Short Communication

THE DIETARY POLYPHENOL ELLAGIC ACID IS A POTENT INHIBITOR OF hOAT1

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Running title: POTENT INHIBITION OF hOAT1 BY ELLAGIC ACID

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ABBREVIATIONS: OAT, organic anion transporter; EA, ellagic acid; OATP, organic anion transporting polypeptide; OR-2, oocyte Ringer medium; PAH, p-aminohippuric acid; BSP, bromosulfophthalein; CSF, cerebrospinal fluid. The prefixes h, r, and m denote human, rat and mouse transporters.
ABSTRACT:

Ellagic acid (EA), a polyphenol present in berries, has been demonstrated to be preventive of esophageal and colon cancer in animals. Here, we have studied the ability of organic anion transporters (OATs) and organic anion transporting polypeptides (OATPs) to transport EA. The accumulation of radiolabeled [14C]EA, [3H]p-aminohippuric acid (PAH), [14C]glutarate, [3H]estrone sulfate, [3H]ochratoxin A, and [3H]taurocholic acid ± inhibitor(s) was tested in OAT- and OATP-expressing oocytes. Oocytes expressing hOAT1, rOat1 and hOAT4 accumulated 6.5-, 7.1-, and 8.9-fold more EA than water-injected oocytes, respectively. This accumulation was prevented by the prototype OAT inhibitors bromosulfophthalein and probenecid. rOatp1, mOat2, hOAT3, and mOat5 showed no EA transport. The uptake of the prototype OAT substrate PAH in hOAT1-expressing oocytes was dose-dependently and potently inhibited by EA with an IC50 of 207 nM. In conclusion, we have demonstrated that the OAT family members hOAT1, rOat1, and hOAT4 mediate transport of EA, with a very high affinity for hOAT1.
Organic anion transporters (OATs) play a critical role in the distribution and elimination of a diverse array of exogenous and endogenous compounds. The substrates of OATs are small organic anions at physiologic pH and include a multitude of clinically used therapeutics such as angiotensin converting enzyme inhibitors, β-lactam antibiotics, and nonsteroidal anti-inflammatory drugs. Certain drugs that are eliminated from the body mainly by the kidneys through the OATs can compete with other OAT substrates for transport. This competition for transport can cause retention of certain drugs leading to longer plasma half-lives (Burckhardt and Burckhardt, 2003). Historically, the interaction was utilized to maintain penicillin plasma levels with the use of probenecid.

Plant polyphenols are the focus of much research for the ability to affect adverse human biological disease states. One of these polyphenols, ellagic acid (EA), found naturally in our diet (Lei et al., 2001) has been demonstrated to be a cancer preventive agent for esophageal cancer in animal models (Stoner and Gupta, 2001). EA is a small organic anion at physiologic pH (Priyadarsini et al., 2002) and its accumulation in human intestinal Caco-2 cells was demonstrated to have OAT-like properties (Whitley et. al., submitted). Studies in mice have noted that the kidney is the primary route of elimination for EA equivalents (Teel and Martin, 1988) and it is also the tissue in which OAT transporters have particular importance. Therefore, the present study was focused on determining the ability of the OATs to transport EA and whether EA could influence OAT transport.
Materials and Methods

Materials. [14C]Ellagic acid was synthesized with a specific activity of 20 mCi/mmol at the Ohio State University Comprehensive Cancer Center (Zeng et al., 1991) and was a kind gift from Dr. Gary Stoner, Ohio State. [3H]Taurocholic acid (3.5 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA). [14C]Glutarate (55 mCi/mmol) was purchased from MP Biomedicals (Orangeburg, NY). [3H]p-aminohippuric acid (20 Ci/mmol), [3H]estrone sulfate (57 Ci/mmol), and [3H]ochratoxin A (15 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

