Breast Cancer Resistance Protein, but not mRNA Expression Correlates with Estrone-3-Sulfate Bi-directional Transport Activity

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Introduction, Aims & Objectives

- Data generated from Caco-2 cell monolayer transport assays can be linked to models that can be used to extrapolate and predict *in vivo* drug absorption and drug-drug interactions. There are few reports of the relationship between absolute protein abundance and transporter activity in Caco-2 cell monolayers[1]. Previously, P-glycoprotein (P-gp) abundances have been correlated to digoxin efflux ratio (ER) in Caco-2 cell monolayers grown for 10 and 29 days[2]. However, no such relationship has been established for Breast Cancer Resistance Protein (BCRP) in Caco-2 cells.
- The purpose of this study was to determine the relationship of BCRP transport activity to mRNA gene expression and absolute protein abundance in Caco-2 cell monolayers. The bi-directional transport of the BCRP probe Estrone-3-Sulfate (E-3-S) was undertaken alongside the BCRP inhibitor Ko143 in 10 and 29d monolayers. BCRP relative mRNA gene expression and protein abundance was also determined in 10 and 29d Caco-2 cell monolayers.

Methods

- Caco-2 cells (ATCC-HTB-37) passage 25-35 were seeded at 2.2x10^5 cells/cm^2 and grown for 10 or 29d on 0.4 µM pore size, 44-cm^2 Transwell filters for BCRP protein abundance analysis and 1.13 cm^2 filters for gene expression and E-3-S transport.
- **BCRP gene expression** was determined by real time PCR using Roche Life Sciences primers and probes and run on a light cycler 480 apparatus relative to the housekeeper protein Cyclophilin A (PPIA).
- Total or plasma membrane proteins were obtained by differential centrifugation. Membrane proteins (typically 50 µg) were shipped to Bertin Pharma (Orleans, France) for protein digestion using the MS2-plex kit and protein abundance quantification[3].
- **Absolute BCRP and Na/K-ATPase abundances** were determined in both membrane fractions by Absolute Quantification (**AQUA**) **LC-MS/MS** methods using the BCRP - SSL[**L**^{13}C,^{15}N]DVLAAR and Na/K-ATPase - AAVPDA[**V**^{13}C,^{15}N]GK standard isotope labelled peptides. Absolute protein abundances are given as femtomol per µg protein.
- **Bi-directional transport of [^{3}-H]-E-3-S** (0.01 µM) with in the presence and absence of the potent BCRP inhibitor Ko143 (2 µM) was performed. The assay was undertaken with pH 7.4 (HBSS-HEPES) buffer in the apical and basolateral chambers, supplemented with 0.05% (w/v) Bovine Serum Albumin (BSA) to limit non-specific binding of E-3-S to the experimental system.
- **E-3-S apparent permeability** (P_{app}) was determined, after correction for E-3-S binding to BSA after determination by ultrafiltration[4], with samples taken at 5, 15, 25, 40, 80 and 120 min.
- Monolayer integrity was monitored by the paracellular marker lucifer yellow (LY, 50 µM). Any filters exhibiting LY P_{app} >1 x 10^{-6} cm.s were discarded from gene expression, protein abundance or transport analysis.

Results

Na/K-ATPase is used as a quality control for membrane harvesting. The magnitude of Na/K-ATPase protein abundances indicate the membrane fraction is of a sufficient quality to measure lower abundance drug transporters. Na/K-ATPase expression is not affected by culture time. Yet, there is limited enrichment in the plasma membrane abundance compared to the preceding total membrane fraction in 10 and 29d monolayers (**Figure 1**).

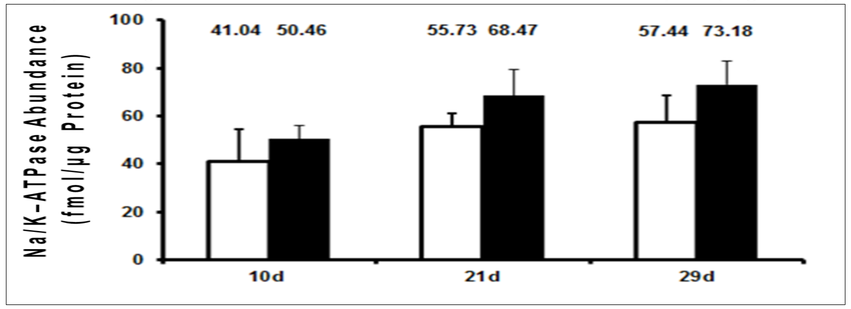

Figure 1. Na/K-ATPase protein abundances in 10 (n=3) and 29d (n=3-6) grown Caco-2 cell monolayer total (white bars) and plasma membrane (black bars) fractions. The bars represent mean ± SD.

BCRP mRNA gene expression in 10 and 29d Caco-2 cell monolayers is shown in **Figure 2A**. There is a non-significant 1.5-fold higher BCRP mRNA gene expression in 29d compared to 10d monolayers. In contrast, there is a lower **BCRP protein abundance** (p=<0.001, unpaired t-test) in 29d compared to 10d Caco-2 cell monolayer total membranes **(Figure 2B)**. Similar to Na/K-ATPase there is limited enrichment in BCRP abundances in plasma compared to the preceding total membrane fraction.

