (Dedrick et al., 1970; Iwatsubo et al., 1997; Ito et al., 1998), the *in vitro* or *in vivo* model for biliary excretion predication is far beyond mature (Mahmood and Sahajwalla, 2002). The remarkable interspecies differences in biliary excretion of xenobiotics and drugs/metabolites (Ishizuka et al., 1999; Shilling et al., 2006) might cause significant overestimation of biliary excretion in human simply by an exponential allometric extrapolation approach (Lave et al., 1999; Pahlman et al., 1999; Ayrton and Morgan, 2001). Therefore, understanding the molecular mechanisms underlying the marked species difference in hepatobiliary elimination of drugs and their metabolites should greatly advance the current allometric scaling models for human pharmacokinetics estimation.

Several ATP-binding cassette efflux transporters are responsible for the hepatobiliary elimination of therapeutics and physiological substances (Keppler and Konig, 1997; Muller and Jansen, 1997; Suzuki and Sugiyama, 1999). Even though the interspecies differences in other transporters involving hepatobiliary elimination remain obscure, the marked species differences in MRP2/Mrp2 transporter activity have been reported in both *in vitro* and *in vivo* models (Ishizuka et al., 1999; Ninomiya et al., 2005; Shilling et al., 2006). For examples, Ishizuka and

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his colleagues found that the *in vitro* transport of DNP-SG into canalicular membrane vesicles (CMVs) was 8 fold higher (V_{max}/K_m: 64.2 vs 7.7) in rat than in dog, while the *in vivo* biliary excretion of temocaprilat demonstrated 40 fold higher in rat than in dog (Ishizuka et al., 1999). Even though species differences in Mrp2 mediated biliary efflux have previously been reported in both *in vivo* and *in vitro* models, agreement on the degree of difference remains to be determined, as the absolute differences of transporters in these models remain unknown.

LC-MS/MS based absolute quantitative method, also termed as AQUA, has been widely utilized in protein quantification in biological and clinical samples (John et al., 2004). However, the application in the measurement of membrane proteins has been delayed due to their hydrophobic nature and extremely low expression level (Barnidge et al., 2003). Recently, a strategy to absolutely quantify the MRP2/Mrp2 across species has been developed in our laboratory, which has been evidenced as a highly sensitive, selective, accurate and precise method (Li et al., 2008b). In the present study, the absolute amount of MRP2/Mrp2 was measured in membrane fraction of liver tissues and isolated hepatocytes from various species. To our knowledge, coupled to the quantitative measurement of mRNA levels and immunologic based protein detection, it was the first time to comprehensively report the absolute differences of MRP2/Mrp2 protein levels across species. The findings could provide a fundamental support for *in vitro/in vivo* correlation, preclinical animals to human pharmacokinetics prediction and further promote the progression of pharmaceutical practice in drug discovery.

Methods and Materials

Chemicals and Reagents

HPLC grade acetonitrile and water were purchased from Burdick & Jackson (Muskegon, MI) and EMD Chemicals, Inc (Gibbstown, NJ), respectively. Formic acid and anti-human MRP2 polyclonal antibody were obtained from Sigma-Aldrich (St. Louis, MO). The protein quantification BCA kit and the in-solution digestion kit were purchased from Pierce Biotechnology (Rockford, IL). ProteoExtract Native Membrane Protein Extraction Kit was purchased from Calbiochem International, Inc (Temecular, CA). Precast Tris-HCl SDS-PAGE gradient gel, R250 coomassie blue and the destaining buffer were purchased from Bio-Rad Laboratories, Inc (Hercules, CA). The enhanced chemiluminescence ECL plus kit was purchased from Amersham (Buckinghamshire, United Kingdom). RNA easy kit and RNase free DNase kit was purchased from Qiagen (Valencia, CA). SYBR supermix was purchased from Applied Biosystem (Foster City, CA).

Snap Frozen Liver Tissues

Snap frozen liver tissues from 15 normal human individual donors aged from 1 to 78 years old (7 males and 8 females), 6 beagle dogs, 4 rhesus monkeys and 7 Cynomolgus monkeys were obtained from the Pfizer Tissue Bank. Rat livers were isolated from 5 Sprague Dawley rats obtained from Charles River Laboratories, Inc (Wlimington, WA). All procedures were approved by the St. Louis Pfizer Institutional Animal Care and Use Committee. The animal care and use program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

Preparation of Cryopreserved and Freshly Isolated Hepatocyte Suspension

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Cryopreserved hepatocytes were from Pfizer central hepatocyte stock (In Vitro Technologies, Baltimore, MA). The lot numbers of cryopreserved hepatocytes used in this study were listed as below: IOE, PXK and OZL for human; FGJ, MON and GWJ for Sprague Dawley rat; FPA and EYS for beagle dog; EHH and FAM for Monkey. The hepatocyte isolation kits were purchased from XenoTech (Kansas City, KS). Frozen cryopreserved hepatocytes were thawed according to the vendor's instructions. Briefly, hepatocytes were thawed at 37°C in shaking water bath for 1.5 minutes and immediately poured into tube A of the kit (supplemented DMEM containing percoll). The tube was then centrifuged for 3 minutes at 85-90g for human and monkey hepatocytes or 70-75g for rat and dog hepatocytes. The supernatant was removed and hepatocyte pellet was then re-suspended with 5 ml medium of tube B of the kit to check the cell viability. Cell viability was assessed by a trypan blue exclusion method. Hepatocytes were then washed with the washing buffer from membrane extraction kit, and applied for membrane fraction extraction.

Freshly isolated hepatocytes were purchased from CellzDirect (Pittsboro, NC). The lot numbers of fresh hepatocytes are human: Hu707, Hu780 and Hu0731; rat: Rs354 and Rs416; Dog:Db176 and Db167; monkey: Cy215, Cy230 and Cy236. Upon arrival, the fresh hepatocytes were centrifuged at 4°C for 4 minutes under the same conditions described above. The supernatant was removed and the cell pellet was re-suspended with 5ml medium to check the cell viability with the trypan blue exclusion method. The hepatocytes were then washed with washing buffer from membrane extraction kit and extracted the membrane fraction under the same procedure as described below. Fresh and cryopreserved hepatocytes with viability greater than 80% were used.

