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Title Page

# Transport of Angiotensin-converting Enzyme Inhibitors by $H^+$/Peptide Transporters Revisited

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**Running Title:** Transport of ACE Inhibitors by Peptide Transporters

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**ABBREVIATIONS:** ACE, Angiotensin-converting enzyme; PEPT1, $H^+$/peptide transporter 1; PEPT2, $H^+$/peptide transporter 2; HRPE, human retinal pigment epithelium; rMVA, recombinant modified vaccinia virus Ankara

## ABSTRACT

Angiotensin-converting enzyme (ACE) inhibitors are often regarded as substrates for the peptide transporters PEPT1 and PEPT2. Even though the conclusions drawn from published data are quite inconsistent, in most review articles PEPT1 is claimed to mediate the intestinal absorption of ACE inhibitors and thus to determine their oral availability. We systematically investigated the interaction of a series of ACE inhibitors with PEPT1 and PEPT2. First, we studied the effect of fourteen ACE inhibitors including new drugs on the uptake of the dipeptide [$^{14}$C]Gly-Sar into human intestinal Caco-2 cells constitutively expressing PEPT1 and rat renal SKPT cells expressing PEPT2. In a second approach, the interaction of ACE inhibitors with heterologously expressed human PEPT1 and PEPT2 was determined. In both assay systems, zofenopril and fosinopril were found to have very high affinity for binding to peptide transporters. Medium to low affinity for transporter interaction was found for benazepril, quinapril, trandolapril, spirapril, cilazapril, ramipril, moexipril, quinaprilat and perindopril. For enalapril, lisinopril and captopril very weak affinity or lack of interaction was found. Transport currents of PEPT1 and PEPT2 expressed in *Xenopus laevis* oocytes were recorded by the two-electrode voltage clamp technique. Statistically significant, but very low currents were only observed for lisinopril, enalapril, quinapril and benazepril at PEPT1 and for spirapril at PEPT2. For the other ACE inhibitors electrogenic transport activity was extremely low or not measurable at all. The present results suggest that peptide transporters do not control intestinal absorption and renal reabsorption of ACE-inhibitors.

## Introduction

Angiotensin-converting enzyme (ACE) inhibitors are effective drugs for the treatment of hypertension, congestive heart failure, post-myocardial infarction and diabetic nephropathy (Bertrand, 2004; Wong et al., 2004). The compounds inhibit the rate-limiting enzyme in the formation of angiotensin II, thereby reducing its capability for binding to its receptor. After oral administration as the primary route, most ACE inhibitors display absorption rates of 30 to 100% of a dose (Steinhilber et al., 2005). Since many ACE inhibitors sterically resemble Ala-Pro dipeptide or Xaa-Ala-Pro tripeptide structures, it was hypothesized that they share the same intestinal transport route as di- and tripeptides (for review see Bai and Amidon, 1992; Amidon and Sadee, 1999). Di- and tripeptides are taken up into intestinal cells by the low-affinity $H^+$/peptide cotransporter PEPT1. In the kidney tubule, di- and tripeptides are reabsorbed by PEPT1 and by the high-affinity $H^+$/peptide cotransporter PEPT2 (for review see Nielsen and Brodin, 2003; Brandsch et al., 2004; Daniel and Kottra, 2004; Terada and Inui, 2004; Biegel et al., 2006). ß-Lactam antibiotics and antivirals such as valacyclovir were unequivocally demonstrated to utilize PEPT1 and PEPT2 for intestinal absorption or renal reabsorption, respectively (Bretschneider et al., 1999; Nielsen and Brodin, 2003; Daniel and Kottra, 2004; Terada and Inui, 2004; Brandsch et al., 2008). The transport of ACE inhibitors, however, is still a matter of controversy. In almost every review on $H^+$/peptide transporters it is stated that ACE inhibitors are peptide transporter substrates. This view originated based on publications from the group of G. L. Amidon suggesting that the intestinal $H^+$/peptide transporter takes up captopril, enalapril, lisinopril, quinapril, benazepril, and ceronapril (for review see Bai and Amidon, 1992; Amidon and Sadee, 1999). In particular captopril and enalapril are considered as prototypical PEPT1 substrates. Reviewing the literature, however, we observed that the affinity constants for enalapril transport attributed to the intestinal peptide transporter differ widely (Bai and Amidon, 1992; Moore et al., 2000; Brandsch et al., 2004). For enalapril, enalaprilat and lisinopril affinity constants for the intestinal peptide

carrier of 0.15, 0.28 and 0.39 mM were reported and those were also used for molecular modeling approaches of peptide transporter substrates (Swaan et al., 1995). Other groups found no or very low affinity of captopril, enalapril, enalaprilat and lisinopril for PEPT1 with $K_i$ values exceeding 20 mM (Moore et al., 2000). Thwaites and coworkers (1995) suggested transport of captopril and enalapril via PEPT1 but observed only a weak interaction of captopril with PEPT1 ($K_i$ > 20 mM). For PEPT2 also only weak interactions with $K_i$ values of 6.2 and 4.3 mM were reported for enalapril (Lin et al., 1999; Zhu et al., 2000). In electrophysiological experiments with PEPT1 and PEPT2 expressed heterologously, enalapril or captopril were found to elicit only low transport currents (Boll et al., 1994; Zhu et al., 2000; Faria et al., 2004). Fosinopril and zofenopril were shown to interact with PEPT1 and PEPT2 with very high affinity (Lin et al., 1999; Moore et al., 2000; Shu et al., 2001) and it has been reported that fosinopril is transported in intact form by proton-coupled peptide transporters (Shu et al., 2001) despite the fact that its structure is not closely related to those of di- and tripeptides. Quinapril was shown to inhibit uptake of reference peptides by PEPT1 and PEPT2 (Akarawut et al., 1998; Zhu et al., 2000) but could not elicit any transport currents in *Xenopus laevis* oocytes expressing the proteins (Chen et al., 1999; Zhu et al., 2000). These findings suggested that quinapril may be a noncompetitive, non-transported inhibitor of peptide transporters but others reported that quinapril is a transported substrate (Bai and Amidon, 1992; Hu et al., 1995).

Since transport functions of peptide transporters but in particular the apparent affinity of substrates depend on a variety of variables from cell type to buffer composition, pH to membrane potential, methodological differences might be responsible for the conflicting data. We therefore assessed in a standardized manner - based on three different approaches and employing fourteen compounds from which three never were studied before - the involvement of PEPT1 and PEPT2 in transport of ACE inhibitors.

## Methods

**Materials.** The human colon carcinoma cell line Caco-2 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The renal cell line SKPT-0193 Cl.2 established from isolated cells of rat proximal tubules was provided by U. Hopfer (Case Western Reserve University, Cleveland, USA). Culture media, media supplements and trypsin solution were purchased from Invitrogen (Karlsruhe, Germany) or PAA (Pasching, Austria). Fetal bovine serum was from Biochrom (Berlin, Germany) and collagenase A from Roche (Mannheim, Germany). The recombinant modified vaccinia virus Ankara (rMVA) was a kind donation of the GSF-Institute (München, Germany). pBluescript II SK(-), pBluescript-hPEPT1 and pBluescript-hPEPT2 were kind donations of V. Ganapathy (Medical College, Augusta, USA). Dexamethasone, apotransferrin, Igepal® Ca-630, Ala-Ala-Ala, Ala-Pro, Gly-Sar and captopril were from Sigma-Aldrich (Deisenhofen, Germany). [Glycine-1-$^{14}$C]Gly-Sar (specific radioactivity 53 mCi/mmol) was custom synthesized by Amersham International (Buckinghamshire, UK). Most ACE inhibitors were gifts from companies: We were supplied with benazepril (Salutas Pharma, Barleben, Germany), cilazapril (Roche, Mannheim, Germany), enalapril maleate (Berlin-Chemie, Berlin, Germany), fosinopril (Solvay, Hannover, Germany), lisinopril and ramipril (Astra-Zeneca, Macclesfield, UK), moexipril (Schwarz Pharma, Zwickau, Germany), perindopril (Servier, München, Germany), quinapril and quinaprilat (Pfizer Pharmaceuticals Group, Groton, USA), spirapril (AWD Pharma, Radebeul, Germany), trandolapril (Abbot, Ludwigshafen, Germany) and zofenopril (Menarini Ricerche S.p.A., Firenze, Italy). Captopril was also from MP-Biomedicals (Heidelberg, Germany). According to the manufactures' HPLC protocols, the purity of the ACE inhibitors was around 100%. All other chemicals were of analytical grade.

**Culture of Caco-2 and SKPT Cells and Uptake Studies.** Caco-2 cells were routinely cultured with Minimum Essential Medium with Earle´s salts and L-Glutamine, supplemented with 10% fetal bovine serum, 1% nonessential amino acid solution and gentamicin (45 µg/ml)

(Knütter et al., 2001; Neumann et al., 2004). SKPT cells were cultured in Dulbecco's Modified Eagle Medium: F12 Nutrient Mixture (Ham) 1:1 and L-glutamine, 10% fetal bovine serum, recombinant insulin (4 µg/ml), epidermal growth factor (10 ng/ml), apotransferrin (5 µg/ml), dexamethasone (5 µg/ml) and gentamicin (45 µg/ml) as described previously (Brandsch et al., 1995; Theis et al., 2002; Neumann et al., 2004). Both cell lines were subcultured in 35-mm disposable petri dishes (Sarstedt, Nümbrecht, Germany) at a seeding density of 0.8 x $10^6$ cells per dish. Uptake of [$^{14}$C]Gly-Sar was measured 7 days (Caco-2) or 4 days (SKPT) after seeding at room temperature as described previously (Knütter et al., 2001; Theis et al., 2002; Neumann et al., 2004). The uptake buffer was 25 mM Mes/Tris (pH 6.0) containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 5 mM glucose. Uptake was initiated after washing the cells for 30 sec in uptake buffer by adding 1 ml of uptake medium containing [$^{14}$C]Gly-Sar (10 µM) and increasing concentrations of the test compounds (0–100 mM). If necessary, the pH of the solutions was corrected before preparing the required dilutions. After incubation for 10 min, the cells were quickly washed four times with ice-cold buffer, solubilized in 1 ml of Igepal® Ca-630 (0.5% v/v) in buffer (50 mM Tris/HCl, pH 9.0, 140 mM NaCl, 1.5 mM $MgSO_4$) and prepared for liquid scintillation spectrometry. For each experiment, the samples for the protein measurements were prepared and measured as described earlier (Knütter et al., 2001).

