The Modified Dipeptide, Enalapril, an Angiotensin-Converting Enzyme Inhibitor, Is Transported by the Rat Liver Organic Anion Transport Protein

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Oatp1, the organic anion transport polypeptide, is an integral membrane protein cloned from rat liver that mediates the uptake of various organic anions such as bromosulfophthalein (BSP) and taurocholate (TCA). Recent studies by others revealed that the thrombin inhibitor, CRC 220, a modified dipeptide, was transported by oatp1. The present study was designed to examine whether another modified peptide, enalapril, an angiotensin-converting enzyme inhibitor, was also a substrate. Transport was studied with enalapril (1 to 800 μmol/L, with [^{3}H]enalapril) in a HeLa cell line stably transfected with oatp1-cDNA under the regulation of a Zn^{2+}-inducible promoter. Noninduced transfected cells (without zinc) that did not express oatp1 failed to take up enalapril. In contrast, cells expressing oatp1 transported enalapril, estrone sulfate (E_1S), taurolithocholic acid sulfate (TLCAS), and the glutathione conjugate of BSP (BSPGSH). Uptake of enalapril by oatp1 at 37°C was substantially higher than that at 4°C. The rate at 37°C (uptake rates for induced - noninduced, transfected cells) was linear over 5 minutes and was concentration-dependent, characterized by a K_m of 214 ± 67 μmol/L and a V_{max} of 0.51 ± 0.15 nmol/min/mg protein. Enalapril uptake was inhibited competitively by BSP (at 1, 5, 10, and 50 μmol/L) and TCA (at 5, 25, and 100 μmol/L) with inhibition constants (K_i) of 2 and 32 μmol/L, respectively. The metabolite enalaprilat was, however, not transported by oatp1. That oatp1 is not a general transporter of anionic compounds was further shown by the lack of transport of harmol sulfate, benzoate, and hippurate. These observations attest to the role of oatp1 as a specific transporter for at least two classes of pharmacologically important peptides. (Hepatology 1998;28:1341-1346.)

Abbreviations: Ntcp, sodium-dependent taurocholate cotransporting polypeptide; oatp1, rat liver organic anion transport polypeptide; BSP, bromosulfophthalein; E_1S, estrone sulfate; TCA, taurocholate; TLCAS, taurolithocholic acid sulfate; BSPGSH, the glutathione conjugate of BSP; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; SFM, serum-free medium.

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Much progress was made within the last decade on the identification of transport proteins residing on the liver cell basolateral membrane. These advances were made possible by the development of expression cloning techniques in *Xenopus* oocytes or cell systems. The comment applies in particular to the sodium-dependent taurocholate cotransporting polypeptide (Ntcp)[1,2] and the sodium-independent, multispecific organic anion transport polypeptide (oatp).[3,4] The rat liver oatp1 (formerly known as oatp) is a bicarbonate/anion exchanger[5] capable of transporting anions such as the amphiphilic anionic dye, bromosulfophthalein (BSP), bile acids,[5-8] and estrogens (estradiol-D-17β-glucuronide and estrone-3-sulfate [E_1S]).[9] The cloned human OATP is closely related to, but is not identical to, oatp1, because it bears 67% amino acid sequence homology[10] and is capable of transporting not only BSP and taurocholate (TCA), but also cationic compounds and neutral steroids.[11,12]

In view of its alleged wide substrate specificity, functional characterization of the organic anion transporting polypeptide is particularly pertinent to the examination of uptake mechanisms of drugs. The peptidomimetic thrombin inhibitor, CRC 220 (4-methoxy-2,3,6-trimethylphenylsulfonyl-L-aspartyl-D-4-amidinophenylalanyl-piperidide), and ochratoxin A, a mycotoxin (dihydroisocoumarin, which is linked to a L-β-phenlyalanine by an amide bond), were found to be transported by oatp1 expressed in oocytes.[13,14]

