Evidence for Overlapping Substrate Specificity Between Large Neutral Amino Acid (LNAA) and Dipeptide (hPEPT1) Transporters for PD 158473, an NMDA Antagonist

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Purpose. The objective of this research was to investigate the substrate specificity of large neutral amino acid carrier (LNAA) and di/tripeptide (hPEPT1) transporters with respect to PD 158473, an NMDA antagonist.

Methods. Cellular uptake studies were carried out using two types of Chinese Hamster Ovary (CHO). CHO-K1 cells represent the wild type with inherent large neutral amino acid (LNAA) activity. CHO-PEPT1 cells were generated by stable transfection of hPEPT1 gene into CHO cells. Therefore, these cells possess both LNAA activity and di/tripeptide transporter activities as a result of the transfection. Cellular uptake of PD 158473 was quantified using a HPLC method previously developed in our laboratory.

Results. The utility of the CHO-PEPT1 cell model was demonstrated by determining the uptake kinetics of Gly-Sar, a prototypical dipeptide transporter substrate. Uptake kinetics of PD 158473 displayed two carrier-mediated transport components in CHO-PEPT1 cells, while in CHO-K1 cells the relationship was consistent with classic one component Michaelis-Menten kinetics. These results confirmed the affinity of PD 158473 for both LNAA and di/tripeptide transporters. Further, results from inhibition experiments using these two cell types indicate that the high affinity-low capacity system was the LNAA carrier and the low affinity-high capacity carrier was the di/tripeptide transporter.

Conclusions. This study demonstrates overlapping substrate specificity between LNAA carrier and di/tripeptide transporter (hPEPT1) for PD 158473, an amino acid analog. Establishing Structure Transport Relationship (STR) for this overlap will aid in a design strategy for increasing oral absorption or targeting specific drugs to selected tissues.

KEY WORDS: large neutral amino acid transporter; di/tripeptide transporter; CHO-PEPT1 cells; NMDA antagonist.

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INTRODUCTION

N-methyl-D-aspartate (NMDA) receptors are members of a family of ionotropic glutamate receptors that function as a major excitatory neurotransmission pathway in the brain (1). Glutamate receptors in general and NMDA receptors in particular have been implicated in excitotoxicity which may accompany acute ischemic events such as stroke or cerebral trauma. This may be a factor in several other neurological disorders including convulsive disorders, neuropathic pain and anxiety (2). Therefore, antagonists of NMDA receptor function can be of therapeutic value in the treatment of numerous neurological disorders.

PD 158473 is a phenylalanine derivative with a phosphonic acid and naphthyl subsitutent on positions 5 and 3 respectively (Fig. 1). It is a potent (ED_{50} = 5 mg/kg) and competitive NMDA antagonist with oral activity in mice (3). Initial studies in Chinese Hamster Ovary cells (CHO-K1) indicated competitive inhibition by PD 158473 on L-Phe uptake, thereby suggesting a potential role for the large neutral amino acid (LNAA) or System L carrier in the transport of this compound (3). Our previous studies using *in-vitro* (Caco-2) and *in-situ* (single-pass rat intestinal perfusion) models has demonstrated a significant involvement of intestinal di/tripeptide carriers in the transport of this compound (4). In addition, in *in-vivo* rat studies designed to evaluate dose-dependency of oral absorption, we demonstrated a linear relationship between dose (0.5 to 5 mg/kg) and $AUC_{0\text{-}inf}$ with PD 158473 demonstrating low oral bioavailability (<5%) (4). Although the relevance of specialized transport mechanisms in the intestinal absorption of PD 158473 was minimal in rats *in-vivo*, it was of interest to investigate the roles of LNAA and di/tripeptide carriers in the cellular uptake of this compound. Towards this end, we evaluated the uptake kinetics of PD 158473 in CHO-K1 cells that possess inherent LNAA activity and in a hPEPT1 (peptide transporter) gene transfected cell line (CHO-PEPT1) that possesses both LNAA and di/tripeptide transporter activities. This cell model (CHO-PEPT1) has been recently developed and characterized, and shown to be an useful screening tool for identifying substrates for the di/tripeptide carrier (5).