**Heterologous Expression of hPEPT1 and hPEPT2 in HRPE Cells and Uptake Studies.** Human retinal pigment epithelium (HRPE) cells (passages 12-25) were cultured in Dulbecco's Modified Eagle Medium: F12 Nutrient Mixture (Ham) 1:1 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Metzner et al., 2008). For subculturing the cells were rinsed with PBS, trypsinated and seeded in 75 $cm^2$ flasks with a cell density of 5-8 · $10^6$ per flask or in 24-well plates (Greiner, Frickenhausen, Germany) with a cell density of 0.5 · $10^6$ (hPEPT1) or 0.7 · $10^6$ (hPEPT2) per well, respectively.

For the vaccinia virus expression of hPEPT1 and hPEPT2 a modified protocol of the procedures described by Ganapathy and coworkers (1995) and Metzner and coworkers (2008) was used. First, HRPE cells were infected 24 h after seeding in 24-well plates with rMVA (50 IU/cell) encoding the T7 RNA polymerase (Sutter et al., 1995) and incubated for 30 min at 37°C. After 30 min incubation with rMVA, for PEPT1 the HRPE cells were transfected with pBluescript hPEPT1 cDNA construct and pBluescript (1µg/well) using Nanofectin® (3.2 µl/well; PAA Laboratories, Cölbe, Germany), whereas for hPEPT2 the HRPE cells were transfected with pBluescript hPEPT2 cDNA construct and pBluescript (1 µg/well) using Metafectene®Pro (2 µl/well; Biontex, Martinsried/Planegg, Germany) according to manufacturers' protocols. HRPE cells transfected with empty plasmid served as control. To minimize toxic effects of the infection/transfection procedure the medium was replaced after 4 h. 24 h post transfection, uptake of [$^{14}$C]Gly-Sar was measured at room temperature. The uptake buffer was 25 mM Mes/Tris (pH 6.0) containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 5 mM glucose. Uptake was initiated after washing the cells once in uptake buffer by adding 0.3 ml of uptake medium containing [$^{14}$C]Gly-Sar (20 µM for PEPT1, 30 µM for PEPT2) and increasing concentrations of the test compounds (0–100 mM). If necessary, the pH of the solutions was corrected before preparing the required dilutions. In HRPE-hPEPT1 cells, [$^{14}$C]Gly-Sar uptake is linear for up to 5 min. In HRPE-hPEPT2 cells, uptake is linear for up to 20 min (data not shown). After incubation for 5 min (hPEPT1) or 10 min (hPEPT2), the cells were quickly washed four times with ice-cold buffer, solubilized in 0.5 ml of 1% SDS in 0.2 M NaOH and prepared for liquid scintillation spectrometry.

***Xenopus laevis* Oocytes expressing PEPT1 and PEPT2 and Electrophysiology.** Female *Xenopus laevis* were purchased from African Xenopus Facility (Knysna, South Africa). Surgically removed oocytes were separated by collagenase treatment and handled as described previously (Boll et al., 1996; Knütter et al., 2001; Theis et al., 2002). Individual oocytes were injected with 30 nl of RNA solution containing 30 ng of rabbit PEPT1 or rabbit

PEPT2 cRNA. All electrophysiological measurements were performed after 3-6 days by incubation of oocytes in buffer composed of 88 mM NaCl, 1 mM KCl, 0.82 mM $CaCl_2$, 0.41 mM $MgCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$ and 10 mM MES/Tris at pH 6.5 (modified Barth-solution). The two-electrode voltage clamp technique was applied to characterize responses in current (*I*) to substrate addition in oocytes expressing PEPT1 or PEPT2 (Boll et al., 1996; Knütter et al., 2001; Theis et al., 2002). In short, oocytes were placed in an open chamber with a volume of 0.5 ml and continuously superfused with modified Barth-solution or with solutions of Gly-Sar and/or the test compound. Electrodes with resistances between 0.5 and 2 MΩ were connected to a TEC-05 amplifier (npi electronic, Tamm, Germany) and oocytes were clamped at -60 mV. Current-voltage ($I$-$V_m$) relationships were measured using short (100 ms) pulses separated by 200 ms pauses in the potential range from –160 to +80 mV. $I$-$V_m$ Measurements were made immediately before and 30 s after substrate application when current flow reached steady state. Currents evoked at -60 mV (PEPT1) or at -160 mV (PEPT2) were calculated as the difference of the currents measured in the presence and the absence of substrate.

**HPLC Analysis.** Benazepril, captopril, enalapril and lisinopril (1 mM, in buffer pH 6.0) were incubated for 10 min with Caco-2 cells. Samples of the extracellular uptake medium were taken at t = 0 min and t = 10 min and the ACE inhibitors were quantified according to the laboratory standard HPLC (La-Chrom®, Merck-Hitachi, Darmstadt, Germany) with a diode array detector and a Purospher® STAR RP-18 endcapped column (125-4, 5 µm). The eluent was 48% acetonitril/52% $H_2O$ with trifluoroacetic acid pH 2.5 for captopril and enalapril, 52% acetonitril/48% $H_2O$ with trifluoroacetic acid pH 2.5 for benazepril and 30% acetonitril/70% $H_2O$ with trifluoroacetic acid pH 2.5 for lisinopril. UV-detection was done at 215 nm. Injection volume was 5 µl and the flow rate 0.5 ml/min.

**Calculations and Statistics.** All data are given as the mean ± S.E. of three to four independent experiments. The kinetic parameters were calculated by non-linear regression

methods (SigmaPlot® program, Systat, Erkrath, Germany) and confirmed by linear regression of the respective Eadie-Hofstee plots. $IC_{50}$ values (i.e. concentration of the unlabeled compound necessary to inhibit 50% of carrier-mediated [$^{14}$C]Gly-Sar uptake) were determined by non-linear regression using the logistical equation for an asymmetric sigmoid (allosteric Hill kinetics): $y = Min + (Max-Min)/(1 + (X/IC_{50})^{-P})$ (equation 1) where Max is the initial Y value, Min the final Y value and the power P represents Hills' coefficient. Inhibition constants ($K_i$) were calculated from $IC_{50}$ values according to the method developed by Cheng and Prusoff (1973).

## Results

**Inhibition of Gly-Sar Uptake by ACE Inhibitors in Caco-2 and SKPT Cells.** Caco-2 and SKPT cells are currently the best native cell models for transport studies on PEPT1 and PEPT2, respectively. We first studied the effect of fourteen ACE inhibitors on the [$^{14}$C]Gly-Sar uptake into Caco-2 cells. Carrier-mediated uptake of Gly-Sar into confluent monolayers of Caco-2 cells is solely mediated by PEPT1 (Knütter et al., 2001; Brandsch et al., 2004; Neumann et al., 2004). For all ACE inhibitors, a concentration-dependent inhibition of [$^{14}$C]Gly-Sar uptake was observed (Fig. 1A, 1B). From the inhibition curves $IC_{50}$ values were obtained and converted into $K_i$ values. These $K_i$ values reflect apparent affinity of the compounds tested. As compiled in Table 1, the $K_i$ values ranged from 0.047 to 46 mM. Most ACE inhibitors tested displayed interaction with PEPT1 with medium affinities (Table 1, for classification of affinity constant at PEPT1 see Brandsch et al., 2004, 2008). Since most ACE inhibitors are derivatives of Ala-Pro we also determined the $K_i$ value of Ala-Pro and for comparison the $K_i$ values of the prototypic PEPT1 substrates Gly-Sar and Ala-Ala-Ala (Table 1). The peptides Ala-Pro and Ala-Ala-Ala and the ACE inhibitors fosinopril and zofenopril were found to be high affinity substrates and/or inhibitors of PEPT1 ($K_i$ < 0.5 mM, Brandsch et al., 2008). Medium affinity was observed for Gly-Sar, benazepril, quinapril, trandolapril, spirapril, cilazapril, ramipril and moexipril (0.5 mM < $K_i$ < 5 mM). Quinaprilat, perindopril and enalapril were inhibitors of the low affinity category (5 mM < $K_i$ < 15 mM). From the very high $K_i$ values of lisinopril (23 mM) and captopril (46 mM) we conclude that these two compounds cannot be considered to interact with PEPT1. Since the $K_i$ value found for captopril here is relatively high compared to values reported by others (e.g. 8.7 mM: Temple and Boyd, 1998) and captopril is considered a transported substrate of PEPT1 (Thwaites et al., 1995; Zhu et al., 2000), we determined the inhibition constant for captopril also by employing two other buffer systems (Sörensen buffer, Hanks balanced salt solution) to rule out that buffer constituents such as Tris or Mes affect affinity. Moreover, we also tested captopril

obtained from different suppliers but in all cases measured $K_i$ values were similar and are highly reproducible (> 40 mM, data not shown). To test the stability of the drugs during the experiment, we analyzed captopril, enalapril, benazepril and lisinopril in the extracellular uptake medium of Caco-2 cells over the incubation period of 10 min by HPLC. 94 to 98% of the drug molecules were found intact after the experiment (captopril 97%, enalapril 94%, benazepril 98%, lisinopril 96%; data not shown). Once inside the cell, hydrolysis-sensitive prodrugs will be hydrolyzed to their corresponding prilates, but this would not interfere with the determination of affinity constants for extracellular binding at the transporters.