RNA extraction and Quantitative RT-PCR

Frozen liver tissues were ground to a fine powder in liquid nitrogen and weighed before processing. For cryopreserved hepatocytes and fresh isolated hepatocytes, RNA was extracted after percoll preparation as described above. The isolated RNA was treated with DNase to digest genomic DNA and quantified by the nano UV-spectrometer (NanoDrop Technology, Wilmington, Delaware). The first strand cDNA was prepared from 200 ng of total RNA by using the Superscript III first-strand synthesis system (Invitrogen) with random hexamer primers according to the manufacturer's introduction. Negative controls were prepared without the presence of reverse transcriptase. All quantitative PCR reactions were prepared using SYBR PCR supermix with the synthesized first strand cDNA and specific primers, and performed using an ABI-PRISM 7500 Fast Detection System (Applied Biosystems, Foster City, CA). The thermal cycling condition comprised 10 minutes at 95 ^{o}C and 40 cycles alternating at 95 ^{o}C for 30 sec, denaturing at 58 ^{o}C for 30 sec and extending at 60 ^{o}C for 30 sec. Quantification of gene expression was performed using $2^{-\Delta\Delta Ct}$ approach to calculate the relative changes normalized to house keeping gene GAPDH.

Extraction of Membrane Fraction

Membrane protein fraction was extracted using the native membrane protein extraction kit according to the manufacture suggested protocol. Briefly, the crashed liver tissues and hepatocyte pellets were homogenized in the extraction buffer I containing the appropriate amount of protease inhibitor cocktail following the incubation at 4 ^{o}C for 10 minutes while rotating. The homogenate was centrifuged at 14,000 rpm for 15 minutes at 4 ^{o}C. The supernatant containing cytosolic protein was removed and the pellets were re-suspended in the extraction

buffer II with proper amount of protease inhibitors. After 30 minutes incubation at 4 ^{o}C with rotating, the suspension was centrifuged at 14,000 rpm for 15 minutes at 4 ^{o}C. The supernatant containing membrane protein was collected and stored at -80 ^{o}C for future analysis. Protein concentrations of obtained membrane fraction were determined by BCA protein assay kit (Pierce Biotechnology, Inc. Rockford, IL)

Western Blotting Analysis

An aliquot of the membrane extraction (30 μg protein) was mixed with an equal volume of Laemmli buffer (Bio-Rad) and incubated at RT for 5 minutes. The denatured samples were then fractionated on 4-20% gradient SDS gel (Bio-Rad) and electrophoretically transferred onto a nitrocellulose membrane (Bio-Rad). After incubating with blocking reagent, the membrane was washed and incubated with anti-human MRP2 polyclonal antibody at 1:500 dilutions overnight at 4^{o}C. Bound antibody was detected with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody, and visualized by enhanced chemiluminescence ECL plus kit (Amersham, Buckinghamshire, United Kingdom). Commasie blue staining of the gel served as a loading control.

Tryptic Digestion and Sample Preparation

The membrane protein samples were diluted to working concentration of 2 μg/μl. 20 μl of the extracted membrane protein (40 μg protein) was subsequently reduced with 10 mM DTT and alkylated with iodoacetamide (IAA) in 50 mM ammonium bicarbonate digestion buffer. After addition of 50 fmol of SIL MRP2 peptide serving as internal standard (Li et al., 2008b), the protein samples were digested by trypsin in a final volume of 40μl at 37^{o}C for 4 hours and then at 30^{o}C for 14 hours. The optimal ratio of trypsin and protein was 1:50. At the end of digestion,

the samples were acidified with equal amount of 50% acetonitrile in H_2O containing 0.1% formic acid, and centrifuged at 3000 rpm for 20 minutes prior to LC-MS/MS analysis. A 16-mer synthetic peptide corresponding to MRP2/Mrp2 tryptic fragment (LTIIPQDPILFSGSLR) and the SIL internal standard (LTIIPQDPILFSGSL[$^{13}C_6$ $^{15}N_1$]R) were obtained from Celtek Bioscience (Nashville, TN) and Sigma-Aldrich, respectively. The calibration curve was prepared at a range of concentration of the synthetic AQUA peptide respectively at the concentration of 31.25, 62.5, 125, 250, 500, 1000 and 2500 pM with the SIL peptide as internal standard at a fixed concentration of 2500 pM. Data were processed by integrating the appropriate peak areas generated from the reconstructed ion chromatograms for the 16-mer analyte peptide and the SIL internal standard peptide by Analyst 1.4.1 (Applied Biosystems, Foster City, CA). The ratio of the peak area of the AQUA peptide to the SIL peptide (y) was plotted against the concentration of the synthetic native peptide (x) for constructing the regression analysis.

LC-MS/MS quantitative measurement of MRP2/Mrp2 protein

Sample quantification was conducted by coupling a triple quadruple mass spectrometer (TQ-MS, API4000, Applied Biosystem, Foster City, CA) to a Shimadzu LC (SLC-10A) system (WoolDale, IL) and HTS PAL Leap autosampler (Carrboro, NC). The LC column used for peptide separation and elution was a 2.1x 100mm C18 column containing 5μm size beads, 300 Å pore size (Vydac EVEREST^{TM}). The mobile phase A is water with 0.1% v/v formic acid while mobile phase B is acetonitrile with 0.1% v/v formic acid. A linear gradient was used to achieve the chromatographic separation starting from 5% B and progressing to 35% B over a period of 30 minutes. A sample volume of 20 μl was injected onto the LC column at a flow rate of 0.4ml/min. The parent-to-product transition for the AQUA peptide monitored represented the

doubly charged parent ion (LTIIPQDPILFSGSLR)2H+ (m/z 886.0) to the single charged product y ion with m/z 1331.6. Similarly, the transition selected for the SIL internal standard peptide is the analogous doubly charged parent ion detected with m/z 889.6 to the single charge product y ion with m/z 1338.6. The instrument settings of the API4000 TQMS were: ion spray voltage: 4 kV; temperature: 400°C; declustering potential (DP): 50V; collision energy (CE) 38 V, entrance potential (EP) 10V and collision cell exit potential (CXP) 11V.

Data Analysis

Data are representative of a minimum of two experiments performed on different days. The MRP2/Mrp2 protein amount across species was statistically analyzed using one-way ANOVA. Comparison of MRP2/Mrp2 expression between isolated hepatocytes and frozen liver tissues or between freshly isolated and cryopreserved hepatocytes of a single species was statistically analyzed using student's *t-test*. A *p* value less than 0.05 was regarded as statistically significant.

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Results

Quantitative mRNA detection as a surrogate protein measurement has been very popular to investigate the differences of transporter protein level in *in vitro* or *in vivo* models (Goh et al., 2002; Hilgendorf et al., 2007). Approaching on that front, we examined the mRNA level in liver tissues and isolated hepatocytes by using the qRT-PCR. Table 1 listed the primers of MRP2/Mrp2 and the control house keeping gene GAPDH of four tested species. In freshly isolated hepatocytes and livers, rat expressed the highest amount of Mrp2 mRNA among the tested species, while monkey was the least. The mRNA level ranked rat > human > dog > monkey ($p<0.05$ by ANOVA) (Figure 1A and 1B). In cryopreserved hepatocytes, the mRNA level of Mrp2 in monkey was the lowest among the tested species, while no significant differences were found among human, rat and dog (Figure 1C).