The results of this study confirm the involvement of both LNAA carrier and di/tripeptide transporter in the cellular uptake of PD 158473 in CHO-PEPT1 cells, whereas, in CHO-K1 cells, its uptake is mediated by the LNAA carrier. To the best of our knowledge, this is the first example of an amino acid analog that appears to possesses a strong affinity to both LNAA and di/tripeptide transporters. The structural and molecular basis for such an overlap in specificity between di/tripeptide and LNAA transporters is not currently known. However, the results of this study highlight the feasibility of targeting drug molecules to nutrient transporters in order to increase their cellular uptake with a view toward improving their oral absorption characteristics or alternatively, to target drugs to selected tissues to maximize local delivery.

MATERIALS AND METHODS

Materials

PD 158473 was synthesized at Parke-Davis Pharmaceutical Research (Ann Arbor, MI). Cephalexin, Gly-Sar, Gly-Phe,

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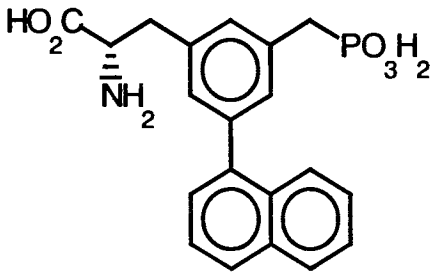

Fig. 1. Structure of PD 158473.

Benzoic acid, L-Glycine, L-Phenylalanine, L-Tyrosine, L-Leucine and L-Aspartic acid were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H] Gly-Sar (specific activity 39 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). All other reagents including buffer components such as MES, MOPS etc. were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC grade solvents were purchased from Mallinckrodt (Kentucky, USA).

Cell Culture

CHO cells transfected with hPept1 gene were used as described previously (5). These cells were termed as CHO-PEPT1. Wild type cells were used as controls (CHO-K1). Cells were routinely cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, non-essential amino acids and L-glutamine in the absence (CHO-K1) or presence (CHO-PEPT1) of G-148 (gentamicin sulfate). Cells were passaged weekly and plated on 6-well clusters at 50,000 cells/cm^2 for uptake studies. Uptake studies were conducted on 5 ± 2 day old monolayers.

Uptake Studies

Uptake of PD 158473 in CHO-PEPT1 and CHO-K1 Cells

To initiate uptake studies where direct accumulation of PD 158473 was determined, cells were gently washed twice with warm (37°C) MES buffer (10 mM MES, 5 mM KCl, 135 mM NaCl and 1.8 mM $CaCl_2$, pH 6.0) and pre-incubated for 15–30 minutes. Cell monolayers of CHO-K1 or CHO-PEPT1 were then incubated with PD 158473 (25 μM) in the absence (control) or presence of other agents (e.g., dipeptides, antibiotics, amino acids etc.) at 37°C for a specific period of time (0–30 min). Thereafter, the incubation media was gently aspirated, cells washed with ice-cold MES buffer (pH 6.0) twice and scraped in water. Subsequently, the cell suspension was sonicated and cell associated proteins precipitated by adding 1–2 volumes of acetonitrile. This mixture was centrifuged at 14,000 g prior to analysis of the supernatant by HPLC. Concentration dependent uptake of PD 15473 was accomplished using the same procedure as described above using 15 min incubations with the concentration ranging from 10 to 2500 μM for CHO-PEPT1 cells and 10 to 1000 μM for CHO-K1 cells.

