Validation of uPA/SCID mouse with humanized liver as a human liver model: protein quantification of transporters, cytochromes P450, and UDP-glucuronosyltransferases by LC-MS/MS

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Non-standard abbreviations: ABC, ATP binding cassette; BCRP, breast cancer resistance protein; BSEP, bile salt export pump; CV, coefficient of variance; CYP, cytochrome P450; ENT, equilibrative nucleoside transporter; γ-GTP, γ-glutamyl transpeptidase; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LLOQ, lower than the limit of quantification; MATE, multidrug and toxin extrusion; MRM, multiplexed multiple reaction monitoring; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; NTCP, sodium/taurocholate cotransporting polypeptide; OAT, organic anion transporter; OATP, organic
anion transporting polypeptide; OCT, organic cation transporter; OST, organic solute transporter; P450R, NADPH-cytochrome P450 reductase; PEPT, oligopeptide transporter; PXR, pregnane X receptor; RI, replacement index; SLC, solute carrier; UGT, UDP-glucuronosyltransferases; uPA/SCID, urokinase-type plasminogen activator/severe combined immunodeficiency;
Abstract

Chimeric mice with humanized liver (PXB mice) have been generated by transplantation of urokinase-type plasminogen activator/severe combined immunodeficiency mice with human hepatocytes. The purpose of the present study was to clarify the protein expression levels of metabolizing enzymes and transporters in humanized liver of PXB mice transplanted with hepatocytes from three different donors, and to compare their protein expressions with those of human livers in order to validate this human liver model. The protein expression levels of metabolizing enzymes and transporters were quantified in microsomal fraction and plasma membrane fraction, respectively, by means of liquid chromatography-tandem mass spectrometry. Protein expression levels of 12 human P450 enzymes, 2 human UDP-glucuronosyltransferases, 8 human ABC transporters and 8 human SLC transporters were determined. The variances of protein expression levels among samples from mice humanized with hepatocytes from all donors were significantly greater than those from samples obtained from mice derived from each individual donor. Compared with the protein expression levels in human livers, all of the quantified metabolizing enzymes and transporters were within a range of 4-fold difference, except for CYP2A6, CYP4A11, BSEP and MDR3, which showed 4- to 5-fold differences between PXB mouse and human livers. The present study indicates that humanized liver of PXB mice is a useful model of human liver from the viewpoint of protein expression of metabolizing enzymes and transporters, but the results are influenced by the characteristics of the human hepatocyte donor.
Introduction

Species differences in drug metabolism and transport in the liver between humans and experimental animals are a critical issue during drug development. To overcome this problem, chimeric mice with humanized liver (PXB mice; PhoenixBio Co., Ltd., Hiroshima, Japan) have been generated by transplantation of human hepatocytes into albumin enhancer/promoter-driven urokinase-type plasminogen activator/severe combined immunodeficiency (uPA⁺/−/SCID) mice; in these mice, approximately 80% of the hepatocytes are human (Tateno et al., 2004). PXB mice generate human-specific metabolites (Inoue et al., 2009; Kamimura et al., 2010; Yamazaki et al., 2010; De Serres et al., 2011), and pregnane X receptor (PXR)-dependent induction of metabolizing enzymes was observed when the mice were treated with a human PXR ligand (Hasegawa et al., 2012). Therefore, the liver of PXB mice is considered to be potentially useful as a model of human liver for studies of drug metabolism.