X. laevis oocyte uptake assay. X. laevis oocytes were obtained as described previously (Youngblood and Sweet, 2004). Substrate uptake assays were performed 3 days after injection with 20 ng of capped cRNA (hOAT1, rOat1, mOat2, hOAT3, hOAT4, mOat5, or rOatp1), as previously described (Cihlar et al., 1999). Oocytes were randomly divided into experimental groups of 10 and incubated for 60 min (Sweet et al., 1997) in 1 ml oocyte Ringer medium (OR-2) containing 20 µM [14C]EA in all types of oocytes, 10 µM [3H]p-aminohippurate (PAH) in hOAT1 and rOat1 oocytes (Pritchard and Miller, 1993), 1 µM [3H]estrone sulfate in hOAT3 oocytes (Cha et al., 2001), 36.4 µM [14C]glutarate in mOat2 oocytes (Kobayashi et al., 2002), 1 µM [3H]ochratoxin A and 1 µM [3H]estrone sulfate in hOAT4 oocytes (Cha et al., 2000), 1 µM [3H]ochratoxin A in mOat5 oocytes (Youngblood and Sweet, 2004), 300 nM [3H]taurocholate in rOatp1 oocytes (Li et al., 1998). The uptake inhibitors used were 1 mM probenecid (Cihlar et al., 1999) or 500 µM bromosulfophthalein (BSP) (Whitley et al., submitted). Other inhibitors used were 0.05 – 35 µM EA or 5 – 200 µM indoxyl sulfate or 50 – 900 µM sodium salicylate (Khamdang et al., 2002; Enomoto et al., 2003). Oocytes were rapidly rinsed 4 times with ice-
cold OR-2 and after digestion with sodium hydroxide, individual oocyte radioactivity was measured by liquid scintillation spectroscopy with external quench correction. Each experiment was repeated in 3 different animals. Water-injected oocytes were included as negative controls in each experiment.

Statistics. Data are expressed as means ± S.E. Statistical differences were determined using ANOVA followed by Dunnett Multiple Comparison Test. Differences were considered significant when $P \leq 0.05$. 
Results

Oocyte accumulation. Experiments in oocytes were conducted to identify specific transporters that recognize EA as a substrate. The uptake of radiolabeled positive markers for each transporter was used to confirm protein expression at the membrane. Probenecid and BSP were used as inhibitors of positive control uptake and exhibited significant inhibition in cRNA-injected oocytes with little or no effect in water-injected oocytes (data not shown).

Accumulation of 20 μM $^{[14C]}$EA was measured in water-injected oocytes and oocytes expressing hOAT1, rOat1, mOat2, hOAT3, hOAT4, mOat5, or rOatp1. As seen in Fig. 1, $^{[14C]}$EA was significantly accumulated in hOAT1 (6.4 ± 1.4 fold), rOat1 (7.0 ± 1.4 fold), and hOAT4 (8.9 ± 1.8 fold) expressing oocytes as compared to water-injected controls (P < 0.0001). The accumulation of $^{[14C]}$EA in hOAT1, rOat1, and hOAT4 expressing oocytes was abolished in the presence of probenecid or BSP (P < 0.05). There was no significant $^{[14C]}$EA accumulation observed in mOat2, hOAT3, mOat5, or rOatp1 expressing oocytes (data not shown).

OAT inhibition. Further experiments in oocytes were performed to define the affinity of the interaction between EA and hOAT1. Because of EA’s limited solubility and the low specific activity of the radiolabel, $^{[14C]}$, identification of a $K_m$ value of EA for hOAT1, rOat1, and hOAT4 was unsuccessful. Thus, to quantify the affinity of the interaction of EA with hOAT1, rOat1, and hOAT4, we determined the inhibitory effect of EA against $^{[3H]}$PAH accumulation in hOAT1- and rOat1-expressing oocytes and against $^{[3H]}$OA in hOAT4-expressing oocytes. EA at 5-35 μM, concentrations that may be attainable in the human plasma after EA-rich sources of berries and fruits, exhibited near complete inhibition of 10 μM $^{[3H]}$PAH, approximate $K_m$ of PAH for hOAT1 (Hosoyamada et al., 1999), accumulation mediated by hOAT1, Fig. 2. Similarly, $^{[3H]}$PAH accumulation mediated by rOat1, the rat hOAT1 ortholog, was also nearly completely inhibited by EA at 5-35 μM. In contrast, EA showed only partial inhibition of
[\textsuperscript{3}H]OA accumulation mediated by hOAT4 with a maximum inhibition of 68% at 35 \textmu M EA. There was no significant inhibition of positive control uptake in presence of negative controls (estrone sulfate for hOAT1 and rOat1 and PAH for hOAT4 – data not shown). As seen in Fig. 3, there was a concentration-dependent, highly potent inhibition of 10 \textmu M [\textsuperscript{3}H]PAH accumulation in hOAT1 oocytes by EA with an IC\textsubscript{50} value of 207 nM. Indoxyl sulfate and sodium salicylate showed inhibition, though much less potent or effective than EA, with IC\textsubscript{50} values of approximately 50 \textmu M and 300 \textmu M respectively (data not shown), in agreement with previous reports (Khamdang et al., 2002; Enomoto et al., 2003) further emphasizing the extraordinary potency of EA as a hOAT1 inhibitor.
Discussion