Uptake of Gly-Sar in CHO-PEPT1 Cells

For studies where the effect of PD 158473 on the uptake of [^{3}H]Gly-Sar was examined, 20 μM Gly-Sar (0.1 μCi/ml) in 3 mM HEPES/MES/TRIS (pH 6.0) containing 10 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ was used. The incubation period was 30 min and concentration of PD 158473 ranged from 0.010 to 10^{-9} M. The IC_{50} values (i.e., concentration of PD 158473 to inhibit 50% of uptake of radiolabelled Gly-Sar) were determined from dose-response inhibition curves. Concentration dependent uptake of [^{3}H]Gly-Sar was determined using the same procedure as described above. Incubations were done for 30 min ranging in concentrations from 0.01 to 50 mM. Quantitation of [^{3}H]Gly-Sar was accomplished using liquid scintillation counting.

HPLC Assay

Quantitation of PD 158473 was accomplished by a sensitive, rapid and reliable procedure developed in our laboratory. The HPLC system consisted of a Spectra-Physics (Fremont, CA, USA) SP 8700 extended range LC pump, SP 8780 autosampler with a 100 μl sample loop, and a Waters (Milford, MA, USA) variable wavelength Lambdamax 481 detector set at 221 nm. The analytical column was Supelcosil LC 18-DB (Bellefonte, PA, USA, 150 mm × 4.6 mm, I.D. 3 mm) and an Alltech (Deerfield, IL, USA) C_{18} guard column. Peak recording and integration was accomplished with a Spectra Physics Chromjet integrator. The mobile phase consisted of 81% (v/v) phosphate buffer, pH 6.5, 19% (v/v) acetonitrile and 0.1% triethylamine. The assay was reproducible with % R.S.D and % R.E. typically within ± 10%. The retention time for PD 158473 was 10 min.

Data Analysis

Uptake Studies in CHO-K1 and CHO-PEPT1 Cells

Uptake of PD 158473 as a function of concentration was fitted to a Michaelis-Menten equation:

$$V = (V_{max} * [S])/(K_m + [S]) + [K_d * S]$$

where V = rate of uptake, V_{max} = maximal rate of uptake, K_m = affinity constant, S = substrate concentration and K_d = non-saturable uptake. Data from CHO-PEPT1 cells were fitted to a two-component model as follows:

$$V = (V_{max1} * [S])/(K_{m1} + [S]) + (V_{max2} * [S])/(K_{m2} + [S]) + [K_d * S]$$

Statistical analysis of data was carried out using SigmaStat 2.0 (Jandel Scientific). Inhibition data from CHO-PEPT1 and CHO-K1 cells were analyzed using one-way ANOVA following a Dunnett's test for pair wise comparison with the control value for uptake in each cell line. Statistical significance was set at $p < 0.05$ for all pair wise comparison.

Inhibition Studies in CHO-PEPT1 Cells

For inhibition studies of [^{3}H]Gly-Sar as a function of concentration of PD 158473, Sigma plot was used to determine the IC_{50} as described by De Lean et al. (6) fitting the following equation.

$$F = (a - d)/(1 + (x/c)^*b) + d$$

where a and d represented the maximum and minimum uptake

of Gly-Sar (in dpm), respectively, x was the concentration of PD 158473 and b the slope factor. F is the response and c denotes the IC_{50}. The IC_{50} reported in the results section is the Mean ± S.E. of 9 independent measurements.

Statistical analysis of all data was carried out using Student's t-test with a significance level set at $p < 0.05$.

RESULTS

Gly-Sar Uptake Studies

Previous work had examined the inhibitory effect of dipeptides and amino acids on Gly-Sar uptake by CHO-PEPT1 cells (5). The system was further characterized by examining the kinetic relationship in these cells. Gly-Sar uptake in CHO-PEPT1 cells was described as follows: K_m = 568 ± 61 μM, V_{max} = 50 ± 2 pmol/min.cm^2 and K_d = 0.00036 ± 0.00004 pmol/min.cm^2.μM. The Michaelis constant for Gly-Sar compared well (1.1 ± 0.1 mM) with the value obtained in Caco-2 cells by Brandsch et al (7). In CHO-PEPT1 cells, PD 158473 was a potent inhibitor of Gly-Sar uptake resulting in a dose-dependent decrease in the uptake of Gly-Sar with an IC_{50} of 52 ± 9 μM (Fig. 2). This value corresponds reasonably with the K_m for this compound in Caco-2 cells (107 μM) as described previously (4).

