Amino Acids (2009) 36:483–492
DOI 10.1007/s00726-008-0106-x

ORIGINAL ARTICLE

Binding specificity of the L-arginine transport systems in mouse macrophages and human cells overexpressing the cationic amino acid transporter hCAT-1

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Received: 2 May 2007 / Accepted: 6 May 2008 / Published online: 27 May 2008

Abstract The uptake of L-arginine into mouse peritoneal macrophages can be inhibited by numerous amino acids and derivatives. Kinetic studies showed an almost entirely competitive inhibition for both cationic and neutral amino acids and derivatives suggesting that the comparison of their binding specificity by using a quantitative structure-activity relationship (QSAR) study is reasonable. The properties of the most efficient inhibitors were the following: the length of the aliphatic side chain, a general structural similarity to L-arginine (>0.79), cationic character, L-configuration, the presence of an α-amino group (with a mean pK_a of 9.41), the van der Waals volume (mean 225 $Å^3$) and a low logP value (mean: −2.99). The significance of four other descriptors (neutral character, presence and the pK_a of an α-carboxyl group, and the presence of a modified guanidino group) is much lower. Similar results were obtained for the hCAT-1 cell line, but the significance of the descriptors was slightly different. The L-configuration, van der Waals volume, the low logP value and the length of aliphatic side chain were the most significant, while the pK_a value of the side chain (mean pK_a = 11.6) was found to be more important than that of the α-amino group. In addition, the general similarity to L-arginine, the presence of an amino group in the terminal position of the side chain (Orn, Lys) and the basic character were significant descriptors, while the significance of the acidity is negligibly low. As a final conclusion, the following descriptors were found to be important generally for the cationic transporters: the van der Waals volume, hydrophobicity (log P); L-configuration; the size of the side chain; the general similarity to L-arginine; the presence of an α-amino group; the general basicity of the molecule; the pK_a values of the α-amino group (in macrophages) or that of the side chain (in CAT-1 cells). These descriptors can be regarded as the general structurally important binding characteristics of the cationic amino transporters.

Keywords Arginine · Cationic amino acid transporter · Binding specificity · Inhibition · Quantitative structure–activity relationship

Abbreviations

ANN	Artificial neural network
ANOVA	Analysis of variances between groups
CAT	Cationic amino acid transporter
HBSS	Hank's buffered salt solution
NOS	Nitric oxide synthase
PBS	Phosphate buffered saline
QSAR	Quantitative structure–activity relationship

Introduction

L-arginine can be metabolized in various biochemical pathways in mammalian cells. The most important routes are catalysed by arginase and nitric oxide synthase (NOS) isoenzymes leading to the production of L-ornithine/urea and L-citrulline/NO. Both of these enzymes are expressed and are functional in rodent macrophages, particularly in mouse peritoneal cells (Currie 1978; Moncada et al. 1991).

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The substrate and inhibitor specificity of these enzymes have been studied and characterized (Marletta 1994; Hrabák et al. 1994a, b; Hey et al. 1997). In order to metabolize this amino acid, it must be taken up by the macrophages because the recycling of arginine from L-citrulline cannot cover the substrate requirement for arginase or NOS (Baydoun et al. 1994; Hrabák et al. 1994a, b). The kinetic properties of the cationic amino acid transporters have been characterized (White 1985; Bogle et al. 1992; Baydoun et al. 1994) and three genes encoding the four CAT proteins (CAT-1, CAT-2A and B, CAT-3) were described (MacLeod and Kakuda 1996; Closs et al. 2006). These isoforms exhibit y^+-transporter activity (i.e. uptake of basic amino acids), described originally by White (1985). In macrophages, the CAT-2 transporter is responsible for the uptake of arginine and other cationic amino acids (Kakuda et al. 1999; Nicholson et al. 2001), while CAT-1 expression may be increased in certain cases as in a BCG-strain infection (Talaue et al. 2006). Numerous investigations were performed to study the inhibitory effect of NOS inhibitors (Closs et al. 1997; Baydoun and Mann 1994; Schmidt et al. 1994) and other amino acids on the y^+ (CAT) transporters. The inducing effect of LPS in macrophages (Sato et al. 1992) and that of cytokines and insulin in cardiac myocytes (Simmons et al. 1996) has also been demonstrated. However, a systematic comparison of the substrate and inhibitor specificity of the arginine transport system and that of the arginyl-tRNA synthetase enzyme characterized by the incorporation of arginine into proteins has not been performed yet.