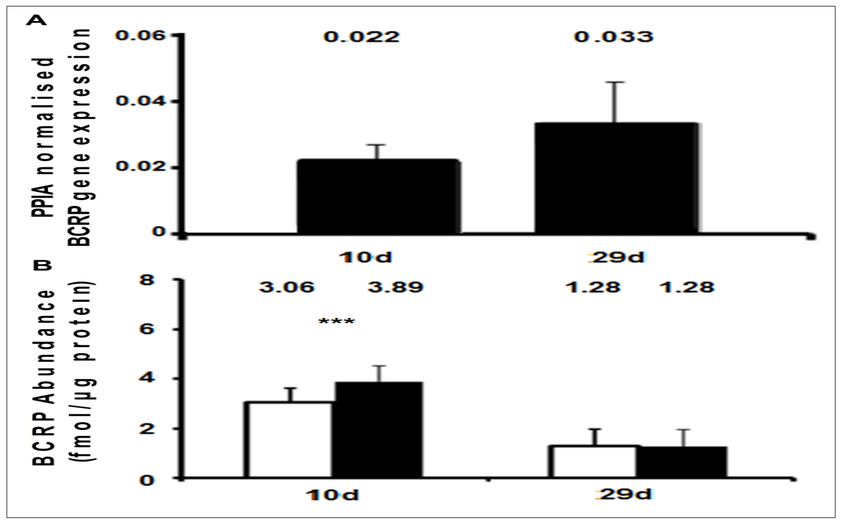

Figure 2. A, Caco-2 cell monolayer (10 & 29d grown) BCRP gene expression normalised to the housekeeper gene PPIA for n=3 separate extractions. Real time PCR assays were run on 2 separate days in duplicate. B, BCRP protein abundances in 10 (n=3) and 29d (n=3-6) grown Caco-2 cell monolayer total (white bars) and plasma membrane (black bars). The text above the bars represents mean expression/abundance values. The bars represent mean ± SD. *** p = <0.001

Figure 3 shows the **bi-directional transport of E-3-S** in 10 and 29d cultured Caco-2 cell monolayers in the presence of Ko143. An increased absorptive and a reduced secretory transport (p=<0.01, unpaired t-test) was observed for 29d compared to 10d monolayers.

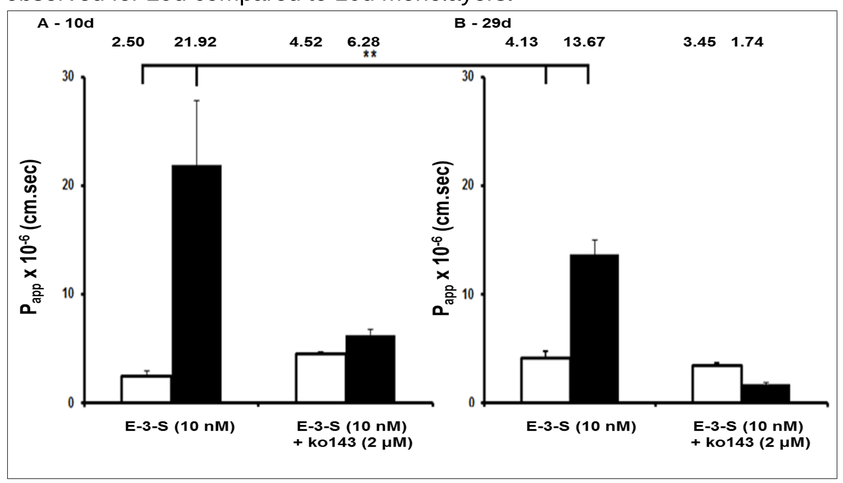

Figure 3. Transport of E-3-S (0.01 µM) in A-to-B (white) and B-to-A (black) transport directions across 10d (A) and 29d (B) cultured Caco-2 cell monolayers in the presence and absence of Ko143 (2 µM). The text above the bars is the mean P_{app}. Values are mean ± SD of a minimum n=6 filters of N=2 experiments. **p = <0.01.

Table 1. The relationship between BCRP mRNA, total membrane protein abundance and E-3-S efflux ratio in 10 and 29d Caco-2 cell monolayers.

Culturing Time	[^{3}H] E-3-S Efflux Ratio (pH7.4/7.4)	BCRP mRNA Expression (Relative to PPIA)	BCRP Absolute Protein Abundance (fmol/µg)
10 days	8.97 (± 2.51)	0.022 (± 0.004)	3.06 (± 0.22)
29 days	3.32 (± 0.67)	0.033 (± 0.013)	1.28 (± 0.33)
Fold Difference (29 days/10 days)	**2.7**	**0.67**	**2.39**

Discussion & Conclusion:

- The expected higher plasma *vs*. total membrane Na/K-ATPase and BCRP abundances was not exhibited. Procedural losses of transporters in steps to obtain the plasma membrane may be responsible for this observation[5].
- Relative BCRP mRNA expression does not correspond to protein abundance.
- Cultivation time influences Caco-2 cell monolayer BCRP protein abundance.
- BCRP protein abundance is a better proxy than relative-mRNA expression for assessing BCRP-dependent E-3-S transport in Caco-2 cell monolayers.

References:

1. Taipalensuu *et al*., 2004., EJPS, 21, 69
2. Miliotis *et al*., 2011, DMD, 39, 2440
3. Kunze *et al.,* 2014, DMD, 42, 1541
4. Neuhoff *et al*., 2006, Pharm Res, 23, 350
5. Harwood *et al*., 2014, DMD, 42, 1766