Recently, a stably transfected HeLa cell system has been devised in which functional oatp1 expression is under regulation of a zinc-inducible metallothionein promoter.[7] In the present study, we took advantage of this system to examine oatp1-mediated transport of the angiotensin-converting enzyme inhibitor, enalapril, a modified dipeptide, and its de-esterified dicarboxylate metabolite, enalaprilat. In addition, we hypothesized that if enalapril or its metabolite enalaprilat is a substrate of oatp1, the transport will be competitively inhibited by BSP and taurocholic acid (TCA). Enalapril, (*S*)-1-[*N*-[1-(ethoxycarbonyl)-3-phenylpropyl]-L-alanyl]-L-proline, is an ACE inhibitor (Fig. 1) that exhibits antihypertensive activity through its de-esterified active di-acid metabolite, enalaprilat.[15] Although the clearance of enalapril by the perfused rat liver is rapid,[16,17] the mechanism for hepatic uptake of this therapeutically important pharmacological agent remains virtually unknown. Evidence for diffusion-limited uptake of the metabolite enalaprilat by the liver, however, has been provided in multiple indicator dilution, rat liver perfusion studies.[18] In addition to study of the transport of enalapril and enalaprilat by oatp1-cDNA transfected HeLa cells, we also examined transport of several

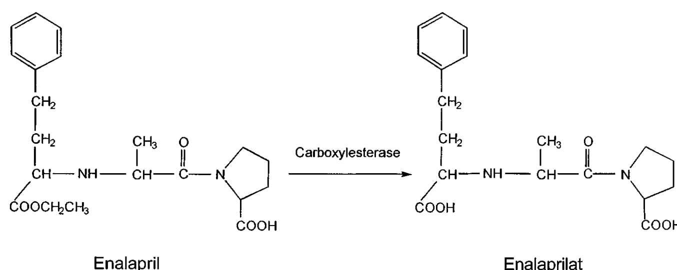

FIG. 1. Enalapril and its conversion to enalaprilat by carboxylesterases.

other tracer organic anions that are known to undergo carrier-mediated transport into the liver. The compounds include benzoate and hippurate,[19-22] E_1S[7,9,23,24] harmol sulfate,[24,25] taurolithocholic acid sulfate (TLCAS),[26] and bromosulfophthalein glutathione conjugate (BSPGSH).[27,28]

MATERIALS AND METHODS

Source of Materials. [^{14}C]Benzoic acid (specific activity: 16 mCi/mmol) was purchased from New England Nuclear (Boston, MA). The radiochemical purity of [^{14}C]benzoic acid was >99%, as judged by high-performance liquid chromatography (HPLC).[29] [^{3}H]E_1S (ammonium salt; specific activity: 50 Ci/mmol) was purchased from Dupont (Boston, MA). [^{3}H]Hippuric acid was synthesized according to Ingersoll and Babcock[30] from [2-^{3}H]glycine (specific activity: 38.8 Ci/mmol; obtained from Dupont) and benzoyl chloride; the resulting radiolabeled product was purified by HPLC and was found to be 96% pure. [^{3}H-22,23]TLCAS (specific activity: 0.79 Ci/mmol) was a gift from Dr. Alan F. Hoffman, UCSD, La Jolla, CA; the radiochemical purity was >97% by thin-layer chromatography (TLC).[31] The tritiated glutathione conjugate of bromosulfophthalein ([^{3}H]BSPGSH) was synthesized from [2-^{3}H]glutathione (250 μCi) and 0.2 μmol of glutathione mixed with 2 μmol of BSP at pH 9 with rat liver glutathione *S*-transferase (Sigma Chemical Co., St. Louis, MO) according to Geng et al.[27]; the resulting specific activity and purity of [^{3}H]BSPGSH as determined by HPLC[32] and TLC (silica gel in a solvent system of 1-butanol:water:acetic acid [75:25:10, vol/vol/vol]) was 215 μCi/mmol and 99% pure, respectively. [^{35}S]BSP was synthesized according to Kurisu et al.[33]; the specific activity was 3 Ci/mmol, and the radiochemical purity determined by TLC was >95%.

[^{3}H]Harmol sulfate was biosynthesized from tracer harmol (98% pure of specific activity 30 mCi/mmol furnished by tritium exchange from New England Nuclear) with the saline-perfused rat liver preparation (40 mL/min) in a procedure described earlier.[34] After biosynthesis, the perfusate, containing mainly [^{3}H]harmol sulfate, was lyophilized and exhaustively extracted against methanol, whose volume was subsequently reduced by distillation under vacuum. The residue was redissolved for preparative TLC Silica Gel GF (1,000 μm) and developed in a solvent system of chloroform: methanol:isopropanol:ammonia (90:10:95:5, vol/vol/vol/vol). After development, the region corresponding to unlabeled authentic harmol visualized under ultraviolet light was scraped and exhaustively extracted with methanol. The radiochemical purity of [^{3}H]harmol sulfate isolated and prepared was >99% as determined by TLC.[35]

