SHORT COMMUNICATION

Role of ABCG2 in transport of the mammalian lignan enterolactone and its secretion into milk in Abcg2 knockout mice

Verónica Miguel, Jon Andoni Otero, Rocío García-Villalba, Francisco Tomás-Barberán, Juan Carlos Espín, Gracia Merino and Ana I. Álvarez,

Department of Biomedical Sciences - Physiology, Veterinary Faculty (V.M.; A.I.A.), INDEGSAL (J.A.O.; G.M University of Leon 2407, Campus de Vegazana, Leon, Spain, and Research Group on Quality, Safety and Bioactivity of Plant Foods, CEBAS-CSIC, 30100 Campus de Espinardo, Murcia, Spain (R.G.V.; F.T.B.; J.C.E.)
Running title: ABCG2 transports enterolactone

Address correspondence to: Dr. Ana I. Alvarez, Department of Biomedical Sciences - Physiology, Veterinary Faculty, University of Leon 2407, Campus de Vegazana, Leon, Spain. Phone: +34-987291265; Fax +34-987 291267; E-mail: aialvf@unileon.es

Text pages: 14

Tables: 1

Figures: 2

References: 35

Number of words in Abstract: 243

Number of words in Introduction: 448

Number of words in Results & Discussion: 1064

Abbreviations: ABC, ATP-binding cassette; ABCG2, ATP-binding cassette subfamily G2; MXR, mitoxantrone; UPLC-qTOF-MS, ultra-performance liquid chromatography quadrupole time of flight mass spectrometry
ABSTRACT

Lignans are phytoestrogens that are metabolized by the gut microbiota to enterodiol and enterolactone, the main biologically active enterolignans. Substantial inter-individual variation in plasma concentration and urinary excretion of enterolignans has been reported, this being determined, at least in part, by the intake of lignan precursors, the gut microbiota, and the host’s Phase II conjugating enzyme activity. However, the role of ATP-binding cassette (ABC) transporters in the transport and disposition of enterolactone has not been reported so far. Active transport assays using parental and MDCKII cells transduced with murine and human ABCG2 showed a significant increase in apically directed translocation of enterolactone in transduced cells, which was confirmed by using the selective ABCG2 inhibitor Ko143. In addition, enterolactone also inhibited transport of the antineoplastic agent mitoxantrone as a model substrate, with inhibition percentages of almost 40% at 200 μM for human ABCG2. Furthermore, the endogenous levels in plasma and milk of enterolactone in wild-type and Abcg2(-/-) knockout female mice were analysed. The milk/plasma ratio decreased significantly in the Abcg2(-/-) phenotype, as compared to the wild mouse group (0.4 ± 0.1 as against. 6.4 ± 2.6). This paper is the first to report that enterolactone is a transported substrate and therefore most probably a competitive inhibitor of ABCG2, which suggests it has a role in the inter-individual variations in the disposition of enterolactone and its secretion into milk. The inhibitory activity identified provides a solid basis for further investigation in possible food–drug interactions.
Introduction

ABCG2 is an apically expressed ATP-binding cassette (ABC) membrane transporter that mediates the active and outward transport of a wide range of anticancer drugs, dietary compounds, food carcinogens and antibiotics from cells (van Herwaarden et al., 2003; Merino et al., 2005; Vlaming et al., 2009). In recent years, its role as a transporter of phytoestrogens and their conjugated metabolites has gained special relevance (Zhu et al., 2010; Alvarez et al., 2011; Tan et al., 2013).

ABCG2 is found in tumor cells, but also in the cells of a variety of normal tissues involved in the uptake and elimination of compounds, such as enterocytes, hepatocytes, and cells of the proximal tubules of the kidney. The apical localization in these cells allows ABCG2 to mediate hepatobiliary and urinary elimination and to function as a barrier to uptake from the gut lumen (van Herwaarden and Schinkel, 2006). In addition, induced expression of ABCG2 in the lactating mammary gland supports its important role in the active secretion of several xenobiotics and beneficial compounds such as vitamins into milk (Jonker et al., 2005; Merino et al., 2006; van Herwaarden et al., 2007).

Dietary intake of lignans and their derivatives, as part of a healthy diet, has been associated with protective effects against a number of chronic diseases (Hu et al., 2007; Adolphe et al., 2010; Guglielmini et al., 2012; Högger, 2013). When consumed, plant lignans are metabolized by the gut microbiota in the upper part of the colon to enterolactone and enterodiol (Del Rio et al., 2013).