The uptake of most drugs from circulating blood into the liver at the sinusoidal membrane of hepatocytes involves active transport. The drugs subsequently undergo biotransformation by intracellular enzymes such as cytochrome P450 (P450) and UDP-glucuronosyltransferase (UGT), and the parent drug or its metabolites are eventually excreted from the hepatocytes by canalicular and/or sinusoidal transporter proteins. Therefore, expression analyses of metabolic enzymes and transporters in the liver of PXB mice are essential to validate the model. For example, it has been established that PXB mice with a high replacement ratio express 8 human P450s and human phase II enzymes, including 3 UGTs, at similar levels to those in human liver, by means of quantitative PCR and quantitative immunoblot analyses (Katoh et al., 2004; Katoh et al., 2005). Gene expression of the human ATP binding cassette (ABC) transporters and human solute carrier (SLC) transporters was also confirmed in humanized liver (Nishimura et al., 2005; Kikuchi et al., 2010). However, protein expression of drug transporters has not yet been quantitatively analyzed.
in PXB mice. This is important, because we recently showed that there is a poor correlation between protein and mRNA expression levels of metabolizing enzymes (except CYP3A4) and transporters in human livers; for example, the correlation coefficients ($r^2$) were less than 0.3 for CYP1A2, 2C9, 2A6, 2E1, UGT1A1, 2B7, MRP2, MDR1, BSEP, MATE1, OCT1, NTCP and OATP1B3 (Ohtsuki et al., 2012). Furthermore, metabolizing activities of P450s, such as CYP2C9, 2C19, 2D6 and 2E1, were correlated to expression levels of protein rather than mRNA (Ohtsuki et al., 2012). In addition, human hepatocytes are transplanted to produce the PXB mice, so it is also important to consider the influence of the donor on the protein expression of metabolizing enzymes and transporters.

We have recently developed a liquid chromatography–tandem mass spectrometry (LC-MS/MS)-based protein quantification method that does not require antibodies (Kamiie et al., 2008). In this method, the target protein concentration in a sample is determined after enzymatic digestion by quantifying one or more peptide fragments specific to the target molecule. By using this method, we have measured protein expression levels of metabolizing enzymes and transporters in human and mouse livers (Kamiie et al., 2008; Kawakami et al., 2011; Ohtsuki et al., 2012). Since the target peptide is identified by mass-weight information, a single amino acid difference can be distinguished. Furthermore, the specificity, accuracy and dynamic range of quantification by LC-MS/MS-based analysis (coefficient of variance (CV) < 20% and 3-order-of-magnitude dynamic range) are greatly superior to those in the case of immunoblot analysis (Kamiie et al., 2008; Kawakami et al., 2011; Ohtsuki et al., 2011). Therefore, this method was considered to be suitable for validating PXB mouse as a human liver model in terms of protein levels in the liver.

The purpose of the present study was to clarify the protein expression levels of metabolizing enzymes and transporters in liver of PXB mice transplanted with human hepatocytes from
different donors by using LC-MS/MS, and to compare the protein expression levels with those of human livers in order to validate PBX mouse as a model of human liver.

Materials and Methods

Generation of PXB mice

The present study was approved by the Ethics Committees of the Graduate School of Pharmaceutical Sciences, Tohoku University and PhoenixBio Co., Ltd. The experiments in this report conformed to the guidelines established by the Animal Care Committee, Graduate School of Pharmaceutical Sciences, Tohoku University and PhoenixBio Co., Ltd. The cryopreserved human hepatocytes from donor BD85 (black, male, 5 years old), BD72 (white, female, 10 years old) and BD87 (white, male, 2 years old) were purchased from BD BioSciences (San Jose, CA) (Supplemental Table 1). The chimeric mice with humanized liver were generated by the method described previously (Tateno et al., 2004). Briefly, uPA+/−/SCID mice were prepared (Tateno et al., 2004), and at 3 weeks after birth they were injected with human hepatocytes through a small left-flank incision into the inferior splenic pole. The concentration of human albumin in the blood of the chimeric mice and the replacement index (RI; the rate of the replacement from mouse to human hepatocytes) were measured using latex agglutination immunonephelometry (LX Reagent ‘Eiken’ Alb II; Eiken Chemical, Tokyo, Japan) and anti-human specific cytokeratin 8 and 18 antibody (Cappel Laboratory, Cochranville, PA), respectively (Supplemental Table 1). There was a good correlation between the human albumin concentration and the RI (Tateno et al., 2004). In this study, the chimeric mice were used at 13 to 14 weeks of age.