We next determined the $K_i$ values of these fourteen ACE inhibitors for the inhibition of [$^{14}$C]Gly-Sar uptake in SKPT cells. These cells express PEPT2 but not PEPT1 (Brandsch et al., 1995; Ganapathy et al., 1995; Shu et al., 2001). The ACE inhibitors reduce [$^{14}$C]Gly-Sar uptake in a dose dependent manner (Fig. 1C, 1D). The apparent $K_i$ values (Table 1) were in a range of 13 μM to 7.9 mM. According to our classification (Luckner and Brandsch, 2005; Brandsch et al., 2008), Ala-Pro, Ala-Ala-Ala, fosinopril and zofenopril are thereby high affinity inhibitors of [$^{14}$C]Gly-Sar uptake ($K_i$ < 0.1 mM). Medium affinity inhibitors (0.1 mM < $K_i$ < 1 mM) are Gly-Sar, benazepril, quinapril, trandolapril, spirapril, ramipril, moexipril and quinaprilat. Interaction of cilazapril, perindopril, enalapril, lisinopril and captopril with PEPT2 was low or very low ($K_i$ > 1 mM or > 5 mM, respectively). In Table 1 we also specify the rank order of $K_i$ values for subsequent correlation analyses (see below).

**Kinetics of Inhibition of Gly-Sar Uptake into Caco-2 and SKPT Cells**. Inhibition of [$^{14}$C]Gly-Sar uptake by ACE inhibitors does not necessarily mean that the drugs are transported. They could represent specific inhibitors or even compounds that affect non-specifically for example membrane integrity, the $H^+$ gradient or membrane voltage as the driving force of [$^{14}$C]Gly-Sar uptake. We therefore determined the type of inhibition for selected compounds. We have chosen quinapril because of controversial reports regarding its type of inhibition and spirapril since – to our knowledge – this interesting drug has never been

studied with regard to transporter interaction. First, we studied the effect of quinapril on the kinetic parameters of Gly-Sar uptake by PEPT1 and PEPT2. Gly-Sar uptake in Caco-2 and in SKPT cells was measured over a concentration range of 0.01-10 mM (Caco-2) or 0.01-5 mM (SKPT), respectively, in the absence or presence of quinapril at a concentration of 1 mM (Caco-2) or 0.5 mM (SKPT). Figures 2A (Caco-2 cells) and 2B (SKPT cells) show the relationship between the Gly-Sar uptake rates and Gly-Sar concentration. In the absence of quinapril, the Michaelis constant, $K_t$, for Gly-Sar uptake at Caco-2 cells was 1.1 ± 0.1 mM and the maximal velocity, $V_{max}$, was 39.4 ± 1.0 nmol · mg of protein$^{-1}$ per 10 min. These data correspond very well to values reported previously (Knütter et al., 2001; Brandsch et al., 2004). The kinetic constants obtained in the presence of 1 mM quinapril were ($K_t$) 2.0 ± 0.5 mM and ($V_{max}$) 25.8 ± 1.9 nmol · mg of protein$^{-1}$ per 10 min. Hence, quinapril at a concentration close to its $K_i$ value increased the $K_t$ value of Gly-Sar uptake 2-fold and decreased $V_{max}$ about 1.5-fold. The situation is quite similar for PEPT2: Nonlinear regression of the curves reveals that in the absence of quinapril the $K_t$ value for Gly-Sar uptake in SKPT was 0.14 ± 0.02 mM and the $V_{max}$ value was 6.9 ± 0.3 nmol · mg of protein$^{-1}$ per 10 min. This too is in agreement with previously reported values (Theis et al., 2002). The corresponding kinetic constants obtained in the presence of 0.5 mM quinapril were ($K_t$) 0.23 ± 0.01 mM and ($V_{max}$) 5.3 ± 0.03 nmol · mg of protein$^{-1}$ per 10 min. Hence, quinapril, again at a concentration close to its $K_i$ value, increased the $K_t$ value of Gly-Sar uptake in SKPT cells 1.6-fold and decreased the $V_{max}$ 1.3-fold. These results are in agreement with the assumption that quinapril does not represent a competitive inhibitor of PEPT1 and PEPT2 as it would have to be expected for a carrier substrate. In the next experiment, we determined the inhibition constant ($K_i$) of quinapril by measuring Gly-Sar uptake in Caco-2 cells at two different Gly-Sar concentrations (50 and 500 µM) in the presence of increasing concentrations of quinapril (0-5 mM). The results are presented as Dixon plot in Figure 2C. They reveal linearity at both Gly-Sar concentrations with lines intersecting on the abscissa as expected for a non-competitive

inhibitor. A $K_i$ value of 0.55 mM for quinapril at Caco-2 cells was calculated from the point of intersection. Such an analysis was also performed with spirapril at Caco-2 cells (Fig. 2D). Again, the lines in the Dixon plot were intersecting on the abscissa. A $K_i$ value of 1.8 mM was determined. The $K_i$ values obtained by this procedure are similar to the $K_i$ values obtained in the competition assays described above.

**Effects of ACE Inhibitors on Gly-Sar Uptake in HRPE-hPEPT1 and HRPE-hPEPT2 Cells.** Caco-2 and SKPT cells originate from different species, man and rat, respectively. To rule out that differences in substrate recognition between PEPT1 and PEPT2 reflect species differences and to confirm the affinity constants obtained in Caco-2 and SKPT cells in a second, independent approach, we performed transport studies with the cloned human PEPT1 and PEPT2. Both transporters were functionally expressed in HRPE cells using the vaccinia virus expression system. The $K_i$ values of Gly-Sar and seven ACE-inhibitors were obtained using competition assays with [$^{14}$C]Gly-Sar as a standard substrate (Fig. 3A, 3B). The $K_i$ values varied between 0.05 and 43 mM for hPEPT1 and between 24 µM and 16 mM for hPEPT2 (Table 2). Whereas Gly-Sar and zofenopril were recognized by hPEPT1 with high affinity, quinapril, benazepril and spirapril displayed medium affinity. Quinaprilat and enalapril were low affinity inhibitors. For captopril no interaction with PEPT1 was found. At hPEPT2, zofenopril and quinapril were high affinity inhibitors. Gly-Sar, benazepril and spirapril displayed medium affinity. For quinaprilat and enalapril low affinity and for captopril no affinity for hPEPT2 was measured.

**Transport of ACE Inhibitors by PEPT1 and PEPT2 Expressed in *Xenopus laevis* Oocytes.** As stated above, the demonstration of [$^{14}$C]Gly-Sar uptake inhibition does not imply that the respective compound is indeed transported. Therefore, the two-electrode voltage clamp technique was applied to *Xenopus laevis* oocytes expressing either rabbit PEPT1 or rabbit PEPT2. The concentrations of ACE-inhibitors or peptides used to determine PEPT1-mediated transport currents was mostly 10 mM except for fosinopril (0.33 mM), zofenopril

(0.1 mM), quinaprilat (3 mM) and trandolapril (5 mM). Captopril was also tested at 40 mM. For PEPT2 the drug concentration used was 2 mM except for fosinopril (0.33 mM) and zofenopril (0.1 mM). The currents elicited by the ACE inhibitors are expressed as percent of the current induced by the dipeptide Gly-Sar applied in saturating concentration (> 10 x $K_t$: PEPT1: 10 mM, PEPT2: 2 mM) measured in the same oocyte (Fig. 4A). For comparison, the currents induced by the peptide transporter substrates Ala-Pro and Ala-Ala-Ala were recorded as well. Importantly, in contrast to the dipeptide-induced currents, all ACE inhibitors generated very low currents. Since some of the inhibitors (e.g. quinapril, trandolapril, spirapril) induced membrane currents also in non-injected oocytes, the following results were corrected for the average current generated in the absence of peptide transporters. Significant current values in case of PEPT1 were recorded for quinapril (10%), lisinopril (9%) benazepril (8%) and enalapril (5%). For the ten other drugs the maximal currents were below or near to 5% and thereby not significantly different from zero. In Figure 4B representative currents elicited by Gly-Sar, fosinopril and quinapril for PEPT1 as a function of membrane potential are shown. For fosinopril no inward currents could be recorded. Interestingly, for quinapril and several other ACE inhibitors the dependence of the transport rate on membrane potential differed from that of Gly-Sar, showing an overproportionally increasing current at more negative membrane potentials (Fig. 4B). Very similar results were obtained with oocytes expressing PEPT2 (Fig. 4C). The different shapes of the I-V relations for different substrates of PEPT1 and the similar differences between PEPT1 and PEPT2 are probably caused by different rate constants during the transport cycle (Sala-Rabanal et al., 2008). Only with lisinopril and spirapril currents above 20% of those generated by Gly-Sar were measured, but due to a larger variability of currents at -160 mV membrane potential, only the current of spirapril turned out to be statistically significant (Fig. 4A). For the other ACE inhibitors, no significant currents were measurable (currents of enalapril, captopril, moexipril and zofenopril < 2%).

## Discussion

In this study we investigated systematically whether ACE inhibitors serve as substrates for PEPT1 and PEPT2. Almost every review written on drug delivery states that ACE inhibitors are substrates of intestinal and renal peptide transporters. However, published data are contradictory (for review see Brandsch et al., 2008) and we felt that the current situation only unsatisfactory describes the role of peptide transporters in the delivery of these drugs. Moreover, the assumption that the activity of PEPT1 mediates oral availability of the drugs appears to hamper the search for other proteins that might be involved in intestinal transport of ACE inhibitors.