Mouse peritoneal macrophages were selected as model cells because at least four arginine-utilizing pathways can be found in them: NO synthase, arginase, arginyl-tRNA synthetase (for protein synthesis) and L-arginine transporter. Therefore, a considerable amount of arginine is required for their functions. The aim of the present study was to test the influence of numerous amino acids and derivatives on the uptake of L-arginine and its incorporation into proteins and to gain information from the comparison of the most important binding requirements of these two systems (i.e. the transporter and arginyl-tRNA synthetase) utilizing L-arginine by the QSAR method. In addition, while macrophages like other cells contain mixed cationic transport systems, a cell line overexpressing the CAT-1 transporter has also been studied.

Materials and methods

Isolation of mouse peritoneal macrophages and CAT-1 cell cultures

CD-CR-1 outbred male mice (purchased from Charles River Hungary) were injected i.p. with 2 ml dephosphorylated casein solution to provoke a high number of elicited macrophages in the peritoneal cavity (Hrabák et al. 1994a, b). After 4 days, mice were killed and peritoneal exudate cells were removed with 15 ml chilled Ca–Mg free Hank's buffered solution (HBSS). Cells were pelleted at 800*g* by centrifugation and resuspended in complete HBSS. Macrophages were separated from other cells through their adherence to 96-well plates (120 min, 37 C, 5% CO_2 atmosphere). Non-adherent cells were then removed and macrophages were cultured further according to the purpose of the experiments.

The CAT-1 cell line was a generous gift from Prof. E. I. Closs' laboratory (Mainz, Germany). U373MG glioblastoma cells were transfected with an expression plasmid encoding a fusion protein of the human CAT-1 and the enhanced green fluorescent protein (Wolf et al. 2002). Overexpression of the hCAT-1 gene makes it possible to study this cationic transporter specifically. Cells were cultured in IMDM Glutamax media containing 10% FBS and 1% antibiotic-antimycotic solution (Invitrogen).

Measurement of the uptake of L-arginine

Incubation times for arginine uptake were selected according to a previous experiment to test the maximum extent of the linear range (data not shown). The uptake of Arg was linear until 120 s in macrophages, and until 60 s in CAT-1 cells; therefore, these incubation times were used in subsequent assays.

Adhered, casein-elicited peritoneal macrophages (10^6) were cultured in 48-well plates (Greiner) for 24 h in RPMI-5% FBS for the induction of NO synthase and arginase. Then media were removed and cells were washed twice with HBSS containing 200 µM L-arginine. Assays were started by adding 50 µl of the same solution containing ^{3}H-labelled L-arginine (200 MBq/ml, final sp. act. 10 kBq/nmole) and the studied inhibitors for 2 min. In kinetic experiments, the final concentration of labelled arginine varied between 50 and 400 µM, but the final volumes were kept at 100 µl. Uptake was stopped by removing the labelled Arg and cells were immediately transferred on ice and washed thrice with ice-cold HBSS (Rotmann et al. 2004). Then cells were lysed in 240 µl 0.5 N NaOH (30 min), neutralized with 0.5 N HCl and the radioactivity of 200-µl aliquots was measured in a toluene-based scintillation mixture in a Packard TriKarb liquid scintillation spectrophotometer. Protein contents of samples were also determined by Bradford's Coomassie Brillant Blue method (Bradford 1976). Background (0 s) labelling was measured by adding at first ice-cold HBSS, then the labelled Arg, removed immediately and washed again three times with ice-cold HBSS.

The uptake into CAT-1 cells has been performed in the same way with minor differences: 5×10^4 cells/well adhered onto 48 multiwell plates, and the incubation time was 60 s instead of 2 min (Rotmann et al. 2004).

Evaluation of the inhibitory effect of tested compounds on L-arginine uptake

Since we tested a high number of amino acids and derivatives, detailed kinetic studies were performed only for a few effectors among the most efficient compounds. The purpose of this test was to present evidence that the mechanism of inhibition is similar for different effectors.

IC_{50} values were determined for each compound using the dose-response curve. IC_{50} value is the concentration causing 50% inhibition, which seems to be sufficient for a comparative study, indicating that the mechanism of the inhibition was similar in case of all tested compounds: an almost entirely competitive inhibition (see Results).