Immunoblotting-based protein detection (e.g. western blotting) has been a widely used assay for protein detection and quantification for decades. By using the commercially available anti-human MRP2 polyclonal antibody, we conducted western blotting assay to detect the MRP2/Mrp2 protein in membrane extractions across species. As shown in Figure 2, anti-human polyclonal antibody was cross-reacted with the MRP2/Mrp2 proteins extracted from liver tissue (Figure 2A), freshly isolated (Figure 2B) or cryopreserved hepatocytes (Figure 2C) of various tested species. Apparently, the MRP2/Mrp2 protein of human and monkey exhibited the highest cross-reactivity with the antibody, while the dog was the least. However, due to lack of the purified MRP2/Mrp2 proteins as calibration standard for each species, we were not able to compare the protein level across species. In addition, the results were not consistent with the mRNA levels of MRP2/Mrp2 and also not in the agreement with the differential transport

activities of MRP2/Mrp2 previously reported (Ishizuka et al., 1999; Mahmood and Sahajwalla, 2002; Shilling et al., 2006; Li et al., 2008a).

Recently, LC-MS/MS mediated MRP2/Mrp2 protein quantification method has been developed in our lab. The method has been validated as a highly sensitive and selective quantitative approach with great accuracy and precision (Li et al., 2008b). In the present study, the LC-MS/MS method was applied to absolutely measure MRP2/Mrp2 protein in membrane fraction extracted from freshly isolated/cryopreserved hepatocytes and frozen liver samples across species. The strategy that we employed was to select the proteotypic peptide representing MRP2/Mrp2 protein. The process includes a combination of comprehensive MS/MS verification of the candidate peptides and *in silico* prediction of the tryptic digested fragments by using the online software http://prospector.ucsf.edu/. To obtain the selective peptide exclusively representing MRP2/Mrp2 across species, further genome wide BLAST search was conducted to ensure the specificity. **Figure 3** shows the alignment of MRP2/Mrp2 sequence across species and highlights the position of the proteotypic peptide. A synthetic peptide (LTIIPQDPILFSGSLR) representing the fragment cleaved from tryptic proteolysis of MRP2/Mrp2 protein serves as the calibration standard for LC-MS/MS quantification. **Figure 4** exhibits the examples of reconstituted ion chromatograms representing the MRP2/Mrp2 peptide produced by tryptic digestion of liver membrane proteins from various species and the internal standard, stable isotope labeled (SIL) peptide added before tryptic digestions. Quality control study was performed to ensure the performance of the MRP2/Mrp2 quantitative method by LC-MS/MS. Both the accuracy (RE) and precision (CV) of all QCs were below 15% (**Table 2**), at two known concentrations (0.625 nM and 1.64 nM) of synthetic AQUA peptide spiked in the biological matrix prepared from liver samples. In addition, the great recovery rate also indicated that the

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potential variations caused by the discrepancy of biological matrix from different species could be omitted (Table 2). In the present study, snap frozen liver tissues of 15 human donors aged from 1 to 78 years old, 5 rats, 6 dogs and 11 monkeys were subjected to LC-MS/MS quantitative analysis. MRP2/Mrp2 protein measurement was also conducted for the pooled samples from 3 lots cryopreserved hepatocytes of human and rats, and 2 lots for dog and monkey. The absolute amount of MRP2/Mrp2 protein in freshly isolated hepatocytes was averaged from the lots prepared from 3 individual donors for human and monkey, 2 donors for rat and dog. **Figure 5A** summarized the results of MRP2/Mrp2 in liver tissues, freshly isolated and cryopreserved hepatocytes of human, rat, dog and monkey. In liver tissue, the MRP2/Mrp2 protein level ranked rat>> monkey>dog ≈human with the average ranged from 0.6 to 5.5 fmol per μg membrane protein. A comparable amount of MRP2/Mrp2 was detected in freshly isolated hepatocytes of all tested species. The absolute amount of Mrp2 protein in rat was 10 fold higher in liver tissue than in human. Impressively, a greater variation of MRP2 expression was observed in human liver donors (6-fold ranged from 0.2 to 1.2 fmol/μg protein) and the animals (monkey and dog, ~4 fold, ranged from 0.6 to 2.7 and 0.5 to 1.7 fmol/μg protein, parallel respectively) that have been tested with discovery compounds prior to tissue collecting, compared to less than 2-fold in rat (ranged from 4.6 to 6.1 fmol/μg protein).

Discussion

MRP2/ABCC2, belonging to the ATP-binding cassette transporter superfamily, is one of the major efflux transporters localized on the hepatic canalicular membrane in livers and has been demonstrated to be responsible for interspecies difference in hepatobiliary secretion (Niinuma et al., 1997; Ishizuka et al., 1999; Shitara et al., 2005). Species differences in

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MRP2/Mrp2 activity have been reported both *in vivo* and *in vitro* models (Ishizuka et al., 1999; Shilling et al., 2006), and are considered to be one of the major causes of the failure in interspecies scaling from preclinical animal to human. As a general rule, two major factors are commonly used to characterize transporter-mediated drug clearance: V_{max} and K_m. While the K_m is the unique property of a certain transporter substrate and usually is a fixed parameter, V_{max} is determined by the expression level of a given transporter in a tissue or the model being applied. The V_{max} varies by tissues and individual species and is sensitive to treatment with inducers and suppressors. During the past decade, various *in vitro* systems have been developed to determine the transport kinetics or modulate recognition in a variety of species. However, direct comparisons of the absolute amount of MRP2/Mrp2 protein across species have not been addressed. In addition, as the *in vitro* "golden tool" for drug metabolism research, freshly isolated and cryopreserved hepatocytes are the most commonly and widely used as *in vitro* liver models in drug discovery. The retention of hepatobiliary transporters remains unknown, when the polarized structure of hepatocytes is disrupted during the hepatocyte isolation/cryopreservation process. Recently, we demonstrated that the elimination rate of an MRP2/Mrp2 specific substrate was 4~6 fold faster in isolated rat hepatocytes than in human hepatocytes (Li et al., 2008a). However, the extrapolation of transporter-mediated drug clearance from *in vitro* to *in vivo* still remains a challenge because the assumption was made that the amount of transporters remains unchanged or underwent a similar degree of loss during the hepatocyte preparation for each species. As another example, the internalization of Mrp2 and P-gp in freshly isolated rat hepatocytes has recently been reported (Bow et al., 2008). The report could be expected to produce obvious discrepancies in the functional efflux observed in isolated hepatocytes (Oude Elferink et al., 1990; Lam and Benet, 2004; Li et al., 2008a). Therefore,

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investigation into the absolute amount of transporter proteins existing in isolated or cultured hepatocytes would be of great value for understanding the protein-activities relationship.