Synthesis of [^{3}H]Enalapril. For the synthesis of *N*-(1-*S*-carboethyoxy-3-phenylpropyl)L-alanine *N*-hydroxysuccinimide ester (or active intermediate), a solution of 0.455 mmol (94 mg) of dicyclohexylcarbodiimide in 0.5 mL dichloromethane was added dropwise into a suspension of 0.455 mmol *N*-(1-*S*-carboethyoxy-3-phenylpropyl)-L-alanine, whose synthesis was described previously in a published procedure,[36,37] and 0.455 mmol (52.5 mg) of *N*-hydroxysuccinimide in 1.5 mL of dichloromethane while stirring at −5°C. The reaction mixture was left stirring at 0°C for 1.5 hours and at 4°C overnight. The solid dicyclohexylurea was removed by filtration and washed with ethyl acetate. The combined filtrate and the washings were chilled, then washed three times with ice-cold 1 mol/L $NaHCO_3$ solution and once with a saturated solution of NaCl. The organic phase was dried over anhydrous magnesium sulfate, filtered, and concentrated under reduced pressure, resulting in 163 mg of oily product. Mass spectral analysis showed that the mass was 377.

For the ultimate synthesis of 1-(*N*-(-*S*-carboethoxy-3-phenylpropyl)-L-alanyl)L-[5-^{3}H]proline or enalapril, water was first removed from L-[5-^{3}H]proline (0.62 μmol, specific activity of 23 Ci/mmol purchased from Amersham) with a rotary evaporator. Sodium bicarbonate (1.32 μmol or 0.109 mg in 20 μL water) and the active intermediate prepared as described above (6.4 μmol or 2.4 mg in 50 μL tetrahydrofurane) were added, while the mixture was stirred and chilled in an ice-water bath for 1 hour. Stirring was then continued at room temperature for another 4 hours, before the second addition of sodium bicarbonate and the active intermediate, with stirring at room temperature for a total of 20 hours. The crude product was purified by preparative TLC on silica gel plate and was developed with 1 propanol:1 mol/L acetic acid:water (10:1:1, vol/vol/vol) and toluene:1-butanol:acetic acid:acetone:water (1:1:1:1:1, vol/vol/vol/vol/vol).

All reagents used were of glass-distilled HPLC grade or were of the highest purity available (Fisher Scientific, Mississauga, Ontario, Canada).

HeLa Cells. HeLa cells (ATCC) stably transfected with pMEP4-oatp1 were cultured and grown in selective medium as previously described.[7] For induction of oatp1, cells were induced for 24 hours in medium containing 100 μmol/L $ZnSO_4$, and then for the next 24 hours with an additional 50 μmol/L (total of 150 μmol/L) of $ZnSO_4$.

Uptake of Anions. Cellular uptake of various radiolabeled anions was examined in zinc-induced (oatp1-containing) and noninduced HeLa cells to explore whether uptake exists. The concentrations of radiolabeled substrates were prepared in serum-free medium (SFM) containing: 135 mmol/L NaCl, 1.2 mmol/L $MgCl_2$, 0.81 mmol/L $MgSO_4$, 27.8 mmol/L glucose, 2.5 mmol/L $CaCl_2$, and 25 mmol/L HEPES adjusted to pH 7.2 with NaOH. These substrates include monocarboxylic acids such as [^{14}C]benzoate and [^{3}H]hippurate, sulfate conjugates exemplified by [^{3}H]E_1S, [^{3}H-22,23]TLCAS, and [^{3}H]harmol sulfate, and other anions: [^{3}H]BSPGSH and the ACE inhibitors, [^{3}H]enalapril and its active dicarboxylate metabolite, [^{3}H]enalaprilat. The transport of [^{35}S]BSP was used routinely to ensure that oatp1 was expressed functionally in zinc-induced, but not in noninduced, HeLa cells.

