

**SHORT
COMMUNICATION**

Analysis of drug transporter expression in human intestinal Caco-2 cells by real-time PCR

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Caco-2 cells,
intestine,
real-time PCR,
transporterReceived 14 May 2007;
revised 12 June 2007;
accepted 13 August 2007*Correspondence and reprints:
olivier.fardel@univ-rennes1.fr**ABSTRACT**

Expression of drug transporters corresponds to a crucial parameter in intestinal Caco-2 cells widely used for investigating drug absorption. In order to characterize it in an accurate, reproducible and comparative manner, we analyzed mRNA levels of 19 influx and efflux drug transporters through real-time quantitative polymerase chain reaction assays combined with the use of a total RNA reference standard. Profiles of transporter expression were found to be significantly correlated in two independent Caco-2 cell clones and in human small intestine, which may support the use of Caco-2 cells for investigating intestinal drug transport. Several transporters were nevertheless quantitatively expressed at higher (MRP2, MRP3, MRP4, MRP5, MRP6, OATP-A, OATP-B, OCT1 and MCT1) or lower (BCRP) levels in Caco-2 cells comparatively to small intestine. Moreover, MDR1, MRP2, OATP-A and PEPT1 mRNA relative expression were found to differ in the two analyzed Caco-2 cell clones by at least a twofold factor, highlighting that some variations in transporter expression may occur in Caco-2 cells depending on cell origin, and therefore underlining the interest of carefully characterizing transporter levels in any Caco-2 cell clone before its use for drug transport assays.

Caco-2 is a human colorectal adenocarcinoma cell line that undergoes spontaneous enterocytic differentiation with time in culture and thus forms monolayers of differential epithelial cells joined by intercellular tight junctions [1]. These cells exhibit remarkable morphological and biochemical similarity to the small intestinal columnar epithelium; they are extremely useful for mechanistic studies of drug absorption and are widely used in pharmaceutical companies as an absorption screening assay for pre-clinical drug selection [2–4].

Influx and efflux drug transporters, expressed at the apical or basal pole of Caco-2 cells and belonging to ATP-binding cassette (ABC) or solute carrier (SLC) transporter superfamilies [5,6], play a crucial role in transport of drugs across Caco-2 cell monolayers [7,8]. It is therefore important to carefully characterize expression levels of

these transporters in Caco-2 cells. A wide range of studies have already been performed in this area [7,9,10], but a standardized method for investigating transporter expression in Caco-2 cells is still lacking, although it may be useful because of the differences reported between Caco-2 cell clones and passages with respect to transporter levels and functional characteristics [1,11]. To gain insights into this point in the present study, we developed reverse-transcription real-time quantitative polymerase chain reaction (RT-qPCR) assays combined with the use of a total RNA reference standard, allowing to determine transporter expression in an accurate and reproducible manner. This method was applied to compare expression of 19 drug transporters in two Caco-2 cell clones and in human small intestine.

Caco-2 cell isolates from two different origins were used in the study: one was from the European Collection of Cell Culture (ECACC number 86010202) whereas the other was from the American Type Culture Collection (ATCC number HTB-37). They were designed as Caco-2_{EU} and Caco-2_{AM}, respectively. Cells were cultured at confluence for 3 weeks as recommended by the suppliers and as previously described [12], at 37 °C under 5% of CO₂; passage numbers between 30 and 40 were used. Enterocytic differentiation of the cells, reached in response to a 3-week culture period at confluence [13], was routinely checked up by measuring trans-epithelial electrical resistance [12].

RNA levels of drug transporters were analyzed using RT-qPCR assays. Total RNA, isolated from cells using a SV total RNA isolation kit (Promega, Madison, WI, USA), was reverse-transcribed and thereafter subjected to qPCR assays using the fluorescent dye SYBR Green methodology and an ABI Prism 7000 detector (Applied Biosystems, Foster City, CA, USA), as previously described [14]. Gene-specific primers, designed with the Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) and taking into account known intron–exon boundary informations to avoid detection of genomic DNA, are indicated in *Table I*. The curves of amplification were read with ABI Prism 7000 SDS software using the comparative cycle threshold method.

Relative quantification of the steady-state target mRNA levels was calculated after normalization of the total amount of cDNA tested to an 18S RNA endogenous reference.