The inhibitory activity of fourteen ACE inhibitors on [$^{14}$C]Gly-Sar influx into Caco-2 cells expressing hPEPT1 and in SKPT cells expressing rPEPT2 was studied. For zofenopril and for fosinopril, a high affinity interaction with both peptide transporters was observed. This result is in good agreement to previous reports (Lin et al., 1999; Moore et al., 2000; Shu et al., 2001). Data for the interaction of trandolapril, spirapril and moexipril with peptide transporters are not available from the literature. For some compounds conflicting data have been reported. This may be a consequence of the different *in vivo* and *in vitro* approaches or different parameters such as buffers, pH or membrane potential. To exemplify this, Bai and Amidon (1992) reported, based on intestinal perfusion experiments in rats, $K_m$ values of 5.9 mM, 70 µM and 75 µM for captopril, enalapril and benazepril and concluded based on competition studies with dipeptides that transport occurred via the peptide transporter. Comparing the affinities with our data it becomes obvious that the activity of the peptide transporter can not explain these findings. It is therefore likely that additional transporters are involved in intestinal uptake of these compounds in the rat intestine. Similarly, Swaan et al. (1995), when defining for the intestinal peptide carrier its substrate template used affinity constants for enalapril, enalaprilat and lisinopril of 0.15, 0.28 and 0.39 mM. Those data were derived from Ussing chamber experiments with rat intestine employing a mucosal buffer pH

of 7.4. In the present study, we detected only a very weak, almost negligible affinity of enalapril and lisinopril for transport by PEPT1 with a $K_i$ of > 14 mM and this value is in good agreement with the one reported by Moore and coworkers (2000) with a $K_i$ > 20 mM also obtained in Caco-2 cells. Based on these very low affinities a major contribution of PEPT1 to the absorption of these drugs seems highly unlikely in particular when taking into account that based on recommended oral doses for an adult of 25 – 75 mg per day for captopril and 5 – 20 mg for enlaparil and lisinopril the mean luminal concentrations in the jejunum would be around 100 µM (captopril) and 10 µM (enalapril, lisinopril).

Another compound of controversy is quinapril. The $K_i$ values measured for interaction of quinapril with PEPT1 (1.0 mM) and PEPT2 (0.39 mM) in the present analysis are in the same range as $K_i$ values reported by other groups (Kitagawa et al., 1997; Akarawut et al., 1998; Lin et al., 1999; Zhu et al., 2000; Moore et al., 2000). However, Zhu and coworkers (2000) observed a non-competitive inhibition of peptide transport and speculated that quinapril may affect the binding and/or translocation of the proton whereas Akarawut and coworkers (1998) favored a different binding site for quinapril in the transporter. In contrast, Kitagawa and coworkers (1997) found a competitive inhibition of peptide transport by quinapril. Our data support a mixed type of inhibition for quinapril but we do not have sufficient mechanistic information to be able to incorporate this into the kinetic 7-state models currently available for PEPT1 and PEPT2 (Sala-Rabanal et al., 2008). For this study the important question is whether quinapril is transported at all (see below) and only when proven that those drugs are transporter substrates they can be included into modeling approaches for defining the pharmacophore of PEPT1 or PEPT2 substrates. It would be interesting to study *in vivo* whether quinapril and other competitive or non-competitors PEPT1/2 inhibitors might interfere with the absorption of simultaneously applied drugs that are PEPT1 and PEPT2 substrates. Such drug-drug interactions with, e.g., orally available ß-lactam antibiotics or

valacyclovir would be a function of both their affinity constants at the transporters and their luminal concentrations.

To assess in more detail the structural elements that may determine their affinity for PEPT1 and PEPT2 we plotted the $K_i$ values over the log D values of the compounds as obtained from the Scifinder database (Table 1). A correlation coefficient of r = 0.69 ($P$ < 0.006) for the $K_{i\ PEPT1}$ values and r = 0.80 ($P$ < 0.0006) for the $K_{i\ PEPT2}$ values of the log D clearly demonstrate that a high affinity is associated with a high hydrophobicity. Similar results were obtained by Lin and coworkers (1999) with nine ACE inhibitors based on inhibition of Gly-Sar uptake into rabbit renal brush border membrane vesicles.

PEPT2 represents the high-affinity $H^+$/peptide cotransporter whereas PEPT1 is the low affinity isoform. For natural dipeptides PEPT2 generally displays an around 10-fold higher affinity than PEPT1 for the same substrates, i.e. the ratios between the $K_i$ of PEPT1 and the $K_i$ of PEPT2 are around 10. In our study, the $K_{i\ PEPT1}/K_{i\ PEPT2}$ ratios vary between 1.3 and 13.1 with an average of 6.3 (Table 1). Hence, PEPT2 recognizes the same ACE inhibitors as PEPT1 but on average with higher affinity. To study possible differences in more detail, a correlation analysis using the affinity constants obtained in Caco-2 cells and in SKPT cells was performed. From this analyses we obtained a very high and significant correlation (r = 0.97, $P$ < 0.0001). Because a clustering of $K_i$ values in certain groups might lead to overestimation of the correlation coefficient, we also calculated the more robust non-parametric Spearman´s rank correlation coefficient using the rank orders of $K_i$ values (Bretschneider et al., 1999, Table 1). This method also revealed a high and significant correlation coefficient ($r_s$) of 0.92 ($P$ < 0.0001). Based on this analysis we conclude that there are no major differences in the substrate recognition pattern of hPEPT1 and rPEPT2 with regard to the ACE inhibitors tested. Experiments in HRPE cells expressing human PEPT1 and human PEPT2, respectively, confirmed as well the $K_i$ values and let us conclude that differences are not due to species differences.

To assess whether the ACE inhibitors not only interfere with the substrate binding sites of the transporters but are indeed translocated by PEPT1 and PEPT2 we employed the two electrode voltage clamp technique to *Xenopus laevis* oocytes expressing either one of the two peptide transporters. The maximal inward currents induced by the drugs were in most cases less than one fifth of the maximal currents elicited by the model peptides Ala-Ala-Ala and Gly-Sar. Despite the fact that there is no gold standard for the judgment on when currents may be taken as relevant and physiologically meaningful we here consider currents as significant when they are: (i) transporter specific, i.e. when no signals are obtained using the same substrate concentration in control oocytes not expressing transporters, (ii) at least 5% of the currents elicited by reference substrates (dipeptides) and (iii) statistically significantly different from zero. Our data suggest very low transport rates (< 10%) of only lisinopril, enalapril, quinapril and benazepril by PEPT1. In case of PEPT2, only spirapril elicited significant currents.

We conclude that the oral availability of the ACE inhibitors that were here shown to generate only very small transport currents and that displayed apparent affinity constants higher than 15 mM cannot be explained by their interaction with the intestinal peptide transporter, especially when considering the low luminal concentrations. For all compounds that failed to show significant interaction and transport by peptide transporters one has to postulate that they may utilize other routes for absorption. Considering the high lipophilicity of most compounds, simple diffusion might be sufficient in many cases. Alternatively other membrane carriers and in particular members of the organic anion transporting family (SLC21, SLC22) seem the relevant candidates to be studied in their capability for transport of ACE inhibitors. For quinapril and enalapril the transport by organic anion transporters, e.g. OATP1B1 or OATP1B3, has already been shown (Akarawut and Smith, 1998; Pang et al., 1998; Liu et al., 2006; Chu et al., 2007).

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**Footnotes:**

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**Legends for Figures:**

**Fig. 1.** Interaction of ACE inhibitors with PEPT1 and PEPT2. Uptake of [$^{14}$C]Gly-Sar (10 µM, pH 6.0, 10 min, n = 4) was measured in Caco-2 cells (A, B) and in SKPT cells (C, D) in the presence of increasing concentrations of ACE inhibitors and, for comparison, Ala-Pro (0 – 100 mM). Uptake rates measured in the absence of inhibitor were taken as 100%.

**Fig. 2.** Type of Gly-Sar uptake inhibition by quinapril and spirapril. A, B: Effect of quinapril on the saturation kinetics of Gly-Sar uptake into Caco-2 cells (A) and SKPT cells (B). Uptake of Gly-Sar (0.01-10 mM at Caco-2 cells, 0.01-5 mM at SKPT cells) was measured at pH 6.0 for 10 min in confluent monolayer cultures. The results represent saturable uptake values after correction for the non-saturable component. If not shown, error bars are smaller than the symbols. Inset: Eadie-Hofstee transformations of the data (v = uptake rate in nmol · 10 min$^{-1}$ · mg of protein$^{-1}$; S = Gly-Sar concentration in mM). n = 4. C, D: Determination of the inhibition constants of quinapril (C) and spirapril (D) at Caco-2 cells in a Dixon type of experiment. Uptake of Gly-Sar was measured at pH 6.0 for 10 min at two Gly-Sar concentrations and at increasing inhibitor concentrations. The linear, non-saturable component of [$^{14}$C]Gly-Sar uptake, measured in the presence of excess amount of Gly-Sar (30 mM and 20 mM, respectively), was subtracted from total uptake to calculate carrier-mediated uptake (n = 4, v = uptake rate in nmol · 10 min$^{-1}$ · mg of protein$^{-1}$).

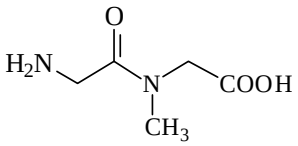
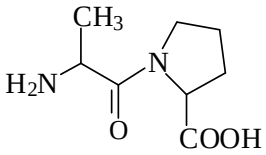
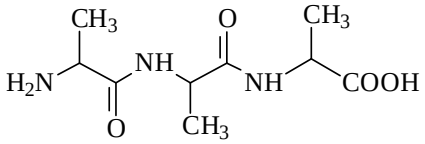
**Fig. 3.** Interaction of ACE-inhibitors with human PEPT1 and human PEPT2 heterologously expressed in HRPE cells. Uptake of [$^{14}$C]Gly-Sar at pH 6.0 (A: 20 µM, 5 min, B: 30 µM, 5 min) was measured in HRPE-hPEPT1 cells (A) and in HRPE-hPEPT2 cells (B) in the presence of increasing concentrations of ACE-inhibitors and, for comparison, Gly-Sar (0–100 mM). Uptake rates measured in the absence of inhibitor were taken as 100%. (n = 4).