Before study, cells were washed three times with 1.5 mL SFM. After the addition of 1 mL SFM, the cells were incubated at 37°C for 15 minutes. Upon substitution with 1 mL of drug solution, uptake was conducted for 5, 10, or 15 minutes. Termination of cell uptake was achieved by placing the cells on ice, rapidly removing the drug solution, and washing three times with 1.5 mL of ice-cold SFM. Cells were then incubated for 5 minutes in 1.5 mL of ice-cold 5% bovine serum albumin in SFM, followed by two washes in 1.5 mL ice-cold SFM. After the addition of 1 mL of SFM, cells were harvested with a rubber policeman, and the radioactivity was quantified by dual-channel counting (Model 1217 liquid scintillation counter, LKB, Gaithersburg, MD). The protein content in each plate was determined using a commercial kit (Bio-Rad, Richmond, CA) and averaged 0.7 ± 0.25 mg protein per 35-mm dish.

Enalapril Uptake Studies. Preliminary studies showed that uptake of [^{3}H]enalapril by zinc-induced HeLa cells was linear for at least 10 minutes. Uptake of [^{3}H]enalapril (1 to 800 μmol/L) was performed at 37°C and 4°C over the initial 5 minutes with zinc-induced and noninduced HeLa cells.

Inhibition Studies. Uptake of [^{3}H]enalapril was also conducted at 37°C in the presence of BSP (100 μmol/L), TCA (100 μmol/L), and E_1S (100 μmol/L), known substrates of oatp1, and BSPGSH (200 μmol/L) as well as enalaprilat (400 μmol/L), the dicarboxylic acid metabolite of enalapril, with zinc-induced and noninduced HeLa cells. Inhibition of enalapril by BSP and TCA with zinc-induced

TABLE 1. Uptake of Radioactive Compounds (75,900 to 214,000 dpm/mL) by oatp1 in Induced (+Zn^{2+}) and Noninduced (−Zn^{2+}) HeLa Cells at 37°C

Compounds Tested	Concentration (μmol/L)	Induced HeLa Cells (pmol/min/mg)	Noninduced HeLa Cells (pmol/min/mg)	oatp1-Mediated Uptake† (pmol/min/mg)	% Uptake per min per mg‡
[^{14}C]Benzoic acid	98	12.7 ± 4.4	6.5 ± 2.1*	—§	—§
[^{3}H]Enalaprilat	0.04	0.008 ± 0.003	0.005 ± 0.001*	—	—
[^{3}H]Hippuric acid	0.05	0.017 ± 0.003	0.007 ± 0.001*	—	—
[^{3}H]Harmol sulfate	0.03	0.011 ± 0.002	0.004 ± 0.0001*	—	—
[^{3}H]BSPGSH	37	14.9 ± 4.8	3.6 ± 1.1	11.3 ± 4.8	0.031
[^{3}H]E_1S	0.05	0.050 ± 0.005	0.011 ± 0.003	0.039 ± 0.005	0.078
[^{3}H]Enalapril	0.04	0.13 ± 0.04	0.007 ± 0.002	0.12 ± 0.04	0.30
[^{3}H]TLCAS	0.03	0.88 ± 0.12	0.073 ± 0.003	0.80 ± 0.12	2.68
[^{35}S]BSP	1.0	62 ± 17	4.6 ± 0.6	58 ± 18	3.34

NOTE. n = 3; mean ± SD.
*Not different from rates for zinc-induced HeLa cells; ANOVA, $P > .05$.
†Difference between rate of enalapril uptake by HeLa cells with zinc induction and that without induction.
‡(Uptake rate/original amount used for incubation) × 100%.
§Uptake rate was virtually zero.

HeLa cells was further characterized in two sets of studies with BSP (0, 1, 5, 10, and 50 μmol/L) and TCA (0, 5, 25, and 100 μmol/L) as inhibitors of enalapril uptake.

Kinetic Analysis. The rate of cellular association of tracers at 37°C or 4°C was expressed as the amount taken up per minute per milligram of protein according to the specific activities of the samples. The uptake rate at 37°C (uptake rate for zinc induced cells − uptake rate for noninduced cells) normalized to the total amount of substrate used was taken as an index of the avidity of uptake by oatp1. Upon presentation of the subsequently derived rate (v) against the substrate concentration [S], kinetic constants: the K_m, Michaelis-Menten constant, and V_{max}, the maximal uptake rate, were obtained by regression of data to the equation:

$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad (1)$$

In the face of competitive inhibition (CI), however, the following equation was used,

$$v^{CI} = \frac{V_{max}[S]}{K_m(1 + [I]/K_i) + [S]} \qquad (2)$$

whereas with noncompetitive inhibition (NI), the following equation was appropriate:

$$v^{NI} = \frac{V_{max}[S]}{(1 + [I]/K_i)\,(K_m + [S])} \qquad (3)$$

In these equations, K_i is the inhibition constant, and [I] is the concentration of the inhibitor.