Several attempts were conducted in our laboratory to elucidate the molecular mechanisms underlying the species differences of MRP2/Mrp2 activities in isolated hepatocytes (Li et al., 2008a). As mRNA quantification has been used widely as an alternate measurement of protein, we firstly evaluated the species difference of MRP2/Mrp2 in mRNA expression. In both liver tissues and isolated hepatocytes, the rank order and the extent of difference in mRNA levels among the species were not consistent with the corresponding activities reported previously (Li et al., 2008a), or the uptake V_{max} of MRP2 substrate, DNP-SG, into CMVs prepared from rat and human liver (1.9 vs 0.23 nmol/min/mg protein)(Ishizuka et al., 1999). The results revealed that the mRNA level of MRP2/Mrp2 in liver tissue or isolated hepatocytes might not directly reflect the functional protein amount existing on plasma membrane. In fact, despite some examples of the concordance between mRNA and protein expression of the drug transporters published in literature, skepticism regarding mRNA as a surrogate protein measurement is frequently a concern (Behrens et al., 2004; Taipalensuu et al., 2004; Jones et al., 2005). It has been reported that the translation of rat Mrp2 mRNA is differentially regulated by the upstream open reading frames (uORF) in the 5' untranslated region (Zhang et al., 2007) and results in the disconnection between mRNA level and protein amounts. Furthermore, posttranscriptional mechanisms might play a more prominent role in LPS-induced regulation of human MRP2 and BSEP compared with the rat transporter proteins (Elferink et al., 2004). Thus, one must exercise caution with regard to utilizing qRT-PCR as an alternative approach to absolute quantification of MRP2/Mrp2 protein in liver or isolated hepatocytes. The different mRNA expression profile observed in cryopreserved hepatocytes (both in individual measurements and rank order), compared to liver

tissues and fresh isolated hepatocytes, was postulated to result from the cryopreservation procedure, which was further evidenced by the absolute measurement of MRP2/Mrp2 protein discussed below. Due to the discrepancy between the mRNA expression level and the corresponding transport activities of MRP2/Mrp2, a more relevant characterization, i.e. direct quantification, of interspecies differences in hepatobiliary transporters should be considered. Therefore, in the present study, we also attempted to quantify the proteins by immunoblotting assay. However, given the unknown affinity of this antibody to MRP2/Mrp2 protein of different species, purified MRP2/Mrp2 proteins are essential as the calibration standard for protein quantification. In this context, the immunoblotting-based approach for protein quantification of MRP2/Mrp2 was excluded due to lack of the purified proteins.

Coupled to one or two dimensional gel electrophoresis, LC-MS/MS has been increasingly used for a variety of biomarker evaluation and quantification (Wu et al., 2002; Barnidge et al., 2004; Kuhn et al., 2004). Recently, we have overcome serial obstacles in developing a method to absolutely quantify multidrug resistance-associated protein (MRP2/ABCC2) for multiple species by LC-MS/MS (Li et al., 2008b). In the present study, we were able to demonstrate the absolute amount of MRP2/Mrp2 protein ranked as rat >> monkey > human ≈ dog in liver tissues and isolated hepatocytes. While level of MRP2/Mrp2 protein in the membrane fraction of freshly isolated hepatocytes was conserved the comparable to that of whole liver tissue, a significant loss (40%) of Mrp2 was found in rat cryopreserved hepatocytes at 3.24 vs. 5.71 or 5.45 fmol/μg in fresh isolated hepatocytes and frozen liver tissues, respectively (Figure 5A). Even though the causes of Mrp2 decrease in rat cryopreserved hepatocytes were not explored here, it is speculated that the cryopreservation process might alter the membrane density of Mrp2 protein. Significantly, the remarkable difference in Mrp2 protein level between rat cryopreserved

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hepatocytes and freshly isolated ones correlated with the previous finding that the elimination half-life of GS-MF (GSH conjugates of 5-Chloromethylfluorescein diacetate, MRP2/Mrp2 substrate) was significantly longer in rat cryopreserved hepatocytes than in its freshly isolated counterpart (Li et al., 2008a).Under the notion that there could be absolute differences of MRP2/Mrp2 in liver tissues, Shilling and his colleagues addressed the uptake clearance of leukotriene C4 (LTC_4, MRP2/Mrp2 substrate) in canalicular membrane vesicles among different species (Shilling et al., 2006). The results suggest that the absolute amount of MRP2/Mrp2 could serve as the key in the biliary excretion activities.

In the present study, a larger variation of MRP2 amount was observed in human samples However, MRP2 expression level in livers among human donors varies in an age-independent manner (**Figure 5B**) and there was also no significant difference between male and female (data not shown). Previous studies evidenced that multiple mechanisms underlie the regulation of MRP2/Mrp2 expression. For example, MRP2 level in human placenta was affected by gestational age with increased MRP2 protein level at later stages of pregnancy (Meyer zu Schwabedissen et al., 2005). In rat, Mrp2 protein started to be detected in livers of 16 and 20-day-old fetuses and tend to increase gradually after birth (Zinchuk et al., 2002). Administration of xenobiotics, including cycloheximidine, 2-acetylaminofluorene and rifampin has been shown to influence on the expression level of Mrp2 protein (Buchler et al., 1996; Fromm et al., 2000; Courtois et al., 2002). Moreover, different endogenous molecules are considered to involve in the regulation of Mrp2 expression (Hartmann et al., 2002). Nevertheless, whether environmental and/or genetic factors affect MRP2/Mrp2 expression still remains largely unknown. Without donor information regarding medication history or disease state, we are yet unable to explain the MRP2 variation within the human liver samples used in our study.