PD 158473 Uptake Studies

An evaluation of time dependent uptake of PD 158473 revealed the uptake of this compound to be linear for up to 30 min in both CHO-K1 and CHO-PEPT1 cells (*results not shown*). Concentration dependent uptake of PD 158473 in CHO-PEPT1 cells is shown in Fig. 3. Uptake was curvilinear with the following parameters: K_{m1} = 14 μM, V_{max1} = 0.2 pmol/min.cm^2, K_{m2} = 950 μM, V_{max2} = 4.2 pmol/min.cm^2 and K_d = 0.002 pmol/min.cm^2.μM. Concentration dependent uptake of PD 158473 in CHO-K1 cells is shown in Fig. 4. Uptake was Michaelis-Menten type with the following parameters: K_m = 10 μM, V_{max} = 0.2 pmol/min.cm^2 and K_d = 0.002 pmol/min.cm^2.μM.

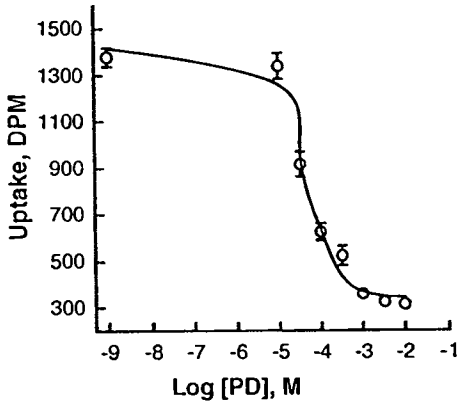

Fig. 2. Representative graph on the effect of PD 158473 on the uptake of [^{3}H] Gly-Sar in CHO-PEPT1 cells. Buffer used was 3 mM HEPES/MES/TRIS (pH 6.0) containing 10 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$. Data reported as Mean ± S.D of triplicate measurements each of n = 3. PD = PD 158473.

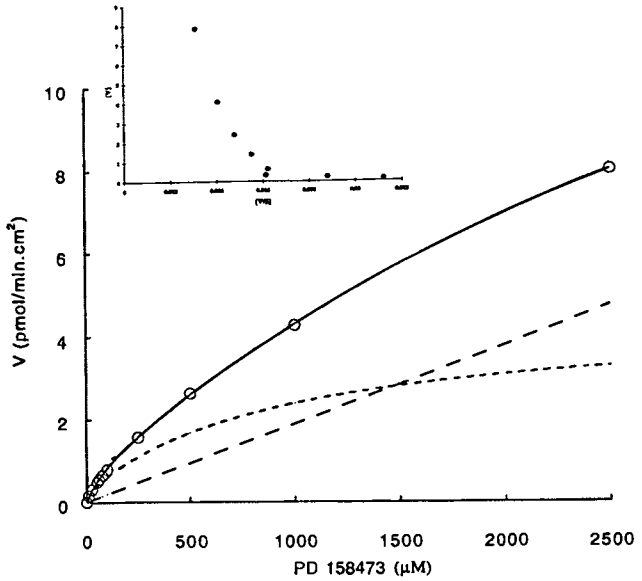

Fig. 3. Concentration dependent uptake of PD 158473 in CHO-PEPT1 cells. Buffer used was MES, pH 6.0. Each value is represented as a mean of three determinations. Inset describes the Eadie-Hofstee plot for PD 158473. Experiments were carried out as described in Materials and Methods. Legend: non-saturable — — —, saturable -------, total transport ____.