Design and validation of the QSAR model

The 79 compounds were categorized into three groups according to their IC_{50} values in both cell types (see Table 1). The datasets were divided into two–two main groups, providing the model-building sets (MBS, 60 molecules), and the external validation sets (EVS, 19 molecules). The corresponding sets contained the same molecules in the case of both targets, possibly with different biological effect. The MBS's were used to build the model while the EVS's were used to validate the obtained models (these molecules were not used in the model building). The EVS's have been selected randomly and contained the following compounds: *N*-iminoethyl-L-ornithine (NIO) and -lysine (NIL) as strong effectors, L-isoleucine, L-glutamine, L-norvaline, tryptamine, L-norleucine, L-threonine, indole, L-serine, phenylethylamine, L-threonine, *S*-ethyl-isothiourea, indole, aspartic acid, proline, taurine, L-serine, ε-aminocaproic acid, indole-3-lactic acid, γ-guanidinobutyric acid *N*-methyl-L-leucine as medium effectors or non-efficient compounds.

Table 1 Division of the tested compounds into three groups in the function of their efficiency

Group symbol	Effect	IC_{50} (mM)	Number of compounds
Macrophages			
0	No	>10	47
1	Medium	3–10	25
2	Strong	<3	7
CAT-1 cells			
0	No	>10	48
1	Medium	3–10	23
2	Strong	<3	8

Most of the tested compounds (70) have the same effects on both cell types; exceptions see in Table 3

Twenty-four molecular parameters (descriptors), which were thought to have some influence on the biological effect were calculated. Certain descriptors were calculated by the MOE program (Table 2). Ionization constants (pK_a) were calculated from the structures by the pK_a calculation module of ACD/Labs ver. 6.0. Lipophilicity (represented by logP values) of the molecules was estimated by three different methods (Labute 1998; Wildman and Crippen 1999; Erős et al. 2002). We let the automatic descriptor selection algorithm to select the most predictive descriptor, while the same or similar molecular property was calculated by different methods (e.g. logP). The descriptors are summarized in Table 2.

Statistical methods

IC_{50} values were determined for each amino acid at least in three experiments and their mean values were used. Results are expressed as average values with standard error of mean. The significance of differences was evaluated by ANOVA and by Mann–Whitney *t* test when only two values were compared.

Results

Kinetic studies on the uptake of L-arginine and selected inhibitors in macrophages

The K_t value (equivalent to the K_M of enzymes) for L-arginine uptake was found to amount 148 μM. In mouse macrophages, four amino acids, N^G-methyl-L-arginine and L-ornithine (basic character) and L-norleucine and L-methionine (neutral aliphatic) were identified as almost purely competitive inhibitors (Fig. 1). This suggests that the observed inhibitory effects may be due to the influence of the tested compounds on the binding of arginine.

Attempts were also made to determine the K_M value for the incorporation of L-arginine into proteins (60 min; radioactivity measured in the acid-precipitable fraction) as a parameter of the arginine-activating enzyme in protein synthesis and to identify the character of its inhibition by various amino acids. However, neither basic nor other amino acids and derivatives could inhibit the incorporation of L-arginine into proteins at all (data not shown).

Comparative test of various amino acids and derivatives on the uptake of L-arginine into macrophages

Numerous amino acids and derivatives were tested to determine whether they can modulate the uptake of