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It is pertinent to note that the difference in protein level of MRP2/Mrp2 measured does not necessarily reflect the difference in transport activity reported in animal models and in *in vitro* systems. For instance, the rank order of the amount of MRP2/Mrp2 protein in livers was rat>> monkey > dog ≈human. The difference in protein level here matched with the respective GS-MF and calcein efflux rate in isolated hepatocytes reported previously (Li et al., 2008a), except a shorter elimination half-life of Mrp2 substrate in dog hepatocytes. The inconsistency of MRP2/Mrp2 protein level with the corresponding transport activities indicates that the difference in MRP2/Mrp2 transporter activity may be ascribed to differential binding affinities of compounds to MRP2/Mrp2 transporters in the different species. In fact, Ninomiya and his colleagues reported that dog Mrp2 transporter contained both high affinity binding site with similar K_m value as rat Mrp2, and an additional low-affinity site, which was the major contributor for some Mrp2 substrates (Ninomiya et al., 2006). Moreover, the difference in binding affinity between human MRP2 and rat Mrp2 may be the mechanism underlying the differential excretion ratio of MPAG (glucuronized form of mycophenolic acid, MRP2/Mrp2 substrate) between human and rats (Takekuma et al., 2007). Taken together, these results reveal that the pronounced interspecies difference in the biliary excretion activity of MRP2/Mrp2 substrates can result from the sum of the protein level (V_{max}) and the intrinsic transporter affinity (K_m) for its substrates (Ishizuka et al., 1999; Ninomiya et al., 2005).

In conclusion, we have systematically investigated the mRNA level and protein level of hepatic efflux transporter MRP2/Mrp2 in freshly isolated, cryopreserved hepatocytes and snap frozen liver tissues from various species. By utilizing LC-MS/MS AQUA method, we quantitatively measured the absolute amount of MRP2/Mrp2 protein across species, with the order ranking rat >> monkey >dog ≈ human in liver tissue. Coupled with the determination of *in*

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vitro transport kinetics parameters (e.g K_m), the absolute comparison of MRP2/Mrp2 protein across species could facilitate the interspecies scaling of pharmacokinetics parameters in drug discovery. The further investigation on protein synthesis and degradation of (functional protein v.s. non-functional protein) hepatic efflux transporter MRP2/Mrp2 will enable an improved understanding of marked differences in biliary excretion across species.

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Legends for Figure

Figure 1. ***Comparison of mRNA level of MRP2 in isolated hepatocytes and liver tissue across species.*** The mRNA level of MRP2/Mrp2 was normalized to GAPDH. The data represents the relative fold of mRNA level of Mrp2 gene to one of human subjects, mean ± SD (n=3 or 4). The panels from top to bottom represent liver tissues (A), freshly isolated hepatocytes (B) and cryopreserved hepatocytes (C). One way ANOVA analysis indicated the difference of mRNA level of MRP2 among four tested species was not statistically significant in cryopreserved hepatocytes; was statistically significant in fresh isolated hepatocytes and liver tissues ($p < 0.05$). The paired comparison among the tested species was conducted by student *t* test. In liver tissues and cryopreserved hepatocytes, the statistic significant pairs were highlighted as * ($p < 0.05$). In fresh cryopreserved hepatocytes, the difference between human and any other tested species was statistically significant.

Figure 2**.** ***Evaluation of MRP2/Mrp2 protein level across species via western blotting***. 30 μg of membrane protein fraction prepared from snap frozen liver tissues (A), freshly isolated hepatocytes (B) and cryopreserved hepatocytes (C) of four different species was separated on SDS-PAGE. MRP2/Mrp2 protein expressed was detected by using anti-human MRP2 polyclonal antibody. The low panels of all of A, B, C are the coomassie blue staining serving as loading control.

Figure 3. ***The schematic representation of membrane topology of MRP2/Mrp2 and protein alignment across species.*** The selected AQUA peptide was highlighted in grey. The

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stable isotope labeled (SIL) internal standard was indicated with a single residue substitution of [[$^{13}C_6$ $^{15}N_1$]] Leu. Genebank number: human MRP2 (NP_000383); rat Mrp2 (NP_036965); dog Mrp2 (NP_001003081); monkey Mrp2 (NP_001028019)

Figure 4. ***Representative of Reconstituted Ion Chromatogram of LC-MS/MS analysis of MRP2 AQUA peptide across species***. Examples of reconstituted ion chromatograms of MRP2/Mrp2 peptide released from tryptic digestion in liver tissues of rat, dog, monkey and human as indicated.

Figure 5. ***Absolute quantification of MRP2/Mrp2 protein level in isolated hepatocytes and liver tissues from different species. A.*** The membrane protein fraction prepared from the pooled cryopreserved hepatocytes from 2-3 lots; the single lot of fresh isolated hepatocytes; and frozen liver tissues of 15 human donors, 5 rats, 6 dogs and 11 monkeys were subjected to SRM analysis for absolute quantification of MRP2/Mrp2 protein. The data represents the mean ± S.D. One way ANOVA analysis indicated the MRP2/Mrp2 protein level among four tested species is statistically significant ($p \leq 0.05$). The student *t* test was conducted to compare the difference of MRP2/Mrp2 protein level in human and other tested species and the difference of Mrp2 level between rat cryopreserved hepatocytes and fresh hepatocytes and liver tissues. * mean $p \leq 0.05$. ***B.*** The correlation between the amount of MRP2 protein and the age of individual donor.

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Table 1. Human, rat, dog and monkey MRP2/Mrp2 and house keeping qRT-PCR Primers

Species	Gene	Genebank #	Sequence	Size (bp)
Human	MRP2	NM_000392	ACAGAGGCTGGTGGCAACC ACCATTACCTTGTCACTGTCCATG A	227
	GAPDH	NM_002046	GGGGAGCCAAAAGGGTCATCATCT GACGCCTGCTTCACCACCTTCTTG	457
Rat	MRP2	NM_012833	TGGAGTTGGCTCACCTCAGATC CTAGAGCTCTGTGTGATTCACATTTTCA	410
	GAPDH	NM_017008	CGGCACAGTCAAGGCTGAGA CTTCTGAGTGGCAGTGATGG	388
Dog	MRP2	NM_001003081	GCACTGTAGGCTCTGGGAAG TAGGAGGGCACAGGCTTCTA	209
	GAPDH	NM_001003142	AACATCATCCCTGCTTCCAC GACCACCTGGTCCTCAGTGT	234
Monkey	MRP2	NM_001032847	TGTGCTCTCCTCCCAGACTT GAGCATCCACAGCAGACAAA	172
	GAPDH	NM_002046	TCAACAGCGACACCCACTC CTTCCTCTTGTGCTCTTGCTG	193

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Table 2. Quality control of synthetic proteotypic peptide in membrane protein matrix across species

Matrix	QC (nM)	n	Statistic Analysis		
			Mean (nM)	RE%	CV%
Human 2	0.625	3	0.644	3.07	8.25
Human 4	0.625	3	0.716	14.52	14.83
Human 5	0.625	3	0.548	-12.3	2.94
Human 15	0.625	3	0.606	-3.05	0.83
Rat 2	0.625	3	0.538	-13.85	9.4
Monkey 1	1.64	3	1.76	7.59	5.22
Monkey 2	1.64	3	1.69	2.87	1.92