LC-MS/MS-based protein quantification analysis
Microsomal and plasma membrane fractions of liver were prepared as described previously (Ohtsuki et al., 2012). For details, see Supplemental Information. Protein quantitation of the target molecules was simultaneously performed by means of HPLC-MS/MS for metabolizing enzymes or nanoLC-MS/MS for transporters with multiplexed multiple reaction monitoring (multiplexed MRM) as described previously (Ohtsuki et al., 2011; Shawahna et al., 2011; Uchida et al., 2011). Protein expression levels were determined by quantifying specific target peptides produced by trypsin digestion (Supplemental Table 2). Absolute amounts of each target peptide were determined by using an internal standard peptide, which is a stable isotope-labeled peptide with the identical amino acid sequence to that of the corresponding target peptide. Details of the quantification procedure are given in Supplemental Information.

One specific peptide was selected for quantification of each target protein, and measured at 4 different MRM transitions. The amount of each peptide was determined as an average of 3 or 4 MRM transitions from one sample. In cases where signal peaks of fewer than 3 transitions were obtained, the amount of peptide in the sample was defined as under the limit of quantification. The absolute expression amount of CYP3A4 was calculated from the quantitative data obtained for a peptide generated from both CYP3A4 and CYP3A43, by subtracting the value obtained for a peptide that is specific for CYP3A43. Since CYP3A43 was under the limit of quantification in all samples, quantification values obtained with CYP3A4/43 peptides were used as those of CYP3A4.

For the comparison of protein expression levels between humanized liver of PXB mice and human liver, the data for microsomal fraction of 17 human liver biopsies were taken from our previous publication (Ohtsuki et al., 2012).

**Statistical analysis**
Statistical significance of differences among donors was determined by one-way analysis of variance followed by the Bonferroni test using Origin9 software (OriginLab Corp., Northampton, MA).

Results

Protein expression levels of metabolizing enzymes in microsomal fraction of PXB mouse liver

Protein expression levels of 12 human P450 enzymes, human NADPH-P450 reductase (P450R), Na\(^+\)/K\(^+\) ATPase and \(\gamma\)-GTP were determined in liver microsomal fraction of PXB mice transplanted with hepatocytes from 3 different donors (Table 1). Two human UGT enzymes were determined in hepatocytes from 2 different donors. Na\(^+\)/K\(^+\) ATPase and \(\gamma\)-GTP are membrane markers, and both the human and mouse molecules were quantified. The coefficients of variance (CV) of their quantification values were 14.5\% and 14.1\%, respectively, among 11 samples, and the values were not significantly different among the 3 donors. This suggests that the purity of the microsomal fraction was similar in all cases.

CYP2E1, P450R and UGT2B7 were expressed most abundantly at 51.8, 31.6, 55.0 pmol/mg protein of microsomal fraction, respectively (Table 1). The highest CV among samples was 76.1\% for CYP2A6. The average of %CV of all samples was 43.4\%. This is significantly greater than those of the individual donors (p < 0.05), which were 26.5\%, 23.9\% and 23.0\% for BD85, BD72 and BD87, respectively, suggesting that differences among the donors contribute substantially to the variances of expression levels of the target proteins in all samples. CYP2C9, 2C8, 2A6, 2C19, 2D6, CYP2B6 and P450R showed significant differences of protein expression
levels among donors (p < 0.05). In addition, CYP3A5 and 3A7 were determined all 5 samples from donor BD85, but were not detected or were detected in only 1 sample from the other donors. CYP2D6 was detected in only 1 sample from donor BD72, but was detected in all samples from BD85 and BD87.

Protein expression levels of transporters in plasma membrane fraction of PXB mouse liver

Protein expression levels of 7 human ABC transporters, 8 human SLC transporters, Na⁺/K⁺ ATPase and γ-GTP were determined in plasma membrane fraction of PXB mouse liver, since these drug transporters function at the plasma membrane (Table 2). Na⁺/K⁺ ATPase and γ-GTP are membrane markers, and the CVs of their quantification values were 29.4% and 40.1%, respectively, among 11 samples. The quantified values were not significantly different among the 3 donors. This suggests that the purity of plasma membrane fraction was similar among donors, although the variability appeared to be greater than that of the microsomal fraction.