Dietary sources of plant lignans are flax grains, seeds, fruit and vegetables, olive oil, and beverages such as tea, coffee and wine (Kuijsten et al., 2005). A significant concentration of enterolactone has been reported in bovine milk (Antignac et al., 2004). In general, under normal dietary conditions, enterolactone is the enterolignan that reaches the highest concentration in plasma (Högger, 2013; Kuijsten et al., 2006) and is the most effective metabolite in the
inhibition of cell proliferation (Corsini et al., 2010; Azrad et al., 2013). Owing to the potential beneficial effects of enterolactone and the complex relationship between the colonic environment and other factors contributing to systemic exposure of such compounds to ABC transporters, a study of the interaction of ABCG2 with enterolactone could be of particular relevance. In this regard, the aim in this research was to assess the potential role of enterolactone as an ABCG2 substrate and/or inhibitor using MCDKII cells transduced with the transporter. The specific \textit{in vivo} role of the ABCG2 transporter in the disposition of this mammalian lignan was evaluated using wild-type and Abcg2\textsuperscript{-/-} knockout female mice through determination of the endogenous enterolactone milk/plasma ratio found in the two types of mice.

\textbf{Materials and Methods}

\textbf{Chemicals.} Mitoxantrone and nitrofurantoin were obtained from Sigma-Aldrich (St. Louis, Missouri, U.S.A.). Enterolactone was purchased from Toronto Research Chemicals (Ontario, Canada). All the other chemicals were of analytical grade and obtained from commercial sources.

\textbf{Cell cultures.} Madin-Darby canine kidney epithelial cell (MDCKII parent cells) and their human ABCG2 and murine Abcg2 transduced sub-clones were kindly provided by Dr. A.H. Schinkel, Netherlands Cancer Institute (Amsterdam, The Netherlands). Culture conditions were as described in a previous publication (Merino et al., 2005). The cells were cultured in DMEM supplied with GlutaMAX™ (Life Technologies, Inc., Carlsbad, California, U.S.A.) and supplemented with penicillin (50 units/mL), streptomycin (50 µg/mL), and 10\% (v/v) fetal calf serum (MP Biomedicals, Solon, Ohio, U.S.A.) at 37°C in the presence of 5\% CO\(_2\). The cells were treated with trypsin every 3 to 4 days for sub-culturing.
**Transport studies.** Transport assays using Transwell plates were carried out as previously described (Merino et al., 2005) with minor modifications. Cells were seeded on microporous polycarbonate membrane filters (3.0 µm pore size, 24 mm diameter; Transwell 3414; Costar) at a density of 1.0 × 10^6 cells per well and grown for 3 days. Transepithelial resistance, measured in each well using a Millicell ERS ohmmeter (Millipore), was used to check the tightness of the monolayer.

Murine Abcg2 and human ABCG2 mediated transport was determined after the addition of enterolactone to the donor compartment at the beginning of the experiment. The appearance of enterolactone in the acceptor compartment was recorded as a fraction of the total (10 µM) added to the donor compartment at the beginning of the experiment. Aliquots of 100 µL were taken from the opposite compartment after 2 hours, and stored at −20°C until analysis. The specific Abcg2/ ABCG2 inhibitor Ko143 (Allen et al., 2002) was added concomitantly with enterolactone, to test the reliability of the efflux of enterolactone by ABCG2.

**Accumulation assays.** *In vitro* accumulation assays were carried out as described elsewhere (Pavek et al., 2005). Mitoxantrone (MXR, 10 µM) was used as fluorescence substrate and enterolactone was used as an inhibitor at different concentrations. The relative cellular accumulation of MXR was determined by flow cytometry using a CYAN cytometer (Beckman Coulter) from histogram plots using the median of fluorescence (MF). ABCG2 inhibition increases the accumulation of MXR in ABCG2-transduced cells and thus increases the MF. Inhibitory potencies were calculated as follows: Inhibitory potency = (MF with tested compound - MF without inhibitor) / (MF with Ko143 - MF without inhibitor) × 100%.