Figure 1

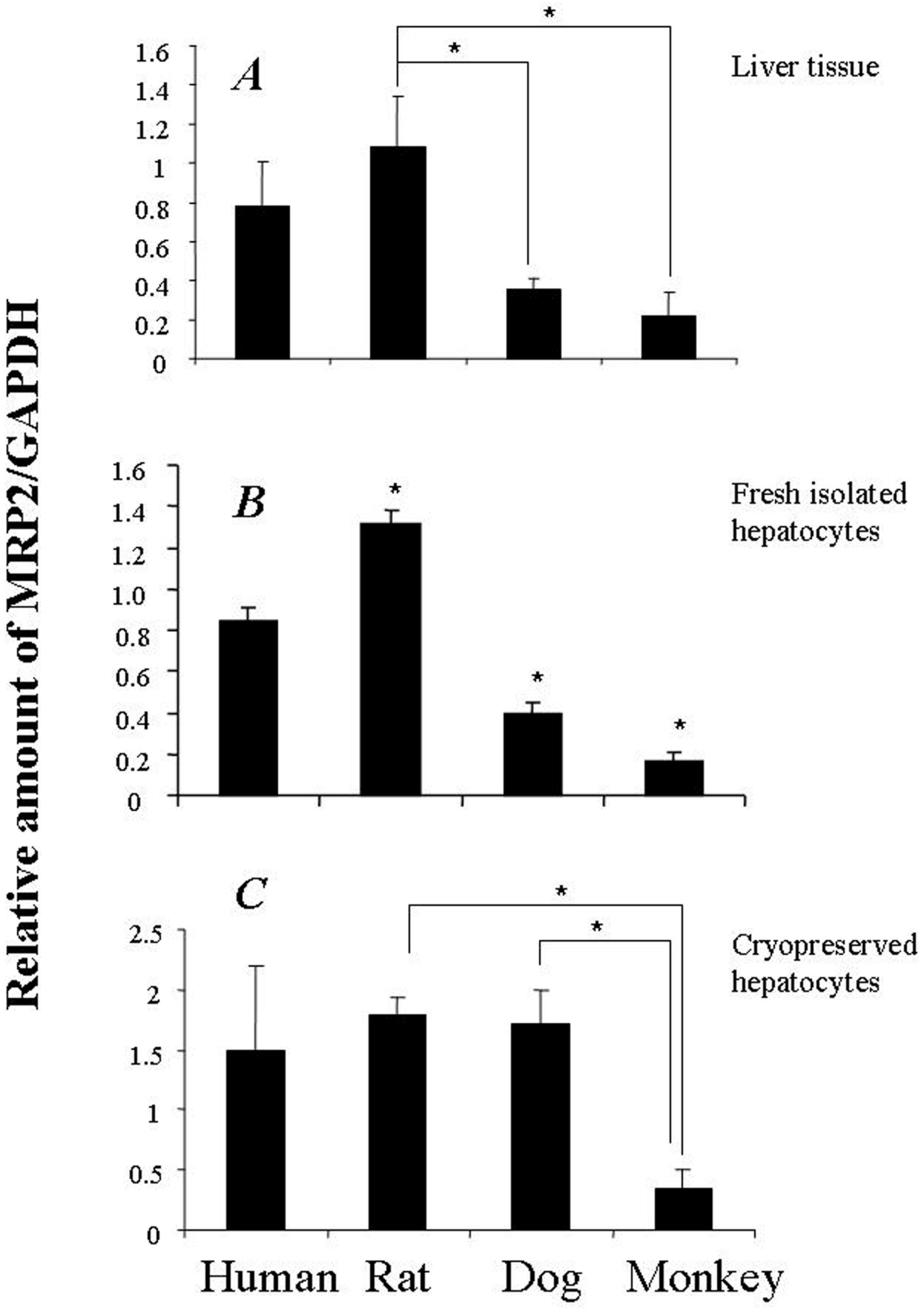

Relative amount of MRP2/GAPDH
A
Liver tissue
B
Fresh isolated hepatocytes
C
Cryopreserved hepatocytes
Human
Rat
Dog
Monkey