PD 158473 Inhibition Studies

The effect of different amino acids, dipeptides or cephalexin on uptake of PD 158473 (25 μM) in CHO-PEPT1 and CHO-K1 cells is shown in Table I. Uptake was inhibited significantly by large neutral amino acids (L-Phe and L-Leu) but not

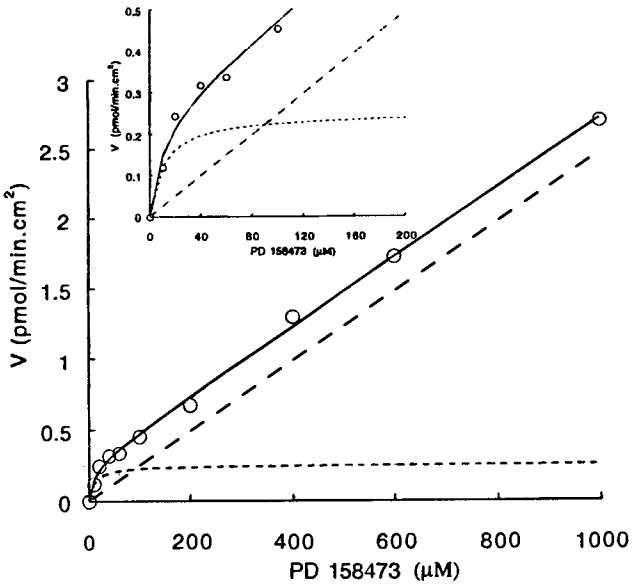

Fig. 4. Concentration dependent uptake of PD 158473 in CHO-K1 cells. Buffer used was MES, pH 6.0. Each value is represented as a mean of three determinations. Inset describes the same plot at lower concentrations (0 to 200 μM). Experiments were carried out as described in Materials and Methods. Legend: non-saturable — — —, saturable ------, total transport ____.

Table I. Summary of Inhibition of Uptake of PD 158473 (25 μM) in CHO-PEPT1 and CHO-K1 Cells by Dipeptides, Amino Acids, and Cephalexin

Inhibitor	CHO-PEPT1	CHO-KI
Control (25 μM)	100.0 ± 13.2	100.0 ± 12.7
Gly-Sar (20 mM)	130.6 ± 21.0*	125.0 ± 7.4
Cephalexin (20 mM)	126.0 ± 12.4*	151.4 ± 18.3*
Gly-Phe(5 mM)	52.0 ± 7.4*	52.2 ± 6.4*
Gly (5 mM)	107.1 ± 8.0	100.0 ± 12.6
Sar (5 mM)	138.6 ± 11.4*	106.9 ± 29.9
Phe (5 mM)	41.2 ± 3.8*	67.3 ± 4.6*
Leu-Phe (5 mM)	51.5 ± 6.8*	64.0 ± 8.8*
Leu (5 mM)	52.0 ± 7.9*	63.5 ± 18.6*

Note: All values expressed as a percent of control., Means ± S.D., n = 3–4. Experiments were conducted in MES buffer, pH 6.0. at 15 minute incubations. Concentration of inhibitors in parenthesis.
* $p < 0.05$ compared to control value using Dunnett's pair wise comparison test following a one-way ANOVA.

by the non-metabolizable dipeptide (Gly-Sar) or cephalexin, in both cell types. The inhibitory effects of agents are similar in both cell types. Presently it is not clear why Sar, cephalexin and Gly-Sar in CHO-PEPT1 cells and cephalexin in CHO-K1 cells led to an increase in the uptake of PD 158473. It should be noted that at this concentration of PD 158473 (25 μM), transport via the LNAA should be predominant mechanism. Inhibitory effects by Gly-Phe and Leu-Phe can be attributed to their breakdown products (Phe, Leu) as opposed to the dipeptides themselves.