Fitting. Data fitting was conducted with the program SCIENTIST v.2 (MicroMath Scientific Software, Salt Lake City, UT). When other curve models (with additional uptake components) were explored for fitting of the control data, the simplest model (Eq. 1) was found to be superior. For the inhibition studies, all data were fitted simultaneously with Eq. 2 or Eq. 3 to arrive at a common K_m, V_{max}, and K_i. Weighting schemes of unity, 1/observation and 1/observation^2 were used; the appropriate weighting scheme that furnished the least coefficient of variation (standard deviation of the parameter estimate/value of estimate) and systematic deviation (shown in residual plot) was chosen. This was found to exist with 1/observation. The appropriate inhibition mechanism (competitive with Eq. 2, or noncompetitive with Eq. 3) was expected to be revealed by the value of the model selection criterion: the greater the value, the better the model.

Statistical Analysis. All data are presented as means ± SD. The paired Student's t test or ANOVA was used for comparison of the means, when appropriate. The level of significance was set at 0.05.

TABLE 2. Inhibition of Tracer [^{3}H]Enalapril Uptake by oatp1 in HeLa Cells

	Ratio +Inhibitor / −Inhibitor
Enalapril + inhibitor	
+Enalaprilat 400 μmol/L	1.15
+BSPGSH 200 μmol/L	0.78
+TCA 100 μmol/L	0.21
+E_1S 100 μmol/L	0.20
+BSP 100 μmol/L	0.06

NOTE. Five-minute incubations.

RESULTS

Uptake of Tracer Radioactive Anions. The monocarboxylic acids, [^{14}C]benozate and [^{3}H]hippurate, and [^{3}H]harmol

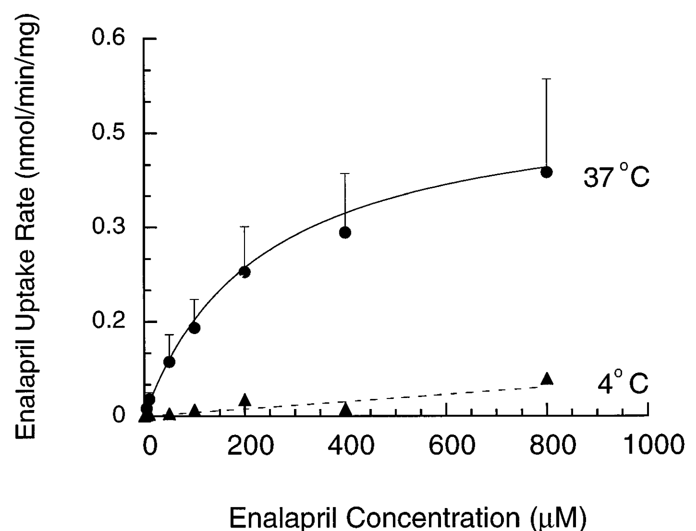

FIG. 2. Temperature- and concentration-dependent uptake of enalapril by oatp1; the rates were estimated as [rate of uptake in induced HeLa cells (with Zn^{2+}) − rate of uptake in noninduced cells (without Zn^{2+})]. The averaged data (each point represents the mean ± SD of 6 to 10 samples) were shown against the rates predicted based on the average kinetic constants shown in Table 3.