Reverse transcriptase real-time quantitative polymerase chain reactions were performed using RNAs from the two Caco-2 cell isolates and also from normal small intestines pooled from five male/female Caucasians and provided by Clontech Laboratories (Mountain View, CA, USA). In order to include a RNA reference standard in our RT-qPCR experiments for facilitating relative quantification of transporter expression and transporter level comparisons, we used the BDTM qPCR total RNA human reference (Clontech Laboratories), corresponding to a standardized mixture of total RNAs from a collection of adult human tissues, and which was routinely included in RT-qPCR assays. Each transporter mRNA level in this RNA reference was arbitrarily set at the value of 1 unit and RT-qPCR data for Caco-2 cells and small intestine were expressed in arbitrary units comparatively to the standardized RNA reference. Transporter expression data in Caco-2 cell clones and small intestine were analyzed using the non-parametric Spearman's rank correlation method; the level of significance was $P < 0.05$.

The most expressed transporters in Caco-2 cells when compared to the RNA reference, i.e. those reaching at least three arbitrary units in at least one Caco-2 clone,

Gene	Forward primer	Reverse primer
MDR1 ^a /ABCB1 ^b	GCCAAAGCCAAAATATCAGC	TTCCAATGTGTTCCGGCAT
BSEP/ABCB11	TGATCCTGATCAAGGGAAGG	TGGTCTCTGGGAAACAA
MRP1/ABCC1	GGGCTGCGGAAAGTCGT	AGCCCTTGATAGCCACGTG
MRP2/ABCC2	TGAGCAAGTTTGAAACGCACAT	AGCTCTTCTCCTGCCGTCTCT
MRP3/ABCC3	GTCCGCAGAATGGACTTGAT	TCACCACTTGGGGATCATTT
MRP4/ABCC4	GCTCAGGTGCCTATGTGCT	CGGTACATTTCTCTCTCCA
MRP5/ABCC5	CGAAGGGTGTGTGGATCTT	GTTTCACCATGAAGGCTGGT
MRP6/ABCC6	TGTCGCTCTTTGAAAAATCC	AGGAACACTGCGAAGCTCAT
BCRP/ABCG2	TGCAACATGTACTGGCGAAGA	TCTCCACAAGCCCCAGG
OCT1/SLC22A1	TAATGGACCACATCGCTCAA	AGCCCTTGATAGACACAGA
OCT2/SLC22A2	AAGAATGGGGATCACAATGG	AGATGTGGACGCCAAGATTC
OCT3/SLC22A3	CCCTGGAATTGCCTACTTCA	GACTCAGGGACCACCCAGTA
OCTN1/SLC22A4	TGCTGCTGCCACTGTTTGCT	TTCAGGAATGAACCACCACA
OCTN2/SLC22A5	GGATTGTTGTGCCTTCCACT	AGGTTGGAAGCAGATCCAGA
OATP-A/SLCO1A2	TGGGGAACTTTGAAATGTGG	AAGGCTGGAACAAAGCTTGA
OATP-B/SLCO2B1	TGATTGGCTATGGGGCTATC	CATATCTCAGGGCTGGTGT
OAT2/SLC22A7	GAGGATGAACCTGCCACAGT	TCTGCTCACACACGAGATCC
MCT1/SLC16A1	CACCGTACAGCAATATACG	CAATGGTCGCCTCTTGTA
PEPT1/SLC15A1	AATGTTCTGGGCCTTGTTTG	CATCTGATCGGGCTGAATTT
18S	CGCCGCTAGAGGTGAAATTC	TTGGCAAATGCTTTCGCTC

Table I Primer sequences for qPCR assays.

^aTrivial name.

^bGene symbol.

were the ABC transporters MDR1 (also known as ABCB1), MRP2 (ABCC2) and MRP3 (ABCC3) (Figure 1) and the SLC transporters OATP-A (SLCO1A2), OATP-B (SLCO2B1), MCT1 (SLC16A1) and PEPT1 (SLC15A1) (Figure 2). Other transporters such as MRP1 (ABCC1), MRP4 (ABCC4), MRP5 (ABCC5), MRP6 (ABCC6), BCRP (ABCG2), OCT1 (SLC22A1), OCTN1 (SLC22A4) and OCTN2 (SLC22A5) were less expressed, with expression levels ranging from 0.4 to 2.3 arbitrary units. With respect to the small intestine, MDR1, MRP3, BCRP and