Previously, we had demonstrated that PD 158473 inhibited the uptake of L-Phe in CHO cells with a K_i of 200 μM (3). In order to evaluate if the converse was true and to confirm the competitive nature of this inhibition, a Dixon-Webb plot of PD 158473 and L-Phe was constructed as shown in Fig. 5. The

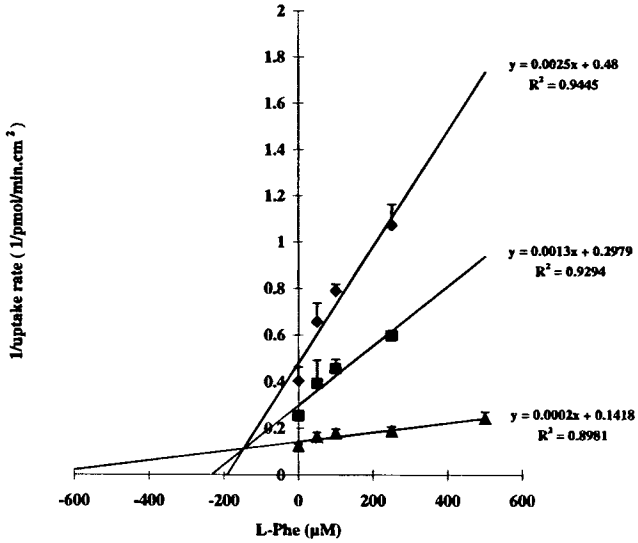

Fig. 5. Dixon-Webb plot of PD 158473 and L-Phe in CHO-PEPT1 cells. Buffer used was MES, pH 6.0. Each value is represented as Means ± S.D., n = 3–4. Concentrations of PD 158473 are as follows: ◆ 20 μM, ■ 40 μM, ▲ 60 μM.

mechanism of inhibition of PD 158473 by L-Phe was shown to be of competitive nature with a K_i of 147 μM.

The results presented above along with our previous studies in *in-vitro* and *in-situ* models clearly demonstrated an overlap in substrate specificity for PD 158473 between di/tripeptide transporter and LNAA carrier. Since the concentration of PD 158473 used for inhibition studies was low (25 μM) and close to the K_m of the high affinity-low capacity LNAA sytem, it was reasoned that the majority of cellular uptake of this compound was mediated via the LNAA carrier. To experimentally confirm this hypothesis, the effect of Gly-Sar on the uptake of PD 158473 in the presence of excess L-Phe was conducted. The rationale for this study was based on the assumption that if the LNAA carrier was saturated, then at concentrations of PD 158473 close to its K_m of 950 μM, a significant portion of its transport would be mediated by the low affinity-high capacity di/tripeptide transporter. The results of these experiments for CHO-PEPT1 and CHO-K1 cells are shown in Fig 6. As expected, uptake of PD 158473 (800 μM) was significantly inhibited by Gly-Sar in the presence of excess L-Phe (2.5 mM) in CHO-PEPT1 cells but not CHO-K1 cells.

DISCUSSION

Kinetics and mechanism of uptake of PD 158473 was examined in two cell types: one possessing inherent LNAA activity but low di/tripeptide transporter activity (CHO-K1), and the other containing both LNAA and di/tripeptide activities as a result of stable transfection with hPEPT1 (CHO-PEPT1). Our first study with the CHO-PEPT1 cell line and PD 158473 consisted of evaluating the effect of this compound on the uptake of [^{3}H] Gly-Sar, a prototypical and non-metabolizable substrate of the intestinal di/tripeptide transporter (7). PD 158473 was an effective inhibitor of Gly-Sar uptake with an IC_{50} of 52 ± 9 μM. This value was remarkable considering that the IC_{50} for self-inhibition of Gly-Sar in this model is approximately 200 μM (5). Although indirect inhibition assays are useful to initially explore the relationship between two substrates, it is known that some compounds can elicit inhibitory action without being transported themselves (8). Encouraged by these findings, a more thorough study was conducted on PD 158473 with both types of CHO cells. When the concentration dependency of PD 158473 uptake was evaluated in both cell types, a two component curvilinear plot resulted for CHO-PEPT1 cells, whereas in the CHO-K1 cells, the relationship was classic Michaelis-Menten type hyperbola. A comparison of the kinetic parameters between cell types suggest that the K_m and V_{max} for the high affinity-low capacity system in both cell types were in close agreement with each other. This system is therefore likely to be the LNAA carrier. It should be noted that the K_m value for this system (10 to 14 μM) was in the range of the K_m typical for LNAA substrates (3). By inference, the second system in CHO-PEPT1 cells must be that of the di/tripeptide transporter. The range of K_m for dipeptides and dipeptide transporter substrates is fairly large (0.5 to 15 mM) and the value of 950 μM was well within this range (9).