**Animals.** Mice (weighing 30 g ± 2 g) were housed and handled in accordance with procedures approved by the Research Committee of Animal Use of the University of Leon,
Spain, which were carried out under the “Principles of Laboratory Animal Care” and the European guidelines described in EC Directive 86/609. The animals used in the experiments were Abcg2(-/-) (n=5) and wild-type female mice (n=5) aged between 9 and 14 weeks, all of them > 99% FVB genetic background. The animals were kept in a temperature-controlled environment with a 12-hour light/12-hour dark cycle. The mice were fed normal rodent feed (Scientific Animal Food & Engineering-SAFE, A04/A04C/R04) from Panlab S.A. Barcelona, Spain, and water was available ad libitum. Pups of approximately 10 days old were separated from their mother approximately 4 hours before milk collection. Oxytocin (200 µl of 1 IU/ml solution) was administered subcutaneously to lactating mothers in order to stimulate milk secretion. Blood was collected by orbital bleeding and milk was obtained from the mammary glands by gentle vacuum suction after anaesthesia with isoflurane. Heparinized blood samples were centrifuged immediately at 1000 × g for 10 min.

**UPLC-qTOF-MS analysis.** Plasma and milk samples (150 and 80 µl, respectively) were mixed with sodium acetate buffer and treated with hydrochloric acid in methanol, following the method of Bolca et al., (2010), re-dissolved in methanol and filtered (0.22 µm) prior to injection into the LC-MS system.

Samples were analysed using an Agilent 1290 Infinity LC system coupled to a 6550 Accurate-Mass qTOF (Agilent Technologies, Waldbronn, Germany) using an electrospray interface (Jet Stream Technology). Data were processed using the Mass Hunter Qualitative Analysis software (B.06.00). Analytes were separated on a reverse phase Poroshell 120 EC-C18 column (3 × 100 mm, 2.7 µm) (Agilent Technologies) operating at 30ºC. The mobile phases used were water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) at a flow rate of 0.4 ml/min and the following gradient conditions: 2 to 25% B at 0 to 10 min, 25 to 40% B at 10 to 16 min; 40 to 95% B at 16 to 19 min; 95% to 2% at 20 to 21 min and the column re-
equilibrated for 5 minutes. A volume of 2 μl of the sample was injected. Electrospray interface parameters were as follows: gas temperature 280ºC, drying gas 9 l/min, nebulizer 35 psi, sheath gas temperature 400ºC and sheath gas flow 12 l/min. Spectra were acquired in the range 100 to 1100 m/z in negative mode and fragmentor voltage was 100 V.

Quantification of enterolactone was carried out by peak area integration of its extracted ion chromatograms (EIC) at m/z 297.1132. The calibration curve was linear over the concentration range from 2.6 to 2,000 nmol/l. The limits of detection and quantification were 0.8 nmol/l and 2.6 nmol/l, respectively. Repeatability, expressed as the relative standard deviation (RSD) of peak area, was 3.3% for intra-day repeatability and 6% for inter-day repeatability. The recovery of enterolactone from plasma and milk reached 60% and 70%, respectively.

Results and Discussion

The enterolignans enterodiol and enterolactone have been reported to exert protective effects through a variety of mechanisms including phytoestrogenic, antioxidant, anti-inflammatory and anticancer effects from in vitro and in vivo studies (Hu et al., 2007; Högger, 2013; Crozier et al., 2009). Recently, an inverse relationship between a decreased mortality risk and enterolactone levels > 10 nmol/l has been found (Guglielmini et al., 2012).

The results obtained here give evidence for a role of ABCG2 in the transport of enterolactone. Active transport assays using parental and human ABCG2 and murine Abcg2 transduced MDCKII cells showed an increase in apically directed translocation of enterolactone in transduced cells, which was confirmed with the use of the selective ABCG2 inhibitor Ko143 (Fig. 1). These data are the first to identify the mammalian lignan enterolactone as a substrate of ABCG2. The metabolites of dietary phenolics, such as enterolignans, reach the systemic bloodstream in the range from nmol/l to low μmol/l concentrations. These levels of bioavailable metabolites can exert modulatory effects in cells through selective actions on different
components of the intracellular signalling cascades, vital for cellular functions such as growth, proliferation and apoptosis (Corsini et al., 2010; Crozier et al., 2009). Therefore, the concentrations used in the present study (10 μm/l) are physiologically relevant (Corsini et al., 2010). In addition, the metabolism and tissue distribution of enterolactone as well as its enterohepatic circulation (Högger, 2013) may be directly influenced by ABCG2 interaction. In fact, in these experiments endogenous milk/plasma concentration ratios pointed to a specific role for ABCG2 in the transport of enterolactone into milk (Table 1). The milk/plasma ratio decreased significantly in the Abcg2(-/-) phenotype as compared with the wild-type mouse group (0.4 ± 0.1 as against 6.4 ± 2.6). In this regard, a noteworthy concentration of enterolactone (234 ± 90 nM) has been reported in bovine milk (Antignac et al., 2004), in agreement with the data obtained here for mouse milk (391 ± 225 nM). In addition, mean plasma concentrations of endogenous enterolactone in wild-type mice (57 ± 13 nM; Table 1) were in agreement with the values reported in humans (Adolphe et al., 2010; Kuijsten et al., 2005). Recently, the secretion of compounds into ruminant milk has been reported to be affected by ABCG2 polymorphisms and ABCG2 inhibition by drugs and flavonoids (Real et al., 2011; Otero et al., 2013). Thus, these ABCG2-related factors presumably could affect enterolactone concentrations in milk and thus the possible health benefits of lignans from the consumption of milk. However, care should be taken since exposure to lignans during early stages of life may adversely alter normal development due to their phytoestrogen nature (Ward et al., 2001).