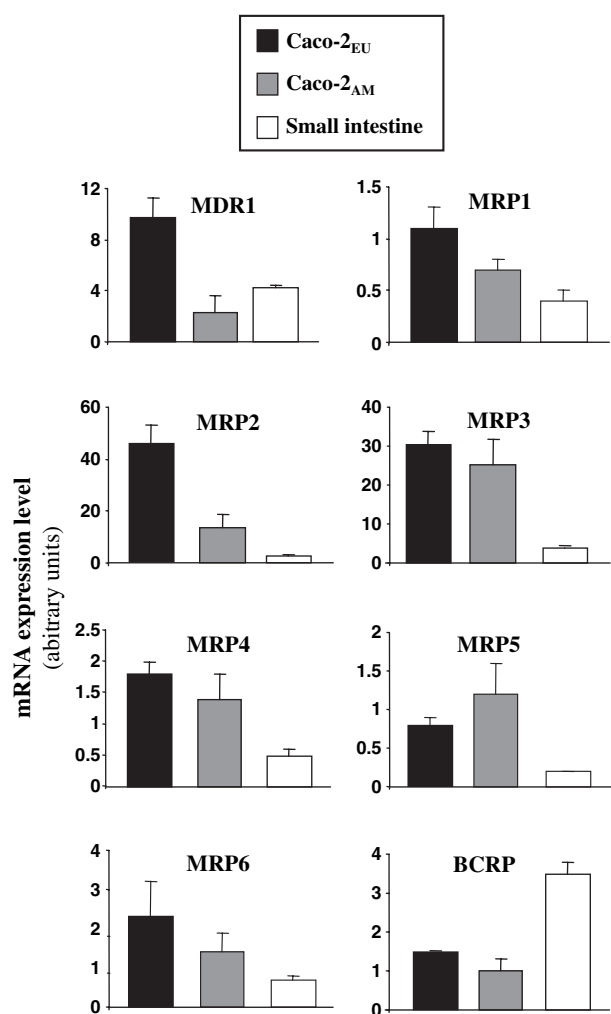


Figure 1 ABC transporter mRNA levels in Caco-2 cells and small intestine. Expression of ABC transporter mRNAs was analyzed by RT-qPCR assays in Caco-2_{EU} and Caco-2_{AM} cells cultured at confluence for 3 weeks and in small intestine. Data are the mean $\pm$ SD of qPCR quantifications from two independent cultures, each being analyzed in triplicate; they are expressed as arbitrary units, 1 arbitrary unit corresponding for each transporter to mRNA level found in the BDTM qPCR human reference total RNA.