Based on these studies, it is clear that while PD 158473 is a substrate for di/tripeptide transporter, it also retains good affinity for LNAA. Due to more than a 50-fold difference in the affinity of PD 158473 for the two types of transporters, PD 158473 will be transported by this carrier in tissue where there

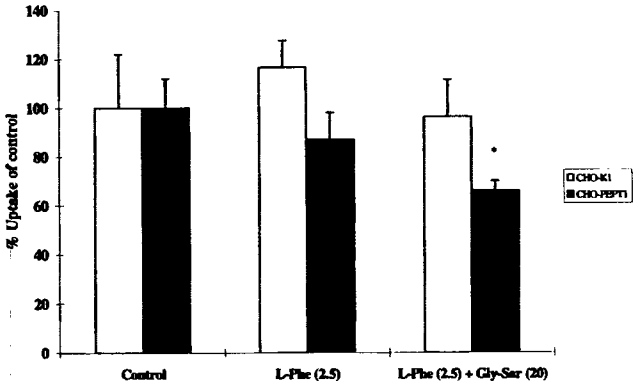

Fig. 6. Effect of Gly-Sar (20 mM) on the uptake of PD 158473 (800 μM) in CHO-K1 and CHO-PEPT1 cells in the presence of excess L-Phe (2.5 mM). Buffer used was MES, pH 6.0. Each value is represented as Means ± S.D., n = 3–4. * $p < 0.05$ compared to control value. Control values: CHO-K1 = 1.57 pmol/min.cm^2 and CHO-PEPT1 = 1.14 pmol/min.cm^2.

is abundance of LNAA carrier (e.g., brain). Cross inhibition between L-Phe and PD 158473 was demonstrated using Dixon-Webb plots to confirm that these two compounds were transported by the same carrier. In addition, the inhibitory relationship was competitive, suggesting that both L-Phe and PD 158473 bind to the same site on the transporter protein. Confirmation of this overlapping substrate specificity was accomplished by examining the cellular uptake of PD 158473 (800 μM) in the presence of excess L-Phe such that the LNAA carrier was saturated. As shown in Fig. 6, in the presence of excess L-Phe, Gly-Sar inhibited the uptake of PD 158473 in CHO-PEPT1 cells but not in CHO-K1 cells.

The structural basis for the utilization of di/tripeptide transporter by this compound is not currently known. In the last few years, numerous laboratories have attempted to elucidate the Structure Transport Relationship (STR) for exploiting the intestinal di/tripeptide transporters in order to increase the oral absorption of drug candidates (10–12). The ability of this compound to be transported by these two disparate nutrient transporters has broad implications to the pharmacokinetics and pharmacodynamics in *in-vivo*. The presence of LNAA carriers in the brain has been well established, while the presence of di/tripeptide is controversial. The presence of peptide transporters has been established in the intestine and kidney, while it is known that these tissues also possess LNAA carriers (13–15). Theoretically, such an overlap in specificity between LNAA carrier and di/tripeptide transporter can be used as a basis for designing small molecule amino acid or peptidomimetic drugs utilizing targeted drug delivery. For e.g., in this scenario, high concentrations of the drug in the intestine would be most efficiently absorbed by the low affinity-high capacity di/tripeptide transporter; subsequently lower concentrations of circulating drug would be transported in to the CNS by the high affinity-low capacity LNAA carrier. Finally, studies are in progress in our laboratory to explore the SAR of LNAA and di/tripeptide transporter in *in-vitro* (Caco-2 and CHO cells) and *in-situ* (rat brain perfusion technique) models to gain insights in to the structural basis for overlap addressed in this research. Results from these studies will form the basis of a future communication.

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