All human transporters, except BCRP, were quantified in all 11 samples (Table 2). MDR3 and BSEP exhibited the highest expression levels among the quantified transporters at 8.68 and 7.57 pmol/mg plasma membrane protein, respectively. Human BCRP was not detected in 5 samples from donor BD85, and was not quantified in samples from BD72 and BD87, in accordance with a report that BCRP expression is very low in human liver plasma membrane fraction (Ohtsuki et al., 2012). The average value of %CV of all samples was 47.0%, which was similar to that of microsomal fraction (43.4%), and significantly greater than that of BD72 (25.5%, p < 0.01) or BD87 (25.6%, p < 0.01). It was also greater, though not significantly, than that of BD85 (35.4%). Therefore, differences among the donors appear to contribute substantially to the variances of protein expression levels in all samples. MDR3, BSEP, MRP2, ABCG8, OCT1 and OATP1B3
showed significantly different protein expression levels, depending on the hepatocyte donors (p < 0.05).

**Comparison of protein expression levels in liver of PXB mice and human.**

The protein expression levels of metabolizing enzymes and transporters in PXB mouse liver shown in Tables 1 and 2 were compared to those in human liver. The protein expression levels determined in 17 human liver biopsies (8 males and 9 females, age 20-74) were taken from our previous report (Ohtsuki et al., 2012). In the human liver biopsies, metabolizing enzymes and transporters were quantified in both microsomal fraction and plasma membrane fraction, as in the case of PXB mice. Expression levels of molecules determined both in PXB mouse and human livers are compared in Fig. 1. The differences between PXB mouse liver and human liver for all compared metabolizing enzymes and transporters were within 4-fold, for except CYP2A6 and CYP4A11 among metabolizing enzymes, and MDR3 and BSEP among transporters. Protein expression levels of CYP2A6 and CYP4A11 in PXB mouse liver microsomal fraction were 5.50- and 4.33-fold lower than those in human liver microsomal fraction, respectively. In contrast, protein expression levels of BSEP and MDR3 in PXB mouse liver plasma membrane fraction were 5.12- and 4.62-fold greater than those in human liver plasma membrane fraction.

**Discussion**

In the present study, we investigated the protein expression levels of human metabolizing enzymes in microsomal fraction and human membrane transporters in plasma membrane of humanized liver of PXB mice using LC-MS/MS based protein quantification. In total, 11 livers of PXB mice humanized with hepatocytes from 3 different donors were quantified. Our results
indicate that differences in the human donors contributed substantially to the variations of measured protein expression levels (Tables 1 and 2). This seems reasonable, because the PXB mice were bred under controlled conditions, whereas the human donors might have been exposed to a variety of different environmental factors, such as food and drug intake, that could affect the expression of metabolizing enzymes and transporters.

Protein expression of CYP3A5 and CYP3A7 was detected in all liver samples from only one donor (BD85), and protein expression of CYP2D6 was detected in all liver samples from two donors (BD72 and BD87) (Table 1). In the previous study with PXB mice from two donors, CYP3A5 protein expression levels were very low in liver from both donors, and the reason for its low expression was genetic polymorphisms of the donor hepatocytes (Katoh et al., 2004). In our previous report, liver biopsies were clearly classified into two groups; a high-CYP3A7 group and a very low CYP3A7 group (Ohtsuki et al., 2012). Hence, the large differences in protein expression levels of these molecules might be attributable to differences in the genetic background of donor hepatocytes, although it is not known whether SNPs were present in the transplanted human hepatocytes in our study. These results suggest that it is important to characterize the donor hepatocytes in studies with PXB mice, and in principle experiments should be performed with PXB mice humanized with hepatocytes from the same donor to ensure comparability of data.