Studies in Caco-2 cells demonstrated that cellular accumulation of EA was dose-dependently and potently inhibited by BSP (Whitley et al., submitted). This suggested that members of the OAT and/or OATP families of transporters may mediate the cellular entry of EA. Therefore, to directly test the ability of OAT and OATP family members to transport EA, EA uptake was measured in oocytes expressing individual members of both families. hOAT4 and the orthologs hOAT1 and rOat1 were identified as transporters of EA. The increase in accumulation of EA when these transporters were expressed was completely inhibited by the commonly used OAT inhibitors, probenecid and BSP. Other members of the OAT family as well as rOatp1 did not transport EA. Upon further investigation of hOAT1 and rOat1, EA displayed a potent ability to inhibit transport of PAH, a prototypical substrate (Pritchard and Miller, 1993).

Although EA per se has been reported to have very low oral bioavailability (Teel and Martin, 1988), significant plasma concentrations may be achieved from natural precursor sources, e.g. ellagitannins, as recently reported (Seeram et al., 2004). The very low IC$_{50}$ of 207 nM for the EA inhibition of hOAT1 transport indicates an extraordinary affinity of this transporter for EA. This very high affinity could lead to EA-drug interactions, as hOAT1 is a key component in the renal secretion of a wide variety of therapeutics and endogenous substrates such as β-lactam antibiotics, ACE inhibitors, nonsteroidal anti-inflammatory drugs, antiviral drugs, prostaglandins, and diuretics. It is interesting to compare the OAT1 IC$_{50}$ for EA and the published OAT1 K$_m$ values of acyclovir (342 µM) and AZT (45.9 µM) (Burckhardt and Burckhardt, 2003). Therefore based on the localization of OAT1, an EA-drug interaction could potentially be utilized to attain higher plasma and/or cerebrospinal fluid (CSF) levels of either acyclovir or AZT by decreasing their renal elimination and/or CSF elimination by the choroid
plexus, respectively. This high affinity interaction may also lead to its development as a useful diagnostic in OAT1 transport research, i.e. its use as a “chemical knockout” of OAT1.

In conclusion, X. laevis oocytes expressing hOAT1, rOat1 and hOAT4 were all demonstrated to mediate efficient inhibitor-sensitive transport of EA. Finally, we showed the interaction between hOAT1 and rOat1 with EA to be one of high affinity. This study demonstrates the potential interaction of EA with therapeutics and/or endogenous substrates through OATs expressed in the kidney and/or blood brain barrier.
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References


Footnote

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Legends to Figures

Fig. 1. Cellular accumulation of EA in OAT-expressing oocytes. Oocytes were incubated for 60 min with 20 µM [14C]EA in the presence/absence of 1 mM probenecid or 500 µM bromosulfophthalein (BSP). *P < 0.01, n ≥ 19 except for BSP treatment where n ≥ 6.

Fig. 2. Cellular accumulation of p-aminohippurate (PAH) in (A) hOAT1- and (B) rOat1-expressing oocytes and ochratoxin A (OA) in (C) hOAT4-expressing oocytes. Oocytes were incubated for 60 min with 10 µM [3H]PAH or 1 µM [3H]OA in the presence/absence of 0.05 – 35 µM EA or 1 mM probenecid. *P < 0.05, **P < 0.01 – Compared to control, n ≥ 9.

Fig. 3. Cellular accumulation of PAH in hOAT1-expressing oocytes in presence of increasing concentrations of EA. Oocytes were incubated for 60 min with 10 µM [3H]PAH in the presence of EA. n ≥ 9.
Fig. 1

- Control
- Probenecid
- BSP

EA uptake
Fold Increase

Water hOAT1 rOat1 hOAT4

* * * * *

Fig. 1
Fig. 2
Fig. 3

IC$_{50}$ = 207 nM

PAH uptake % of control

EA, Log(M)

Fig. 3