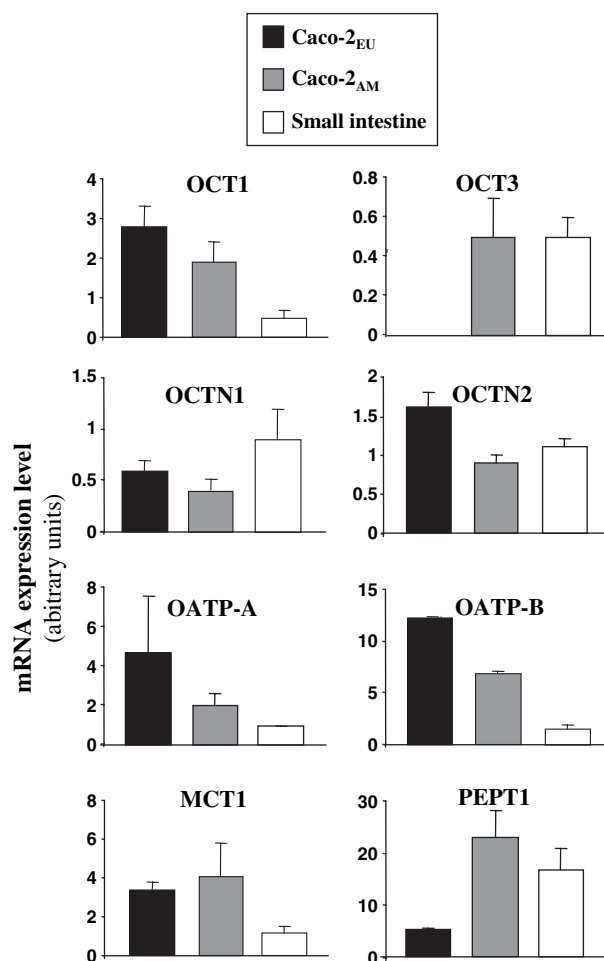


Figure 2 SLC transporter mRNA levels in Caco-2 cells and small intestine. Expression of SLC transporter mRNAs was analyzed by RT-qPCR assays in Caco-2_{EU} and Caco-2_{AM} cells cultured at confluence for 3 weeks and in small intestine. Data are the mean $\pm$ SD of qPCR quantifications from two independent cultures, each being analyzed in triplicate; they are expressed as arbitrary units, 1 arbitrary unit corresponding for each transporter to mRNA level found in the BDTM qPCR human reference total RNA.

PEPT1 were found to be the most expressed transporters, i.e. those reaching at least 3 arbitrary units (Figures 1 and 2); notable expressions of MRP2 and OATP-B were also detected. BSEP (ABCB11), OCT2 (SLC22A2) and OAT2 (SLC22A7) were not expressed in the two Caco-2 cell clones and in small intestine (data not shown) whereas OCT3 (SLC22A3) was detected only in Caco-2_{AM} and small intestine (Figure 2).

Comparison of Caco-2 cells and small intestine reveals qualitative similarities with major transporters present at substantial levels (MDR1, MRP2, MRP3, OATP-B and PEPT1) or absent (BSEP, OCT2 and OAT2), which may

support the use of Caco-2 cells for investigating intestinal drug transport as already underlined [13,15–17]. In addition, transporter ranking according to their expression levels and Spearman's rank correlation method revealed that transporter expressions in Caco-2_{EU} and Caco-2_{AM} were found to be significantly correlated to that found in small intestine (Figure 3). Moreover, Spearman's rank correlation coefficients were close for Caco-2_{EU}/small intestine ($\rho = 0.69$) and Caco-2_{AM}/small intestine ($\rho = 0.65$) (Figure 3), indicating that the two Caco-2 clones gave similar transporter expression correlation with small intestine. It is however noteworthy that

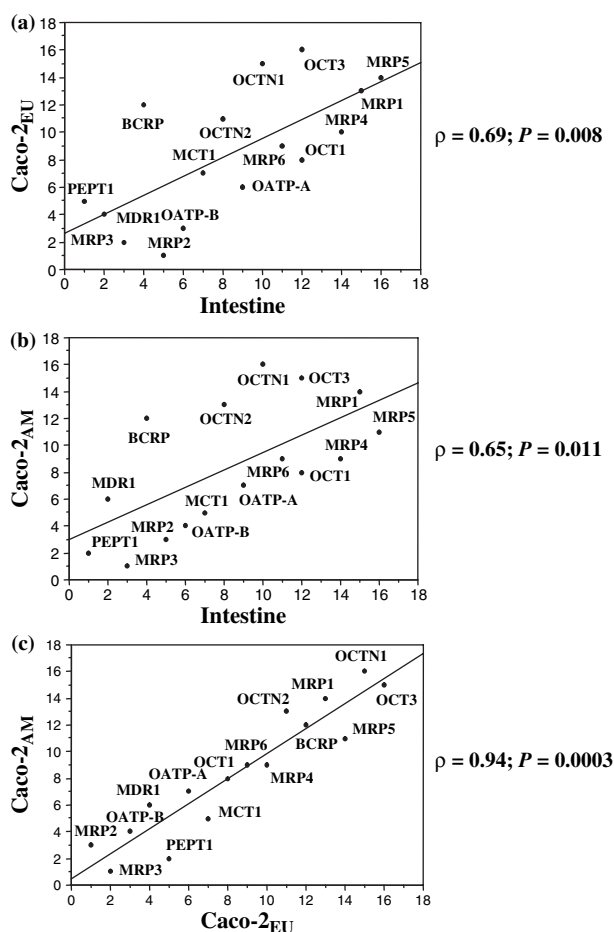


Figure 3 Rank correlation analysis of drug transporter expression in (a) Caco-2_{EU}/small intestine, (b) Caco-2_{AM}/small intestine and (c) Caco-2_{AM}/Caco-2_{EU} clones. Transporters were ranked according to their expression levels in Caco-2 cell clones and in small intestine. Correlations were analyzed using the Spearman's rank correlation method. Spearman's rank coefficients (ρ) and P-values are provided on the right of the correlation graphs.