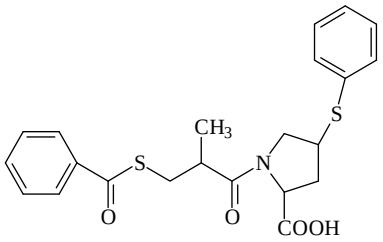
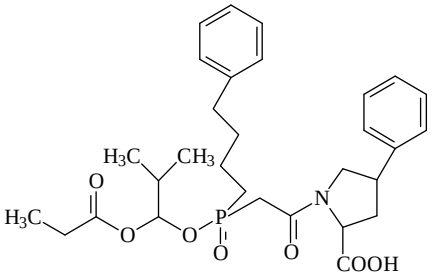
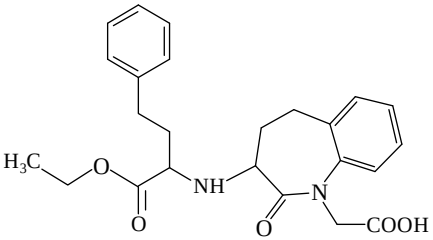
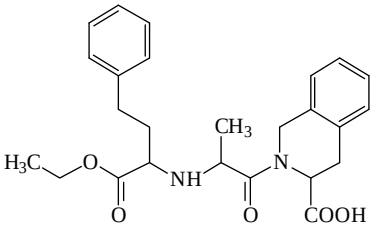
**Fig. 4.** ACE inhibitor-induced inward currents in *Xenopus laevis* oocytes expressing rabbit PEPT1 or PEPT2. A: Currents induced by Ala-Ala-Ala, Ala-Pro and 14 ACE inhibitors as the percentage of the current induced by 10 mM (PEPT1, -60 mV) or 2 mM (PEPT2, -160 mV) Gly-Sar. The concentration of ACE inhibitors or peptides at PEPT1 was mostly 10 mM except for fosinopril (0.33 mM), zofenopril (0.1 mM), quinaprilat (3 mM) and trandolapril (5 mM), and at PEPT2 2 mM except for fosinopril (0.33 mM) and zofenopril (0.1 mM). Values were, if necessary, corrected for the shifts of the zero line due to the presence of DMSO and were corrected for the average current generated in the absence of peptide transporters. Negative values mean inhibition of the basal membrane conductance. Mean values of 2 to 8 oocytes. * $p < 0.05$; ** $p < 0.01$. B, C: Steady-state I-V relationships were measured by the two-electrode voltage clamp technique in oocytes expressing PEPT1 (B) or PEPT2 (C) superfused with modified Barth-solution at pH 6.5 and 10 mM (PEPT1) or 2 mM (PEPT2) peptide or ACE inhibitor (except for fosinopril: 0.33 mM). The membrane potential was stepped symmetrically to the test potentials shown and substrate-dependent currents were recorded as the difference measured in the absence and the presence of substrates.

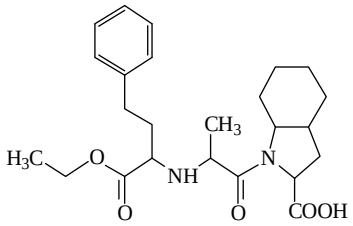
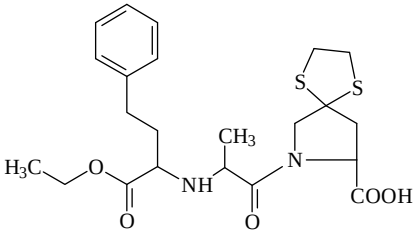
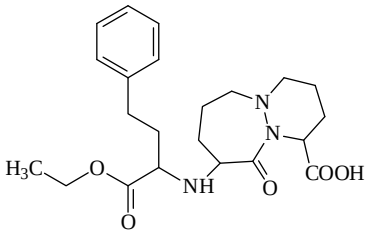
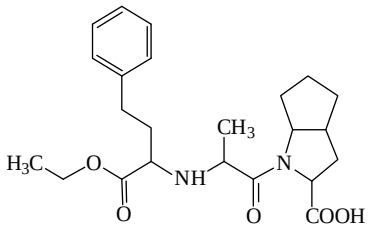
TABLE 1

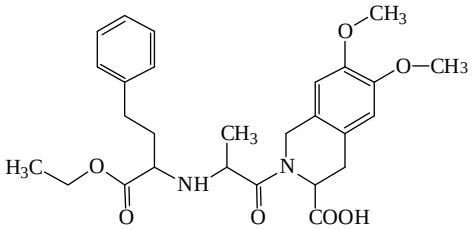
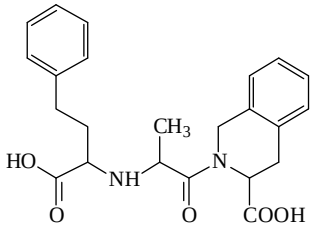
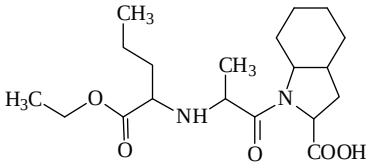
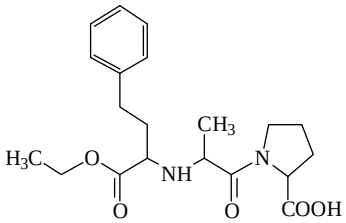
Inhibition constants ($K_i$) of three reference peptides and fourteen ACE inhibitors for the inhibition of [$^{14}$C]Gly-Sar uptake in Caco-2 cells (hPEPT1) and in SKPT cells (rPEPT2).

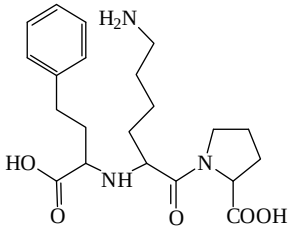
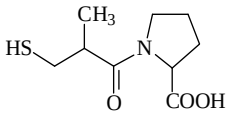
Uptake of [$^{14}$C]Gly-Sar (10 µM, 10 min) was measured at pH 6.0 with increasing concentrations of the test compounds (0-100 mM). Constants were derived from the competition curves shown in Figure 1. Parameters are shown ± S.E. ($n$ = 4).

| Compound | Structure | log D* | $K_{i\ PEPT1}$ (mM) | Rank at PEPT1 | $K_{i\ PEPT2}$ (mM) | Rank at PEPT2 | $K_{i\ PEPT1}$/ $K_{i\ PEPT2}$ |
|---|---|---|---|---|---|---|---|
| Gly-Sar |  | | 0.72 ± 0.02 | | 0.10 ± 0.01 | | 7.2 |
| Ala-Pro |  | | 0.14 ± 0.01 | | 0.014 ± 0.001 | | 10 |
| Ala-Ala-Ala |  | | 0.20 ± 0.01 | | 0.020 ± 0.001 | | 10 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Zofenopril |  | 1.21 | 0.047 ± 0.001 | 1 | 0.037 ± 0.003 | 2 | 1.3 |
| Fosinopril |  | 3.73 | 0.17 ± 0.01 | 2 | 0.013 ± 0.001 | 1 | 13 |
| Benazepril |  | 1.29 | 0.96 ± 0.07 | 3 | 0.34 ± 0.04 | 3 | 2.8 |
| Quinapril |  | 1.87 | 1.0 ± 0.1 | 4 | 0.39 ± 0.03 | 4 | 2.6 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Trandolapril |  | 1.65 | 1.7 ± 0.3 | 5 | 0.45 ± 0.03 | 7 | 3.8 |
| Spirapril |  | 0.54 | 2.2 ± 0.1 | 6 | 0.44 ± 0.06 | 6 | 5.0 |
| Cilazapril |  | -1.77 | 3.0 ± 0.3 | 7 | 1.3 ± 0.1 | 10 | 2.3 |
| Ramipril |  | 1.08 | 3.4 ± 0.2 | 8 | 0.48 ± 0.05 | 8 | 7.1 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Moexipril |  | 1.59 | 5.0 ± 0.5 | 9 | 0.41 ± 0.04 | 5 | 12 |
| Quinaprilat |  | -0.06 | >3.16 (≈ $6^{\#}$) | 10 | 0.73 ± 0.04 | 9 | 8.6 |
| Perindopril |  | 1.02 | 6.9 ± 1.5 | 11 | 2.2 ± 0.6 | 11 | 3.1 |
| Enalapril |  | 0.08 | 14 ± 1 | 12 | 3.6 ± 0.3 | 13 | 3.9 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Lisinopril |  | -1.32 | 23 ± 1 | 13 | 3.4 ± 0.1 | 12 | 6.8 |
| Captopril |  | -2.02 | 46 ± 2 | 14 | 7.9 ± 0.9 | 14 | 5.8 |

[*]Log D values and structures were obtained from the Scifinder data base. [#]$K_i$ value extrapolated beyond measurement range because of limited solubility of compound. See Figure 1 for maximal substrate concentrations used.