Enterolactone exposure is partly determined by intake of lignan precursors, intestinal bacterial activity and Phase-II conjugating enzyme activity (Kuijsten et al., 2005; Adlercreutz, 2002; Lampe et al., 2006). Conjugation of enterolignans with sulphate and glucuronic acid occurs in the intestinal wall and liver, with the predominant conjugates being glucuronides. Both sulfate and glucuronide conjugates have been identified as ABCC2 and ABCG2 substrates (Lampe et al., 2006; van de Wetering and Sapthu, 2012; Krumpochova et al., 2012). These
authors found that ABCG2 was able to transport the enterolactone sulfate with low affinity, and its disposition in Abcg2−/− mice was altered, highlighting the physiological relevance of this result. The role of ABCG2 in the transport of phenolic compounds and their conjugates is well known (Alvarez et al., 2011). ABCG2 is not only present at a considerable level in the main organs involved in the conjugation of enterolactone, the intestinal wall and liver (Vlaming et al., 2009), but is also located in the brush border membrane of kidney proximal tubule cells (Huls et al., 2008). Controlled feeding studies have demonstrated dose-dependent urinary lignan excretion with substantial variation in the systemic disposition and urinary excretion of enterolignans (Lampe et al., 2006), thus, the interaction of enterolactone and its conjugates with ABCG2 may be a factor that contributes to these variations, together with interaction with the colonic environment.

Although enterolactone is an ABCG2 substrate, in the present case, no statistically significant differences between Abcg2 knockout and wild-type mice in endogenous levels in plasma were noted. Some authors have reported no ABCG2-mediated effect in plasma systemic profile of some ABCG2 substrates, while it has proved possible to demonstrate local effects in compound transfer in the placenta or the mammary glands (Jonker et al., 2005; Zhou et al., 2008). Additional factors affecting systemic disposition, such as other transporters or metabolism, might preclude an ABCG2 effect at this level.

In addition, to characterize further the interaction of enterolactone with mouse ABCG2 and human ABCG2, the ability of this compound to modulate the accumulation of the antineoplastic agent mitoxantrone as a model ABCG2 substrate in murine Abcg2 and human ABCG2 expressing cell lines was tested in flow cytometry experiments. ABCG2 inhibition increased the accumulation of mitoxantrone in transduced cells, reaching 38% ± 2% of inhibition (200 μM) (Fig. 2). These results agree with Tan et al., (2013) who described significant inhibitory activities of sinapic and ellagic acids against ABCG2-mediated transport of methotrexate, with IC₅₀ values
of 117.5 and 20.7 μM, respectively, values which were considerably less than those reported for other flavonoids. The concentrations used in the present study were physiologically significant, as the concentrations achievable in the colon lumen have been estimated to be 10 to 1000 μM (Kitts et al., 1999). Moreover, it should also be kept in mind that enterohepatic circulation and enteric recycling might lead to longer residence times, and thus to accumulation of these compounds in the body, in particular with repeated intake. Therefore, enterolactone inhibition of the ABCG2 function should be considered related to potential food-drug interactions affecting the pharmacokinetics of other ABCG2 substrates, as reported previously for dietary flavonoids (Vlaming et al., 2009; Tan et al., 2013). In addition, ABCG2 inhibitors may be useful in other fields of application, for instance in resistance reversal in chemotherapy (Allen et al., 2002; Robey et al., 2009). Further studies are needed to establish the in vivo application of this compound as an ABCG2 inhibitor.