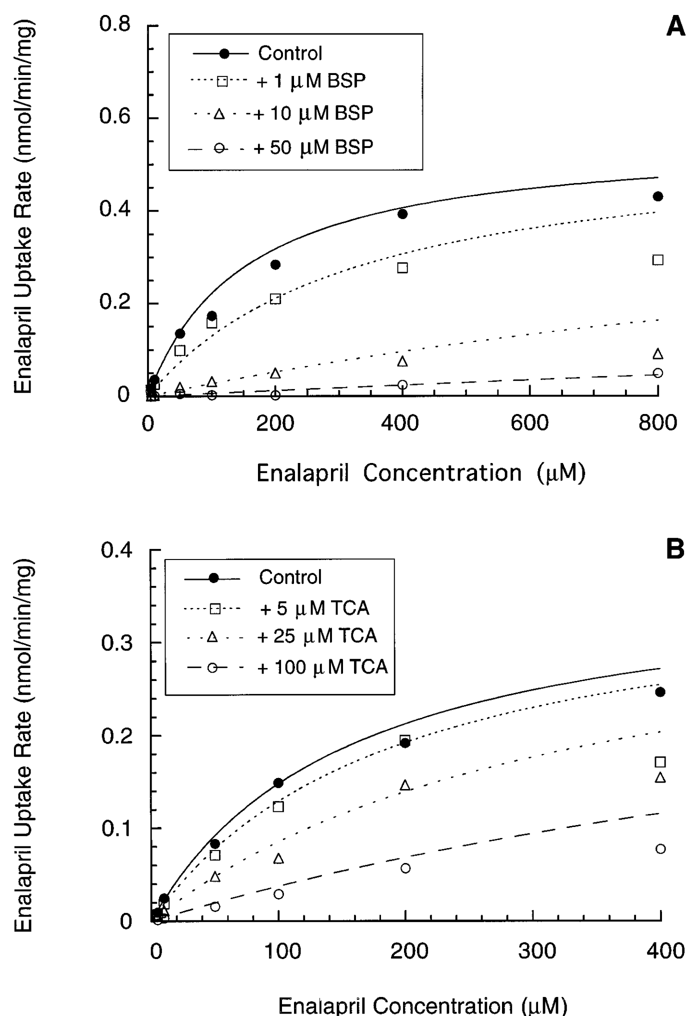

FIG. 3. Concentration-dependent inhibition of the uptake of enalapril by (A) BSP and (B) TCA by oatp1 at 37°C [rate of uptake in induced HeLa cells (with Zn^{2+}) − rate of uptake in noninduced cells (without Zn^{2+})] for representative studies. The uptake rates were fitted simultaneous to Eqs. 2 or 3, and the mode of inhibition was found to be competitive inhibition. Enalapril uptake rates were reduced in a concentration-dependent fashion as a result of the presence of the competitive inhibitors, BSP and TCA. The lines are fitted lines according to competitive inhibition.

sulfate were not transported by oatp1 (Table 1). [^{3}H]Enalaprilat was not transported, and the observation contrasted with its ethoxy ester precursor, [^{3}H]enalapril. [^{35}S]BSP, [^{3}H]E_1S, [^{3}H]TLCAS, and [^{3}H]BSPGSH were all found to be transported by oatp1. Moreover, *cis*-inhibition of [^{3}H]enalapril was found with BSP and TCA (both at 100 µmol/L), BSPGSH (200 µmol/L), and E_1S (100 µmol/L), but not with enalaprilat (400 µmol/L) (Table 2).

Concentration-Dependent Uptake of Enalapril. Because the transport of [^{3}H]enalapril remained linear up to 10 minutes, all uptake studies for enalapril (1 to 800 µmol/L) were conducted for 5 minutes. The uptake rate was found to be temperature-dependent: the rate at 4°C was low, but was substantially greater at 37°C. There was a demonstrable trend toward saturation with increasing concentration (Fig. 2).

Inhibition of Enalapril Uptake by BSP and TCA. The *cis*-inhibition of enalapril (5 to 800 µmol/L) uptake by BSP (0, 1, 5, 10, and 50 µmol/L) at 37°C was concentration-dependent: the greater the concentration of BSP, the greater the extent of inhibition on enalapril uptake (Fig. 3A). The same existed with TCA (0, 5, 25, and 100 µmol/L) (Fig. 3B). Results for enalapril alone (data fitted to Eq. 1) and in the presence of BSP or TCA (fitted simultaneously to Eq. 2 for competitive inhibition, or Eq. 3 for noncompetitive inhibition) are summarized in Table 3. The K_m and V_{max} of oatp1 for the uptake of enalapril (252 ± 82 µmol/L and 0.41 ± 0.13 nmol/min/mg protein) for controls were similar to those (156 ± 26 and 228 ± 53 µmol/L; 0.49 ± 0.07 and 0.61 ± 0.16 nmol/min/mg protein) obtained in the presence of BSP or TCA as competitive inhibitors ($P > .05$, ANOVA). The parameters were averaged to yield a K_m of 214 ± 67 µmol/L and a V_{max} of 0.51 ± 0.15 nmol/min/mg protein (mean ± SD, n = 10). The same comment applied to the estimates obtained with the noncompetitive inhibition model, which yielded an averaged K_m of 283 ± 78 µmol/L and a V_{max} of 0.59 ± 0.18 nmol/min/mg. The values for noncompetitive inhibition were significantly greater than those for competitive inhibition ($P < .05$, paired *t* statistic).