In our previous reports, individual differences of BSEP protein expression were not large in plasma membrane fraction of human liver; the % CV was 29.7% among 17 liver biopsies (Ohtsuki et al., 2012). In contrast, % CV of BSEP protein expression was 105% in PXB liver (Table 2). A possible explanation is that BSEP expression is highly regulated in human liver, but lack of human-specific regulation in PXB mice resulted in a very high variance of protein expression. Interestingly, differences in MDR3 protein levels according to donor showed a
similar trend to those of BSEP (Table 2). BSEP and MDR3 are cooperatively involved in excretion of lipids, and are regulated by FXR (Oude Elferink and Paulusma, 2007). Interspecies differences in FXR response have been reported (Cui et al., 2002; Song et al., 2013). Although further studies are necessary, it was possible that a shared regulatory mechanism of BSEP and MDR3 played some role in the inter-donor differences.

The present study demonstrated that protein expression levels of most of the quantified metabolizing enzymes and transporters were within a 4-fold range from those in the same fraction of human liver reported previously (Ohtsuki et al., 2012) (Fig 1). This range of difference was the same as that in average expression levels among the 3 donors. The enzyme activities of P450 enzymes were reported to be better correlated to protein expression levels in the microsomal fraction than to mRNA expression levels (Ohtsuki et al., 2012). We compared the protein expression levels of transporters between PXB mice and human in plasma membrane fraction, where the quantified transporters function. A large species difference was reported in protein expression levels of BCRP in plasma membrane fraction: 0.419 pmol/mg protein in human liver and 8.51 pmol/mg protein in mouse liver (Kamiie et al., 2008; Ohtsuki et al., 2012). Here, human BCRP was under the limit of quantification in humanized liver of PXB mice, indicating that human hepatocytes retain the characteristic low expression of BCRP in PXB mice.

Overall, our results indicate that humanized liver in PXB mice well retains the protein expression pattern of metabolizing enzymes and transporters of human liver.

Our previous report demonstrated that, in sandwich-cultured human hepatocytes, differences of protein expression levels of metabolizing enzymes and transporters compared with those in human liver were within a 5-fold range (Schaefer et al., 2012). A similar range of difference was found in liver of PXB mice in this study, though in cultured human hepatocytes, the protein expression levels of transporters tended to be greater than those in human liver. Since humanized
liver retains the structure of human liver tissue (Tateno et al., 2013), our results suggest that humanized liver of PXB mice is a useful model for analyzing human hepatic drug metabolism in vivo.

In conclusion, we measured the protein expression levels of metabolizing enzymes in microsomal fraction and transporters in plasma membrane fraction of humanized liver of chimeric PXB mice. The protein expression levels of the quantified metabolizing enzymes and transporters well reflected those of the donor human liver. The protein expression in humanized liver was significantly affected by background of each individual donor. Our results indicate that humanized liver of PXB mice is a useful model of human liver, but it is important to note that interpretation of the results obtained from PXB mice requires a knowledge of not only the value of the replacement index, but also information about the background of the hepatocyte donor.

**Authorship contributions**

Participated in research design: Ohtsuki, Tateno, Kamiie, Horie and Terasaki.

Conducted experiments: Kawakami, Inoue, Nakamura, Katsukura.

Contributed new reagents or analytic tools: Obuchi, Uchida.

Performed data analysis: Ohtsuki, Kawakami, Inoue, Nakamura.

Wrote or contributed to the writing of the manuscript: Ohtsuki, Tateno, Horie and Terasaki.
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Oude Elferink RP and Paulusma CC (2007) Function and pathophysiological importance of


Footnotes

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Legends for Figures

Figure 1 Comparison of protein expression levels of metabolizing enzymes in microsomal fraction and transporters in plasma membrane fraction between humanized liver of PXB mice and human liver.