TABLE 2

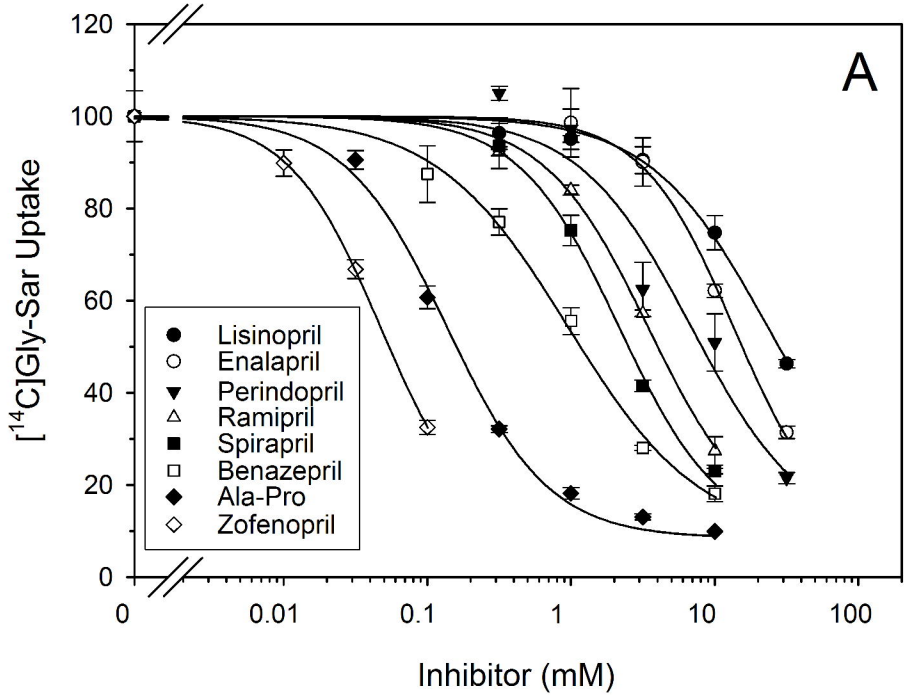
Inhibition constants ($K_i$) of Gly-Sar and seven ACE inhibitors for the inhibition of [$^{14}$C]Gly-Sar uptake in HRPE-hPEPT1 and HRPE-hPEPT2 cells.

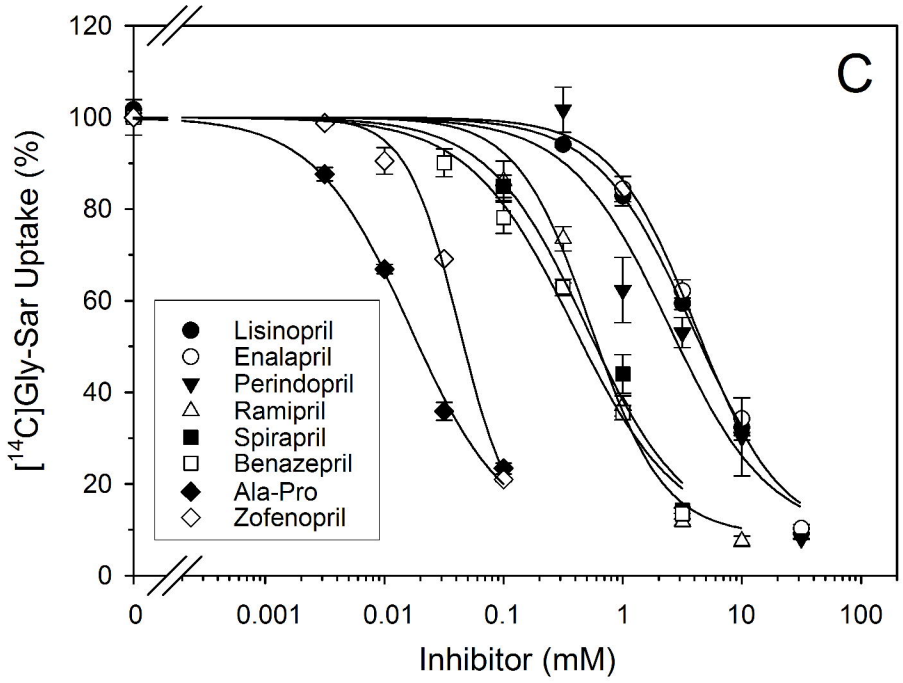
Uptake of [$^{14}$C]Gly-Sar (20 or 30 µM, 5 or 10 min, respectively) was measured at pH 6.0 with increasing concentrations of the test compounds (0-100 mM). Constants were derived from the competition curves shown in Figure 3. Parameters are shown ± S.E. ($n$ = 4).

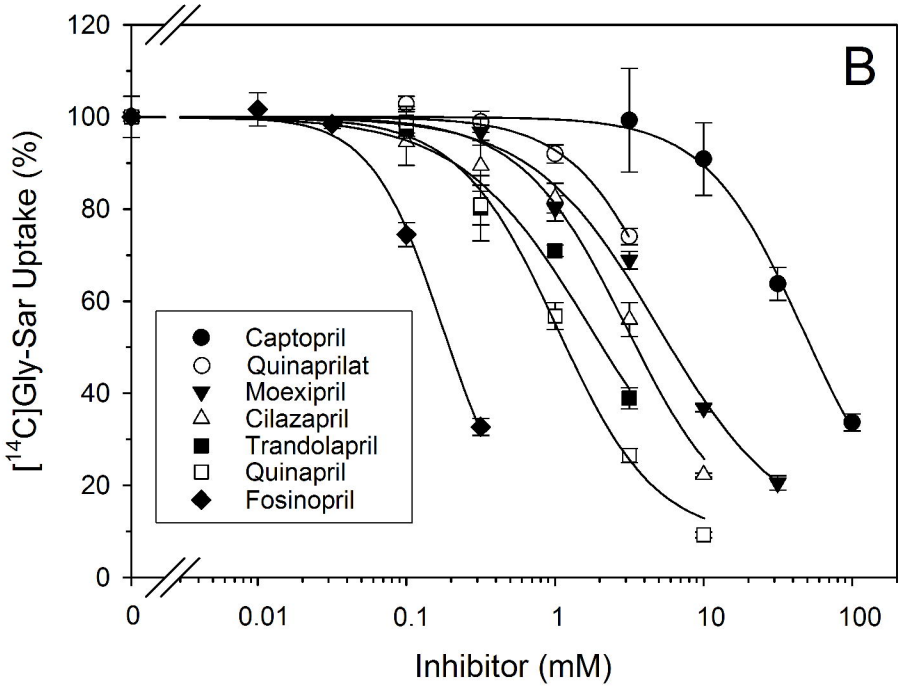
| Compound | $K_{i\ hPEPT1}$ (mM) | Rank at hPEPT1 | $K_{i\ hPEPT2}$ (mM) | Rank at hPEPT2 | $K_{i\ hPEPT1}$/ $K_{i\ hPEPT2}$ |
|---|---|---|---|---|---|
| Gly-Sar | 0.44 ± 0.01 | | 0.12 ± 0.02 | | 3.6 |
| Zofenopril | 0.05 ± 0.01 | 1 | 0.024 ± 0.002 | 1 | 2.1 |
| Quinapril | 0.71 ± 0.03 | 2 | 0.09 ± 0.02 | 2 | 7.9 |
| Benazepril | 0.91 ± 0.05 | 3 | 0.18 ± 0.01 | 4 | 5.1 |
| Spirapril | 1.9 ± 0.2 | 4 | 0.17 ± 0.02 | 3 | 11 |
| Quinaprilat | >3.16 (≈ 6$^{\#}$) | 5 | 1.6 ± 0.3 | 5 | 3.7 |
| Enalapril | 10 ± 1 | 6 | 3.6 ± 0.2 | 6 | 2.8 |
| Captopril | 43 ± 7 | 7 | 16 ± 1 | 7 | 2.7 |

$^{\#}K_i$ value extrapolated beyond measurement range because of limited solubility of compound. See Figure 3 for maximal substrate concentrations used.

Fig. 1


A
[14C]Gly-Sar Uptake
Inhibitor (mM)
Lisinopril
Enalapril
Perindopril
Ramipril
Spirapril
Benazepril
Ala-Pro
Zofenopril


C
[14C]Gly-Sar Uptake (%)
Inhibitor (mM)
Lisinopril
Enalapril
Perindopril
Ramipril
Spirapril
Benazepril
Ala-Pro
Zofenopril


B
[14C]Gly-Sar Uptake (%)
Inhibitor (mM)
Captopril
Quinaprilat
Moexipril
Cilazapril
Trandolapril
Quinapril
Fosinopril