some quantitative differences between Caco-2 cells and small intestine occurred. For example, several transporters such as MRP4, MRP5, MRP6, OATP-A, OCT1, MCT1, and more strikingly, MRP2, MRP3 and OATP-B, exhibited higher expression in Caco-2 cells than in small intestine. MRP2 and OATP-B have been similarly reported to be more expressed in Caco-2 cells than in jejunum or ileum [15,16]. By contrast, a lower expression of transporters, especially PEPT1 and BCRP, has been recently reported to occur in Caco-2 cells comparatively to jejunal mucosa [16,18]. BCRP was also less present in both Caco-2_{EU} and Caco-2_{AM} clones than in small intestine (Figure 1). Such differences, corresponding to either increased or decreased transporter expression in Caco-2 cells when compared to intestine, may have to be taken into account for precisely interpreting drug transport data from the Caco-2 cell model.

Transporter expression ranks in the two Caco-2 clones were found to be significantly highly correlated (Figure 3). However, precise comparison of transporter expression in the two cell clones Caco-2_{EU} and Caco-2_{AM} revealed some differences. Indeed, the rank order of transporter mRNA prevalence was MRP2 > MRP3 > OATP-B > MDR1 > PEPT1 > OATP-A > MCT1 and MRP3 > PEPT1 > MRP2 > OATP-B > MCT1 > MDR1 > OATP-A in Caco-2_{EU} and Caco-2_{AM} cells, respectively. Moreover, four transporters (MDR1, MRP2, OATP-A and PEPT1) were found to be differently expressed in the two Caco-2 clones by at least a 2-fold factor. Such data fully corroborate the transporter expression variations depending on Caco-2 cell origin or cell passage, already reported for the transporters PEPT1 and BCRP [11,19]. This highlights the interest of carefully characterizing transporter levels in any Caco-2 cell clone before its use for drug transport assays and, for this purpose, to use a standardized method. In this context, RT-qPCR assays associated with the use of a RNA reference, as reported in the present study, are likely to be interesting since (i) RT-qPCR assays allow accurate quantitative determination of gene expression at mRNA level and (ii) the RNA reference easily permits comparison between Caco-2 cell isolates or passages or with other cell types or tissues. The use of the common RNA reference may also facilitate intra- and inter-laboratory comparisons, which are likely not possible when using study-specific references such as human intestinal segments, as described in recently published works [15,16].

Reverse transcriptase real-time quantitative polymerase chain reaction analysis of transporter mRNA levels

in Caco-2 cells should be ideally completed by the characterization of transporter expression at protein and activity level. Unfortunately, antibodies against transporters, especially SLC transporters, are often not commercially available, making it difficult to perform western-blots for routine quantification of transporter expression in Caco-2 cells. In addition, accurate interpretation of functional transport assays may be hampered by the lack of specific substrates for most of the transporters. It should however be kept in mind that transporter mRNA abundance has been proposed as a good predictor of transporter activity, at least for MDR1-related efflux, in Caco-2 cells [20]. This suggests that mRNA level analysis through RT-qPCR assays is likely to be a valuable first approach to characterize transporter expression in the Caco-2-related model of intestinal drug absorption.

In summary, we have developed RT-qPCR assays combined with the use of a total RNA reference standard, in order to accurately and comparatively determine drug transporter mRNA expression in Caco-2 cells. Although profiles of transporter expression were found to be qualitatively similar between two Caco-2 cell clones and small intestine, some quantitative differences were demonstrated, highlighting the interest of carefully characterizing transporter levels in any Caco-2 cell clone before its use for drug transport assays.