Protein expression levels of metabolizing enzymes and transporters were determined in microsomal fraction (A) and plasma membrane fraction (B), respectively, of humanized liver of PXB mice, as shown in Tables 2 and 3 (mean ± SD). The data for microsomal fraction of 17 human liver biopsies (mean ± SD) were taken from our previous report (Ohtsuki et al., 2012). The solid line passing through the origin represents the line of identity, and the broken lines represent 4-fold differences. Open symbols indicated quantification values obtained with peptides that are conserved in the human and mouse proteins.
Table 1 Protein expression levels of metabolizing enzymes in microsomal fraction of humanized liver of PXB mice

<table>
<thead>
<tr>
<th>Donor:</th>
<th>ALL (n=11)</th>
<th>BD85 (n=5)</th>
<th>BD72 (n=3)</th>
<th>BD87 (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>S.D.</td>
<td>%CV</td>
<td>n</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>51.8</td>
<td>9.7</td>
<td>18.7%</td>
<td>11</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>27.8</td>
<td>12.4</td>
<td>44.5%</td>
<td>11</td>
</tr>
<tr>
<td>CYP2C9*</td>
<td>21.7</td>
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<td>65.5%</td>
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</tr>
<tr>
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<td>11</td>
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<tr>
<td>CYP2C8*</td>
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<td>6.3</td>
<td>39.8%</td>
<td>11</td>
</tr>
<tr>
<td>CYP2A6*</td>
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<tr>
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<tr>
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<tr>
<td>P450R*</td>
<td>31.6</td>
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<tr>
<td>UGT2B7</td>
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<tr>
<td>UGT1A1</td>
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<td>6</td>
</tr>
<tr>
<td>Na'/K+ ATPase</td>
<td>10.4</td>
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<tr>
<td>γ-GTP</td>
<td>2.64</td>
<td>0.37</td>
<td>14.1%</td>
<td>11</td>
</tr>
</tbody>
</table>

Unit of protein expression is pmol/mg protein of plasma membrane fraction. n, number of determined samples, LLOQ, lower than the limit of quantification; N.D., not determined. *: significant difference (p < 0.05) in protein expression levels among donors (BD85, BD72 and BD87).
Table 2 Protein expression levels of transporters in plasma membrane fraction of humanized liver of PXB mice

<table>
<thead>
<tr>
<th>Donor:</th>
<th>ALL (n=11)</th>
<th>BD85 (n=5)</th>
<th>BD72 (n=3)</th>
<th>BD87 (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>S.D.</td>
<td>%CV</td>
<td>Mean</td>
<td>S.D.</td>
</tr>
<tr>
<td><strong>Canalicular localized transporter</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDR3/ABCB4*</td>
<td>8.68</td>
<td>7.32</td>
<td>84.3%</td>
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</tr>
<tr>
<td>BSEP/ABCB11*</td>
<td>7.57</td>
<td>7.91</td>
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<tr>
<td>MDR1/ABCB1</td>
<td>4.71</td>
<td>1.78</td>
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<td>MRP2/ABCC2*</td>
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<td>0.88</td>
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<tr>
<td>MATE1</td>
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<td>ABCG8*</td>
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<td>0.701</td>
<td>73.3%</td>
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<tr>
<td>BCRP/ABCG2 LLOQ</td>
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<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>γ-GTP</td>
<td>2.22</td>
<td>0.89</td>
<td>40.1%</td>
<td>11</td>
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<td><strong>Sinusoidal localized transporter</strong></td>
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<tr>
<td>OCT1*</td>
<td>4.21</td>
<td>3.41</td>
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<tr>
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<tr>
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<tr>
<td>OATP1B3*</td>
<td>1.03</td>
<td>0.47</td>
<td>45.9%</td>
<td>11</td>
</tr>
<tr>
<td>OATP2B1</td>
<td>0.578</td>
<td>0.241</td>
<td>42.5%</td>
<td>11</td>
</tr>
<tr>
<td>Na+/K+ ATPase</td>
<td>22.6</td>
<td>6.65</td>
<td>29.4%</td>
<td>11</td>
</tr>
<tr>
<td>Localization unknown</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENT1</td>
<td>1.55</td>
<td>0.50</td>
<td>32.6%</td>
<td>11</td>
</tr>
</tbody>
</table>

Unit of protein expression is pmol/mg protein of plasma membrane fraction. n, number of determined samples, LLOQ, lower than the limit of quantification; N.D., not determined. *: significant difference (p < 0.05) in protein expression levels among donors (BD85, BD72 and BD87).
Figure 1