Values for the inhibition constants (K_i) for BSP and TCA were 2 and 32 µmol/L, respectively, with the competitive model (Table 3), and were similar to the K_ms reported for BSP

TABLE 3. Mode of Inhibition of Enalapril Uptake by oatp1 in HeLa Cells Is Competitive

	Enalapril (n = 3)		Enalapril + BSP (n = 3)				Enalapril + TCA (n = 4)			
Models Fitted	V_{max} (nmol/min/mg)	K_m (µmol/L)	V_{max} (nmol/min/mg)	K_m (µmol/L)	K_i (µmol/L)	MSC†	V_{max} (nmol/min/mg)	K_m (µmol/L)	K_i (µmol/L)	MSC
Control	0.41 ± 0.13*	252 ± 86								
Competitive inhibition			0.49 ± 0.07	156 ± 26	1.98 ± 1.33	1.81 ± 0.26	0.61 ± 0.16	228 ± 53	32 ± 11	2.91 ± 0.13
Noncompetitive inhibition			0.63 ± 0.05	290 ± 105	5.35 ± 3.60	1.36 ± 0.31	0.69 ± 0.23	297 ± 81	52 ± 7	2.75 ± 0.19
Paired *t* statistic‡			<.05	NS	NS	<.05	<.05	<.05	<.05	<.05

NOTE. Data fitting was performed with the optimal weighting scheme, 1/observation. The coefficients of variation (standard deviation of parameter estimate/parameter value) × 100% were <11% for V_{max} and <27% for K_m for data fit to the competitive inhibition model (Eq. 2), and were <18% for V_{max} and <30% for K_m for data fit to the noncompetitive inhibition model (Eq. 3). The kinetic parameters among the pooled data (control and with BSP or TCA for competitive model) were not different (ANOVA, $P > .05$). When averaged, the parameters were: V_{max}, 0.51 ± 0.15 nmol/min/mg and K_m, 214 ± 67 µmol/L. The same comment applies to the data sets for the control and noncompetitive inhibition (ANOVA, $P > .05$); the averaged parameters were: V_{max} of 0.59 ± 0.18 nmol/min/mg and K_m of 283 ± 78 µmol/L. However, values of V_{max} and K_m for noncompetitive inhibition were greater than those for competitive inhibition, by the paired *t* statistic ($P < .05$).

*SD of mean.

†Model selection criterion; the larger the value, the better the fit to the model.

‡Competitive vs. noncompetitive inhibition; NS = not significant.

and TCA[5-7] for oatp1. The inhibition constants (K_i) for BSP and TCA on enalapril uptake were greater for noncompetitive inhibition (2.7 and 2.0 times those for competitive inhibition, although only the K_i values for TCA were statistically different [$P < .05$, paired *t* statistic]). A greater model selection criterion and lower coefficient of variation (Table 3), as well as improved residual plots (data not shown), were obtained with the competitive model.

DISCUSSION

In addition to the expected rapid uptake of BSP and E_1S, we have now found that oatp1 also mediates transport of BSPGSH and TLCAS (Table 1). The percent uptake was highest for BSP, then TLCAS, enalapril, and E_1S, for which low or tracer concentrations were used. The ranking for BSPGSH was lowest, but the extent of uptake might have been dramatically underestimated in view of the higher concentration (low specific activity of the radiolabel) employed for study. Although concentration-dependence has not been explored in detail in this study, the transport of BSPGSH by oatp1 appears to be less than that of BSP as a result of their differential inhibition potentials on enalapril uptake (Table 2). The uptake kinetics of BSPGSH by oatp1 remain to be clarified inasmuch as a low K_m (1.5 μmol/L) for influx, ordinarily implying rapid transport, has been found for BSPGSH in multiple indicator dilution perfused rat liver studies.[27,28] The transport of TLCAS by oatp1 was efficient. Carrier-mediated transport of sulfated bile acids has been described previously in isolated rat hepatocytes, in which two high-affinity systems of similar K_ms (4.2 and 11 μmol/L) were found for TLCAS.[26] Rapid transport of other sulfated bile acids, 3α- and 7α-chenodeoxycholic acid sulfates, has also been reported.[38] Our studies also confirmed previous reports of efficient uptake of E_1S by oatp1.[9,11,12,39] These transported substrates of oatp1 also exhibited *cis*-inhibition on the transport of enalapril (Table 2).