In this study the enterolignan enterolactone was identified as an ABCG2 substrate, and its Abcg2-mediated secretion into milk was demonstrated, this potentially affecting the health benefits related to the lignan content of milk. Furthermore, the inhibitory activity of this mammalian lignan that was identified provides a base for further investigations into possible food–drug interactions.

Acknowledgements. The Abcg2(−/−) mice were kindly supplied by Dr. AH Schinkel from the Netherlands Cancer Institute.

Authorship Contributions. Participated in research design: Álvarez and Merino

Conducted experiments: Miguel, Otero, García-Villalba

Performed data analysis: Tomás-Barberán and Espín

Wrote or contributed to the writing of the manuscript:

Álvarez, Merino and Espín
References


Footnotes
This work was supported by the Spanish Ministry of Economy and Competitiveness and the European Regional Development Fund [Projects CICYT AGL2012-31116, AGL2011-22447]; and the Seneca Foundation of the Murcia Region, Spain [Excellence Group GERM 06 04486, 05556/PI/04, Consolider Ingenio 2010, CSD2007-00063 (Fun-C-Food)]. J.A.O. holds a pre-doctoral fellowship of the Basque Government and R.G.V. is the holder of a JAE-DOC contract from the Spanish Higher Council for Scientific Research (CSIC, Spain) co-financed by the European Social Fund (ESF).
Legends for Figures

**Figure 1.** Transepithelial transport of enterolactone (10 μM) in MDCKII (parent), MDCKII-Abcg2 and MDCKII-ABCG2 monolayers. Two hours before the start of the experiment, cells were pre-incubated with or without the ABCG2 inhibitor Ko143 (1 μM) (Ko). The experiment started with the addition of enterolactone to one compartment (basolateral or apical), either with or without 1 μM Ko143. After 2 hours, the percentage of drug appearing in the opposite compartment was measured using UPLC-qTOF-MS. The results are means; error bars indicate S.D. (n = 3 to 4).

**Figure 2.** Effect of the enterolignan enterolactone on the accumulation of mitoxantrone (at 10 μM) in parent MDCKII cells and in their murine Abcg2- and Human ABCG2-transduced derivatives. Cells were pre-incubated with or without Ko143 (1 μM) or enterolactone at the indicated concentrations. A) Units of fluorescence, (median). B) Percentage of inhibition of the different concentrations of enterolactone. Data are expressed as mean values ± SD (n = 3). Percentage of inhibition was related to the effect of reference inhibitor Ko143 (set at 100% inhibition of ABCG2).
**TABLE 1**

Enterolactone concentration (nM) ± SD in plasma and milk samples from wild-type and Abcg2\(^{(-/-)}\) mice fed on standard feed (n = 5).

<table>
<thead>
<tr>
<th>ENTEROLACTONE</th>
<th><strong>Wild-type</strong></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Milk</td>
<td>Plasma</td>
<td>Milk/Plasma Ratio</td>
<td></td>
</tr>
<tr>
<td>136.23</td>
<td>39.85</td>
<td>3.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>382.16</td>
<td>52.88</td>
<td>7.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>210.38</td>
<td>52.66</td>
<td>4.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>567.67</td>
<td>72.55</td>
<td>7.82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>663.13</td>
<td>69.03</td>
<td>9.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>391±225</td>
<td>57±13</td>
<td>6.4±2.6</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Abcg2(^{(-/-)})</strong></th>
<th>Milk</th>
<th>Plasma</th>
<th>Milk/Plasma Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>30.06</td>
<td>57.47</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>57.17</td>
<td>118.29</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>29.16</td>
<td>87.08</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>15.87</td>
<td>34.79</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>14.16</td>
<td>85.9</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>29±17*</td>
<td>77±32</td>
<td>0.4±0.1*</td>
</tr>
</tbody>
</table>

*P < 0.05, significantly different between wild-type and knockout mice
Figure 2

A) Arbitrary units of fluorescence (median)

B) % inhibition compared to 100% Ko143 inhibition

- MXR
- MXR + Ko143
- MXR + Ent 100 µM
- MXR + Ent 200 µM

- Abcg2
- ABCG2

MH + Ent 100 µM  |  MH + Ent 200 µM