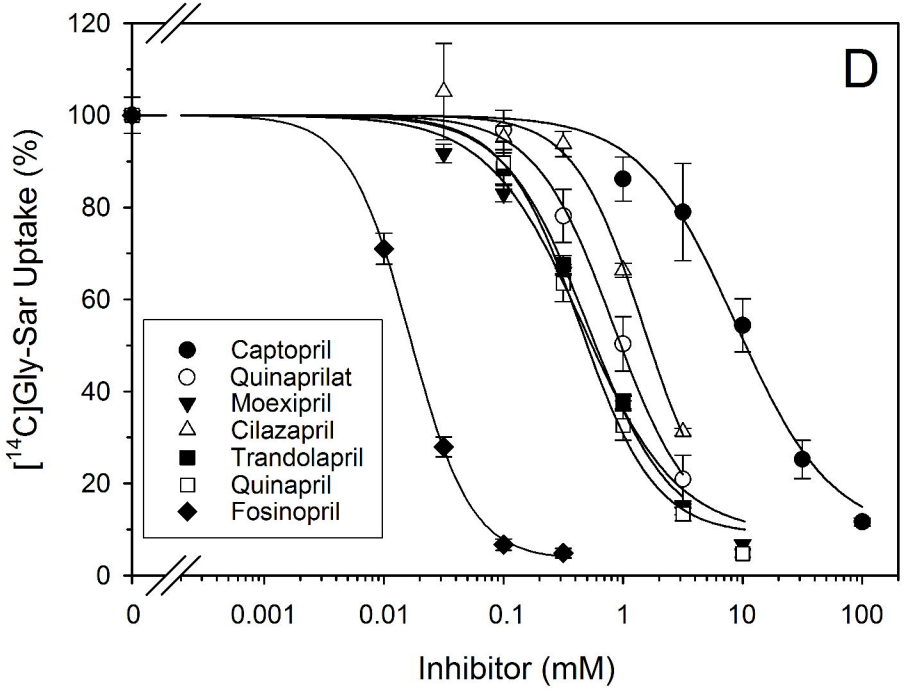
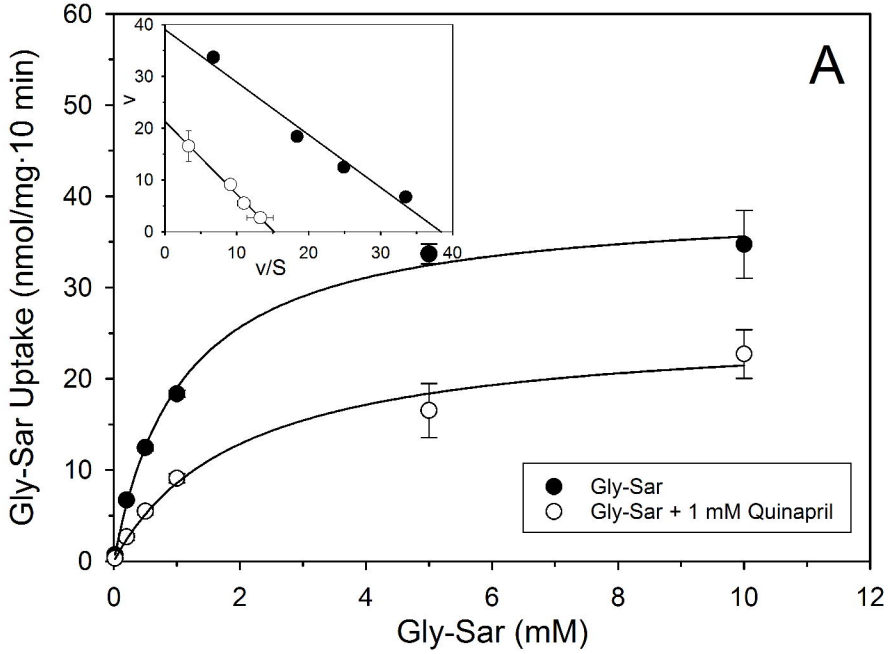
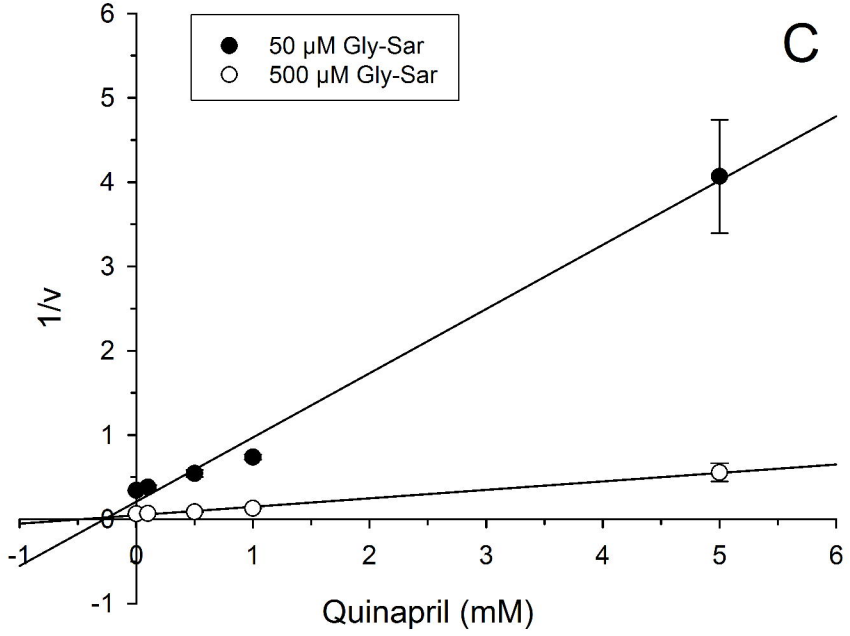
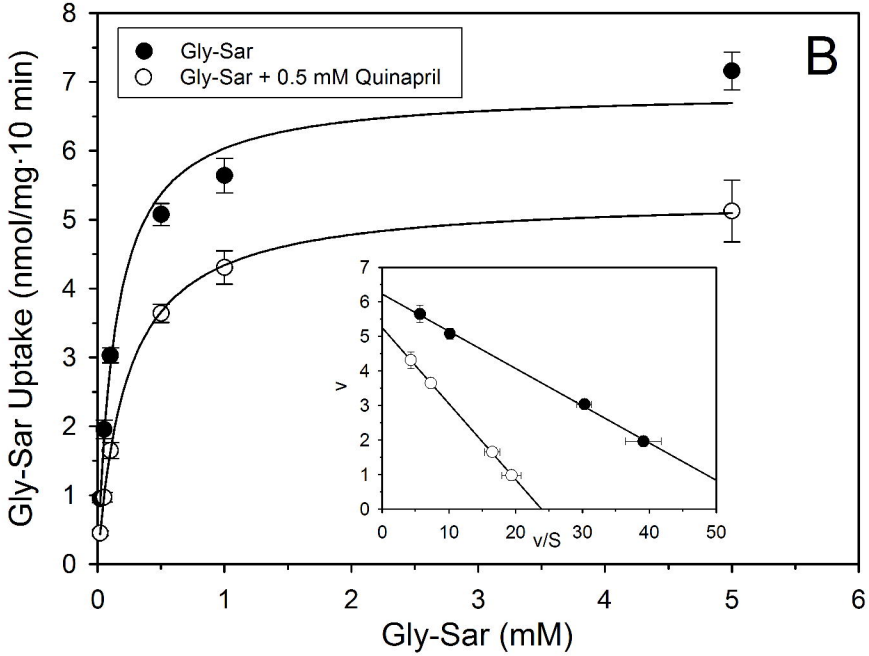

D
[14C]Gly-Sar Uptake (%)
Inhibitor (mM)
Captopril
Quinaprilat
Moexipril
Cilazapril
Trandolapril
Quinapril
Fosinopril

Fig. 2


A
Gly-Sar Uptake (nmol/mg·10 min)
Gly-Sar (mM)
v
v/S
Gly-Sar
Gly-Sar + 1 mM Quinapril


C
50 µM Gly-Sar
500 µM Gly-Sar
1/v
Quinapril (mM)


B
Gly-Sar
Gly-Sar + 0.5 mM Quinapril
Gly-Sar Uptake (nmol/mg·10 min)
Gly-Sar (mM)
v
v/S

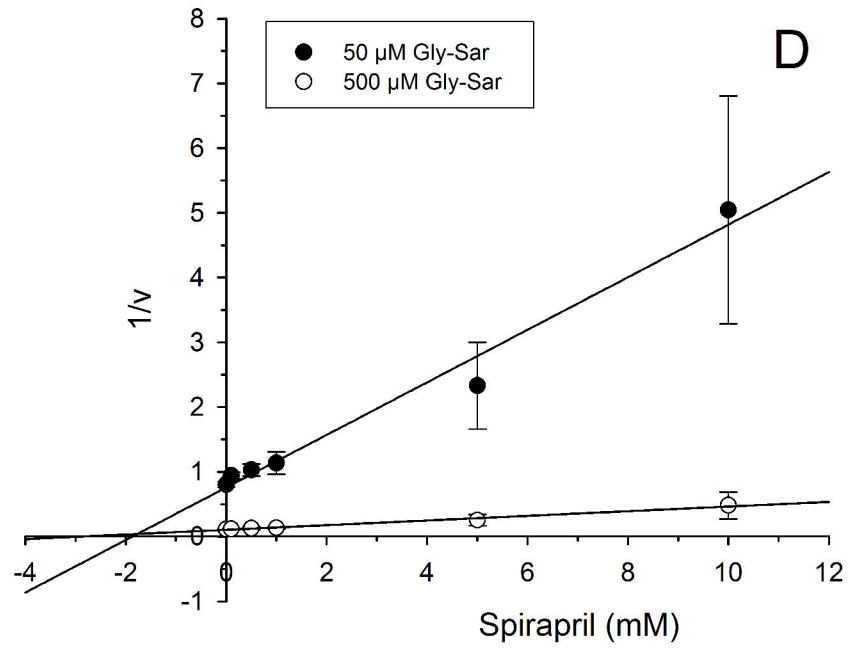

D
50 µM Gly-Sar
500 µM Gly-Sar
1/v
Spirapril (mM)