REFERENCES

- 1 Sambuy Y., De Angelis I., Ranaldi G., Scarino M.L., Stamatii A., Zucco F. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol. Toxicol.* (2005) **21** 1–26.
- 2 Sun D., Yu L. X., Hussain M.A., Wall D. A., Smith R. L., Amidon G. L. In vitro testing of drug absorption for drug 'developability' assessment: forming an interface between in vitro preclinical data and clinical outcome. *Curr. Opin. Drug Discov. Dev.* (2004) **7** 75–85.
- 3 Shah P., Jogani V., Bagchi T., Misra A. Role of Caco-2 cell monolayers in prediction of intestinal drug absorption. *Bio-technol. Prog.* (2006) **22** 186–198.
- 4 Balimane P.V., Han Y.H., Chong S. Current industrial practices of assessing permeability and P-glycoprotein interaction. *AAPS J.* (2006) **8** E1–13.
- 5 Petzinger E., Geyer J. Drug transporters in pharmacokinetics. *Naunyn Schmiedeberg's Arch. Pharmacol.* (2006) **372** 465–475.
- 6 Ware J.A. Membrane transporters in drug discovery and development: a new mechanistic ADME era. *Mol. Pharm.* (2006) **3** 1–2.
- 7 Anderle P., Huang Y., Sadee W. Intestinal membrane transport of drugs and nutrients: genomics of membrane transporters using expression microarrays. *Eur. J. Pharm. Sci.* (2004) **21** 17–24.
- 8 Suzuki H., Sugiyama Y. Role of metabolic enzymes and efflux transporters in the absorption of drugs from the small intestine. *Eur. J. Pharm. Sci.* (2000) **12** 3–12.
- 9 Hunter J., Jepson M.A., Tsuruo T., Simmons N.L., Hirst B.H. Functional expression of P-glycoprotein in apical membranes of human intestinal Caco-2 cells. Kinetics of vinblastine secretion and interaction with modulators. *J. Biol. Chem.* (1993) **268** 14991–14997.
- 10 Hirohashi T., Suzuki H., Chu X.Y., Tamai I., Tsuji A., Sugiyama Y. Function and expression of multidrug resistance-associated protein family in human colon adenocarcinoma cells (Caco-2). *J. Pharmacol. Exp. Ther.* (2000) **292** 265–270.
- 11 Behrens I., Kamm W., Dantzig A.H., Kissel T. Variation of peptide transporter (PepT1 and HPT1) expression in Caco-2 cells as a function of cell origin. *J. Pharm. Sci.* (2004) **93** 1743–1754.
- 12 Le Ferrec E., Chesne C., Artusson P. et al. In vitro models of the intestinal barrier. The report and recommendations of ECVAM Workshop 46. European Centre for the Validation of Alternative methods. *Altern. Lab. Anim.* (2001) **29** 649–668.
- 13 Artursson P., Borchardt R.T. Intestinal drug absorption and metabolism in cell cultures: Caco-2 and beyond. *Pharm. Res.* (1997) **14** 1655–1658.
- 14 Le Vee M., Jigorel E., Glaise D., Gripon P., Guguen-Guillouzo C., Fardel O. Functional expression of sinusoidal and canalicular hepatic drug transporters in the differentiated human hepatoma HepaRG cell line. *Eur. J. Pharm. Sci.* (2006) **28** 109–117.
- 15 Seithel A., Karlsson J., Hilgendorf C., Bjorquist A., Ungell A. L. Variability in mRNA expression of ABC- and SLC-transporters in human intestinal cells: comparison between human segments and Caco-2 cells. *Eur. J. Pharm. Sci.* (2006) **28** 291–299.
- 16 Englund G., Rorsman F., Ronnblom A. et al. Regional levels of drug transporters along the human intestinal tract: co-expression of ABC and SLC transporters and comparison with Caco-2 cells. *Eur. J. Pharm. Sci.* (2006) **29** 269–277.
- 17 Sai Y., Kaneko Y., Ito S. et al. Predominant contribution of organic anion transporting polypeptide OATP-B (OATP2B1) to apical uptake of estrone-3-sulfate by human intestinal Caco-2 cells. *Drug Metab. Dispos.* (2006) **34** 1423–1431.
- 18 Hilgendorf C., Ahlin G., Seithel A., Artursson P., Ungell A.L., Karlsson J. Expression of 36 drug transporter genes in human intestine, liver, kidney and organotypic cell lines. *Drug Metab. Dispos.* (2007) **35** 1333–1340.
- 19 Xia C.Q., Liu N., Yang D., Miwa G., Gan L.S. Expression, localization, and functional characteristics of breast cancer resistance protein in Caco-2 cells. *Drug Metab. Dispos.* (2005) **33** 637–643.
- 20 Taipalensuu J., Tavelin S., Lazorova L., Svensson A.C., Artursson P. Exploring the quantitative relationship between the level of MDR1 transcript, protein and function using digoxin as a marker of MDR1-dependent drug efflux activity. *Eur. J. Pharm. Sci.* (2004) **21** 69–75.