Among the other tracers screened, simple aromatic carboxylic acids such as benzoate and hippurate, as well as harmol sulfate, were not transported by oatp1 (Table 1). Similarly, another simple aromatic acid, para-aminohippurate, was not transported by oatp1[40] nor exhibited *cis*-inhibition.[6] All these point to the possibility that additional organic anion transporters must exist in the hepatocyte, as suggested in recent reviews[39,41] and in the transport studies of bumetanide[42] and BSP,[43] in which basolateral transporters other than Ntcp and oatp1 are implicated.

Inasmuch as tracer enalapril was avidly transported by oatp1 (Table 1), further exploration over a concentration range revealed saturable uptake at 37°C, defined by the K_m of 252 ± 86 μmol/L, that was drastically reduced at 4°C (Fig. 2). That enalapril is an oatp1 substrate was further confirmed by finding *cis*-inhibition of its uptake by BSP and TCA. The inhibition was highly concentration-dependent (Fig. 3, Table 3) and was best described by competitive inhibition, revealing similar K_ms (156 ± 26 and 228 ± 53 μmol/L) and V_{max}s (0.49 and 0.61 nmol/min/mg protein) for enalapril uptake and inhibition constants for BSP and TCA that are similar to the K_ms of the respective inhibitors: 2 μmol/L for BSP and 32 μmol/L for TCA.[5-7] Similar K_m values for enalapril uptake were also obtained with isolated rat hepaotcytes in which sodium ion was either present or absent,[44] excluding the role of the sodium-dependent Ntcp. The lack of transport of enalapril by Ntcp was confirmed in the newly developed system of Ntcp-cDNA–transfected HeLa cells in Dr. Wolkoff's laboratory (data not shown). The same was observed for ochratoxin A[14] and the peptide-based thrombin inhibitor, CRC 220,[13] which are substrates of oatp1 and not Ntcp.

The oatp1-mediated sinusoidal influx of enalapril, though modest, is enough to bring the substrate into the liver cell for metabolism and excretion, explaining the measurable hepatocellular extraction of enalapril in liver perfusion studies.[16,17] The moderately high K_m for enalapril uptake, however, predisposes its transport process to inhibition by other oatp1 substrates of lower K_ms. The most likely physiological inhibitors *in vivo* are the bile acids and various steroidal hormones.[6,9,39] This was indeed observed for CRC 220, wherein inhibition of uptake occurred in the presence of high concentrations of bile acids and BSP, thereby reducing the extraction of the compound.[45]

By contrast, the metabolite enalaprilat is not a substrate of oatp1 because of its lack of transport in HeLa cells and absence of inhibitory effect on enalapril uptake (Tables 1 and 2). This observation is consistent with the poor basolateral transport of enalaprilat found previously in rat liver perfusion studies.[16,18,46] Because blockade of the carboxylate anion via formation of the ester is the only structural difference between enalapril and enalaprilat (Fig. 1), protection of this anionic site appears to be an essential requirement for oatp1 uptake. The effect of the negative charge is somewhat revealed in the comparison of inhibitory constants of the various estrogens and estrone/estradiol conjugates on the transport of tracer estradiol-17β-glucuronide transport by oatp1. It appears that inclusion of a negative charge reduces the K_m for oatp1.[9]

In summary, the present study demonstrates that enalapril, but not its metabolite, enalaprilat, is a substrate for oatp1. This agrees with previous studies in the perfused rat liver in which there was a relatively high extraction of the precursor but poor extraction of the metabolite.[16,18,46] The high K_m for enalapril uptake, however, predisposes it toward *cis*-inhibition by other oatp1 substrates of lower K_ms. During the accumulation of bile acids that occurs with cholestasis, perturbation in enalapril uptake is expected. Alteration of the kinetics of this important precursor and reduced bioactivation to the pharmacologically active metabolite, enalaprilat, are anticipated to be direct consequences of the inhibition.

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