Figure 2

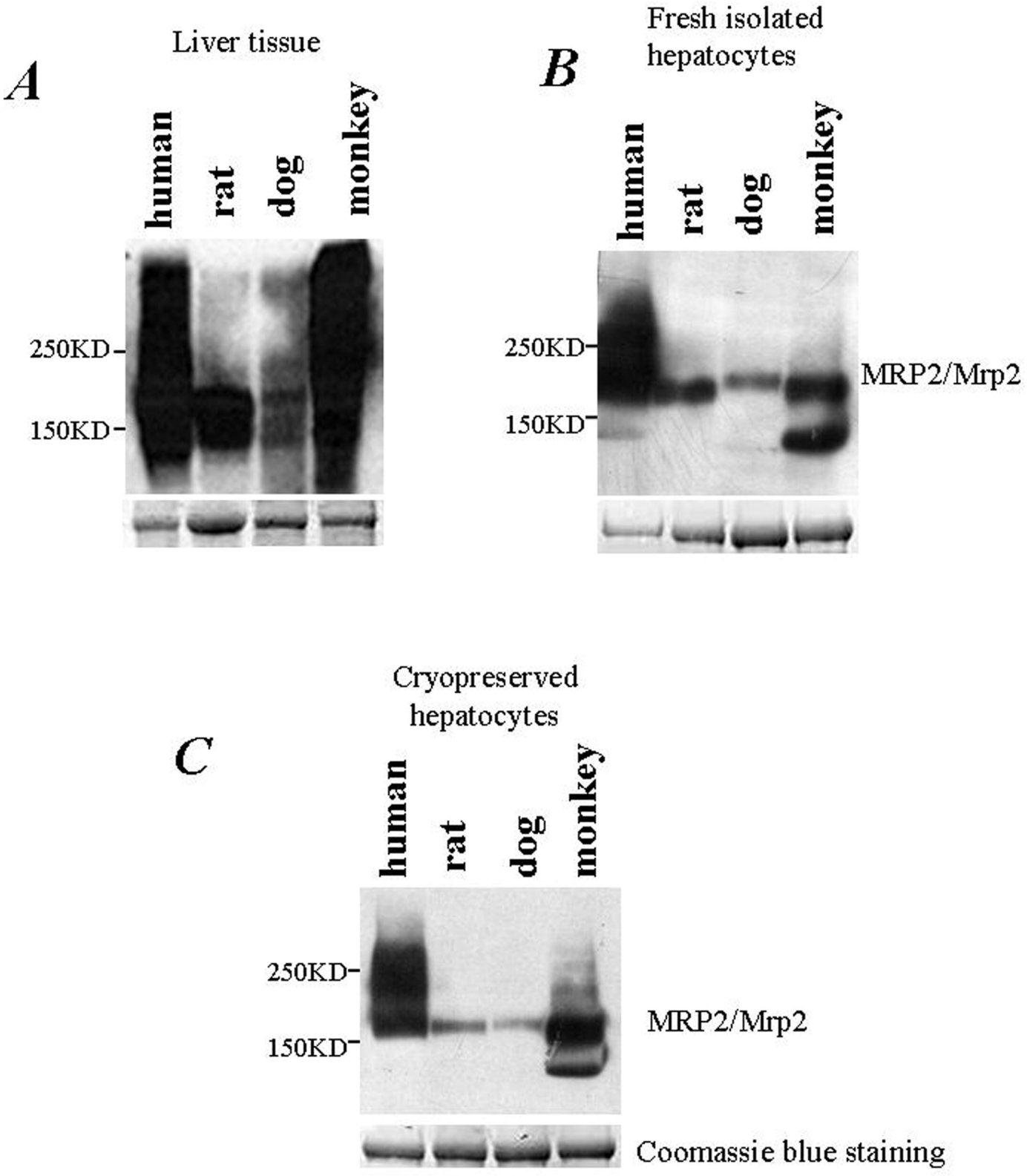
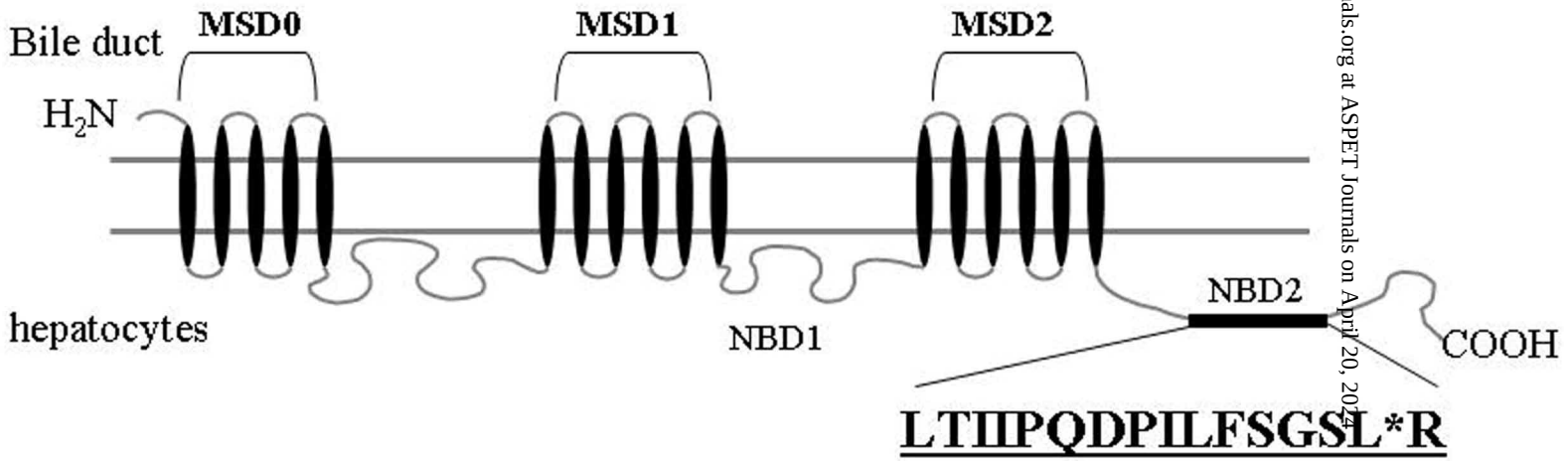

A
Liver tissue
human
rat
dog
monkey
250KD
150KD
B
Fresh isolated
hepatocytes
human
rat
dog
monkey
250KD
150KD
MRP2/Mrp2
C
Cryopreserved
hepatocytes
human
rat
dog
monkey
250KD
150KD
MRP2/Mrp2
Coomassie blue staining

Figure 3




```
human_MRP2      1361  DGVDIASIGLHDLREHLTIIPQDPILFSGSLRMNLDPFNYSDEEIWKALELAHLKSFVA  1420
rat_MRP2        1357  DGIDVASIGLHDLREHLTIIPQDPILFSGSLRMNLDPFNKYSDEEVWRALELAHLRSFVS  1416
dog_MRP2        1360  DGVDIASIGLHDLREHLTIIPQDPILFSGSLRMNLDPFNHYSDGEIWKALELAHLKTFVA  1419
monkey_MRP2     1361  DGVDIASIGLHDLREHLTIIPQDPILFSGSLRMNLDPFNNYSDEEIWKALELAHLKSFVA  1420
```