Fig. 3

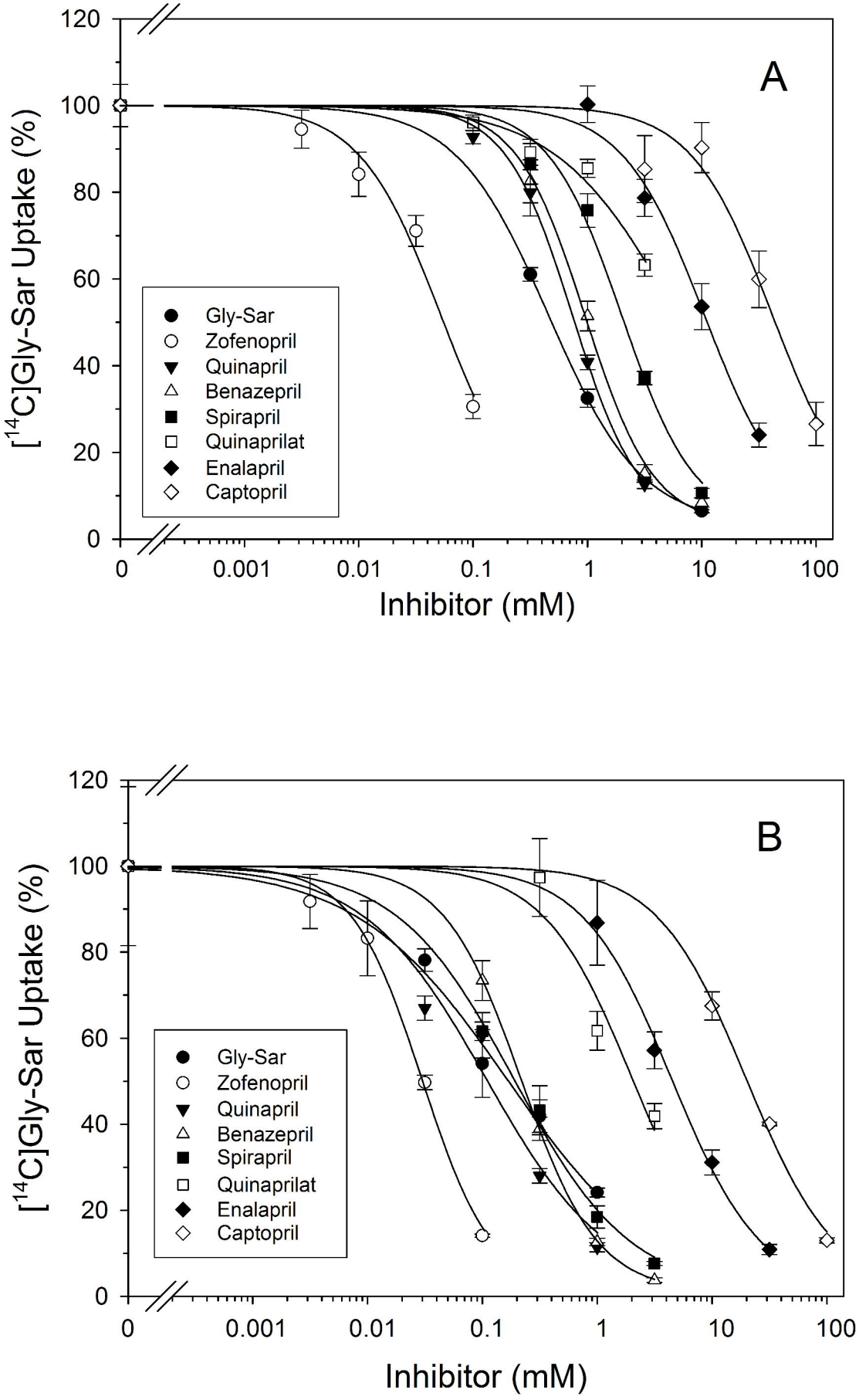
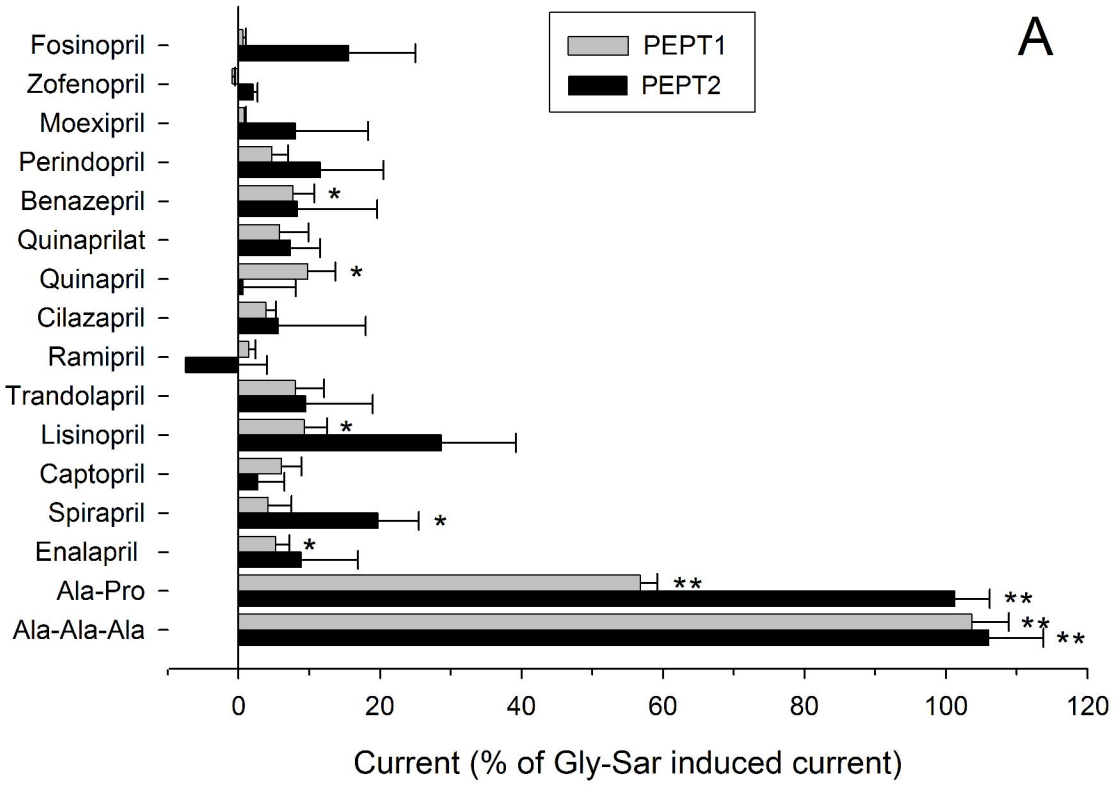
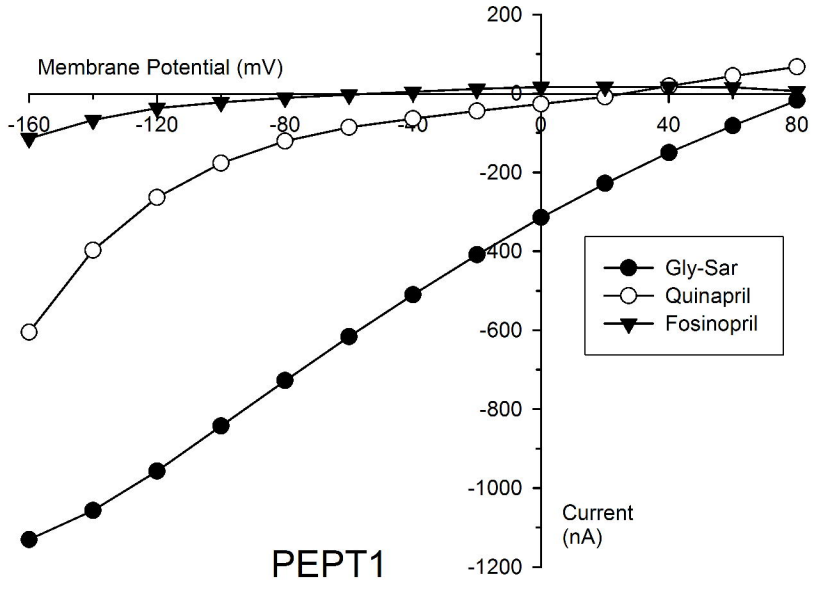
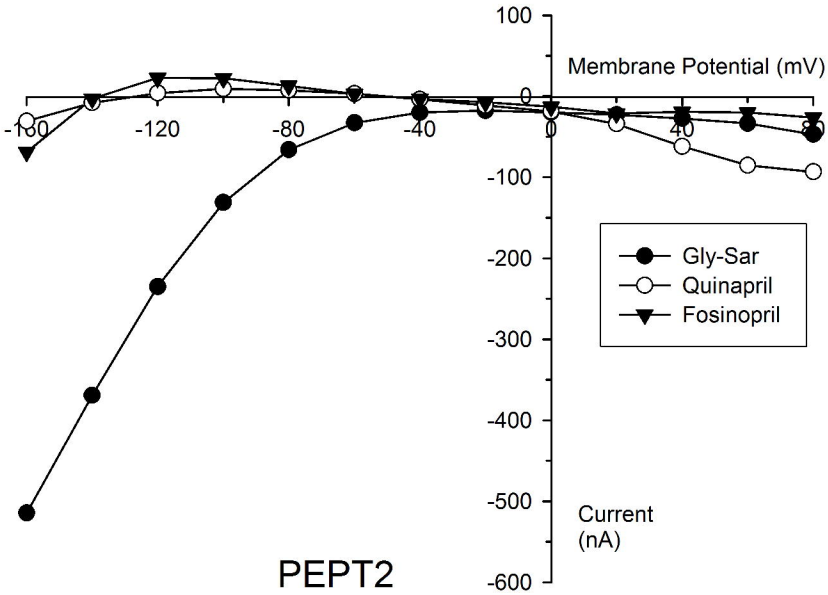

A
B
[14C]Gly-Sar Uptake (%)
Inhibitor (mM)
0
20
40
60
80
100
120
0
0.001
0.01
0.1
1
10
100
Gly-Sar
Zofenopril
Quinapril
Benazepril
Spirapril
Quinaprilat
Enalapril
Captopril

Fig. 4


A
PEPT1
PEPT2
Fosinopril
Zofenopril
Moexipril
Perindopril
Benazepril
Quinaprilat
Quinapril
Cilazapril
Ramipril
Trandolapril
Lisinopril
Captopril
Spirapril
Enalapril
Ala-Pro
Ala-Ala-Ala
*
**
0
20
40
60
80
100
120
Current (% of Gly-Sar induced current)


B
Membrane Potential (mV)
200
0
-160
-120
-80
-40
40
80
-200
-400
-600
-800
-1000
-1200
Gly-Sar
Quinapril
Fosinopril
Current
(nA)
PEPT1


C
100
Membrane Potential (mV)
0
-120
-80
-40
-100
-200
-300
-400
-500
-600
Gly-Sar
Quinapril
Fosinopril
Current
(nA)
PEPT2