Figure 4

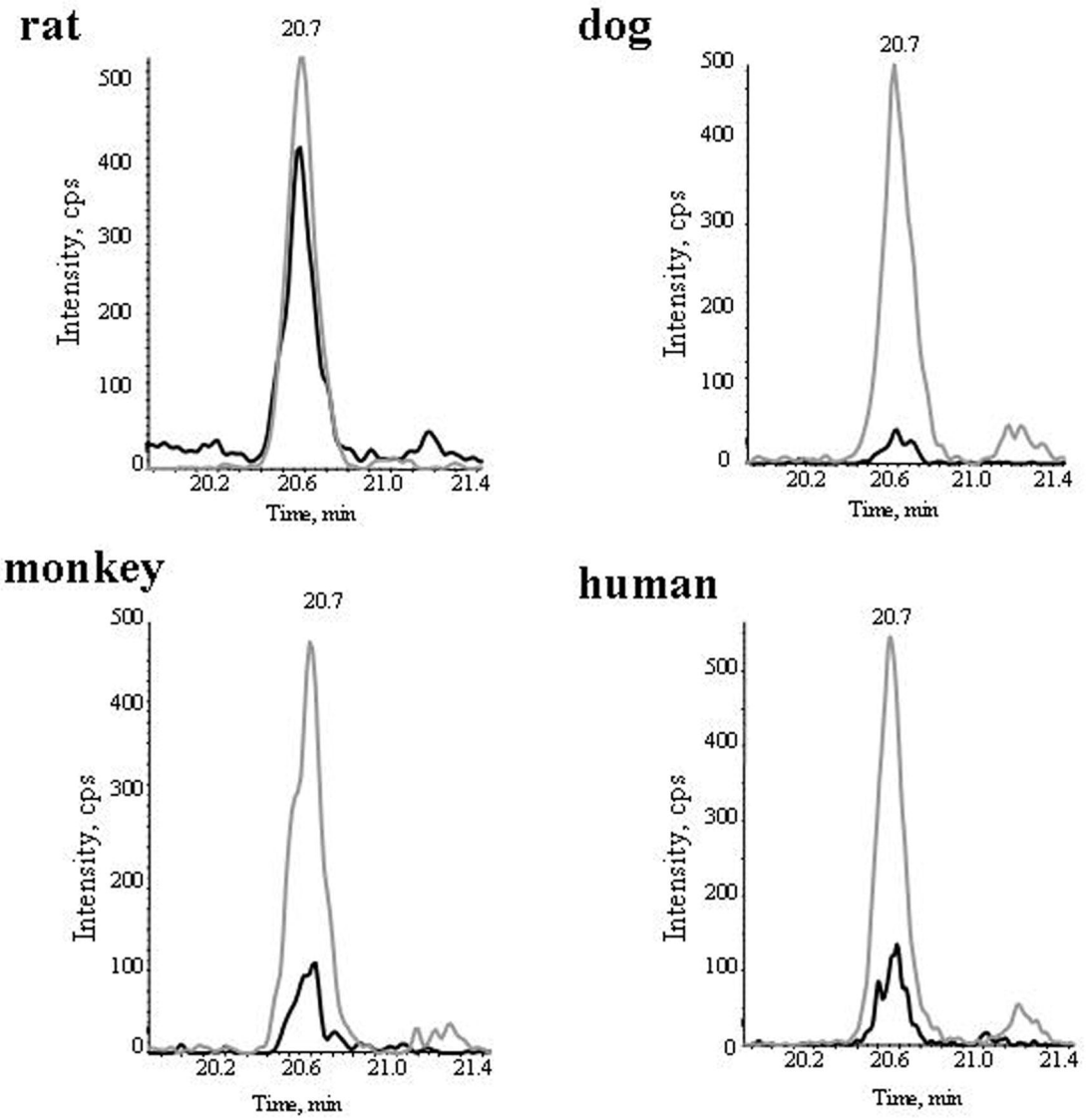
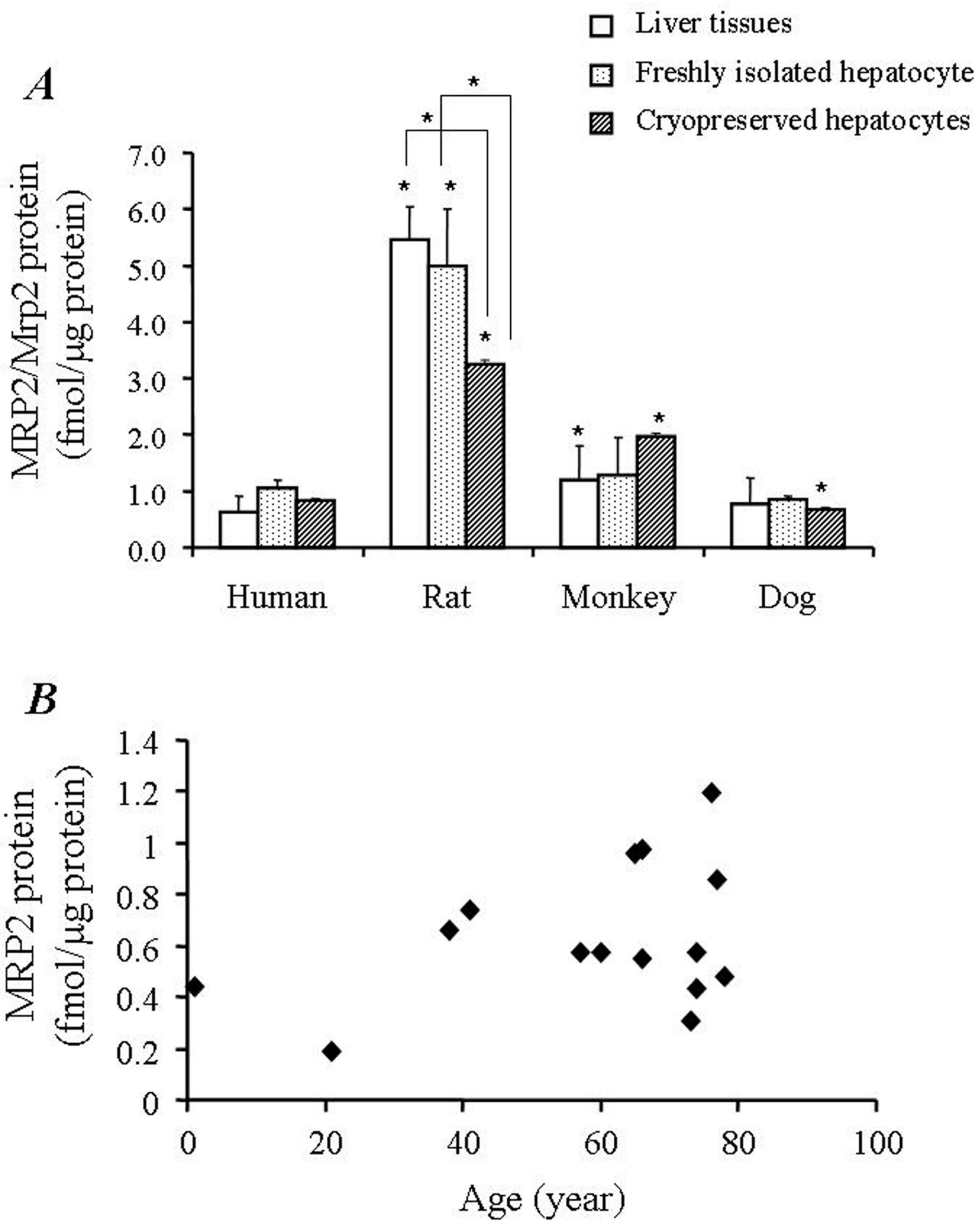

rat
20.7
Intensity, cps
Time, min
dog
20.7
Intensity, cps
Time, min
monkey
20.7
Intensity, cps
Time, min
human
20.7
Intensity, cps
Time, min
MS/MS transition 886.0-1331.6 889.6-1338.6
XIC of Tryptic digested fragment of MRP2/ABCC2
XIC of SIL internal standard

Figure 5


A
Liver tissues
Freshly isolated hepatocyte
Cryopreserved hepatocytes
MRP2/Mrp2 protein (fmol/μg protein)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Human
Rat
Monkey
Dog
B
MRP2 protein (fmol/μg protein)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Age (year)