Table 2 List and explanation of calculated descriptors

Descriptor group	Descriptor code	Comment	Reference
Calculated physico-chemical parameters	PKA_O	Calculated pK_a value of the carboxyl group (value is set to 20, if not applicable)	ACD/Labs ver. 6.0 (2002)
	PKA_N	Calculated pK_a value of the α-amino group (value is set to 20, if not applicable)	
	PKA_SIDE	Calculated pK_a value of the side-chain basic group (value is set to 20, if not applicable)	
	3DNET4 W_logP	Calculated logP by the 3DNET4 W program	Erős et al.
	LogP(o/w)	Calculated logP values by the MOE program	MOE 2006.08
	SlogP		MOE 2006.08
Size descriptors	vdw_vol	van der Waals volume calculated using a connection table approximation	MOE, Labute
	Vol	van der Waals volume calculated using a grid approximation	MOE, Wildman
	C_NUM	Number of carbon atoms in the side chain	
	C_LEN	Length of the side chain (carbon atoms)	
Similarity score	Similarity	Similarity score (Tanimoto similarity) to L-arginine. Calculations are based on molecular fingerprints.	
Indicator variables	ALIF	1 if molecule is aliphatic, 0 if aromatic	
	CARBOX	1 if α-carboxyl group is present, 0 otherwise	
	AAM	1 if α-amino group is present, 0 otherwise	
	AOH	1 if α-hydroxyl group is present, 0 otherwise	
	GUA	1 if guanidino group is present in the side chain, 0 otherwise	
	GUA_MOD	1 if modified guanidino group (e.g. nitro-guanidino) is present in the side chain, 0 otherwise	
	TERM_AM	1 if amino group is present in the side chain, 0 otherwise	
	Acid	1 if molecule is acidic, 0 otherwise	
	Base	1 if molecule is basic, 0 otherwise	
	Neutral	1 if molecule is neutral, 0 otherwise	
	AAA	1 if molecule is α-amino acid, 0 otherwise	
	D	1 if the chirality of α-carbon is D, 0.5 if racemic, 0 if chirality is L or molecule is achiral	
	L	1 if the chirality of α-carbon is L, 0.5 if racemic, 0 if chirality is D or molecule is achiral	

arginine. The efficiency of tested compounds was characterized by their IC_{50} values (the calculated concentrations for 50% inhibition, Fig. 2, Table 3). The most efficient inhibitors were *N*-iminoethyl-L-lysine, *N*-iminoethyl-L-ornithine, L-homoarginine, N^G-methyl-L-arginine, L-canavanine, L-ornithine and L-lysine, their IC_{50} values varied between 0.5 and 3 mM. The next group (IC_{50} between 3 and 10 mM) consisted of 25 mainly neutral apolar amino acids and arginine analogues, for example L-histidine, L-citrulline, L-glutamine, tryptamine, L-norvaline, L-norleucine, L-methionine, L-valine, L-leucine and isoleucine. Most of the tested amino acids and derivatives (n = 47) were considered inefficient, since their IC_{50} values exceeded 10 mM. A surprising observation was the weak, but reproducible inhibitory effect of some tested D-amino acids, namely D-arginine, D-lysine, D-ornithine (in macrophage its IC_{50} value was over 10 mM) and D-leucine.

Comparative test of various amino acids and derivatives on the uptake of L-arginine into CAT-1 cells

Since the kinetic properties of CAT-1 cells were known (K_t = 150 μM), 200 μM of final arginine concentrations was used for comparative experiments. These cells are derived from U373 cells, which express only the CAT-1, CAT-3 and y + LAT2 transporters (Rotmann et al. 2007, 2006). CAT-3 transporter was originally identified and cloned as a brain-specific cationic amino acid transporter (Hosokawa et al. 1997; Ito and Groudine 1997). We compared the arginine uptake of non-transfected U373 MG

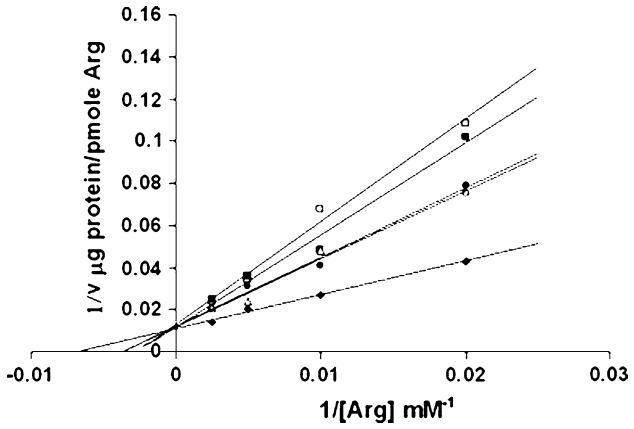

Fig. 1 Kinetics of the inhibition of L-arginine transport into macrophages. 10^6 peritoneal cells were adhered onto 96-well plates and tested for inhibition in 200 µl HBSS during for 2 min. For other details, see Methods. A double-reciprocal plot is used for the determination of K_t and V_{max} values. Uptake was expressed in pmole per µg cellular protein unit; its reciprocal value is shown on the vertical axis. The inhibitory effects of 2 mM N^G-methyl-L-arginine (*open square*), 5 mM L-methionine (*filled circle*), 2 mM L-ornithine (*filled square*) and 5 mM L-norvaline (*open circle*) are shown. Control (non-inhibited): *filled diamond*

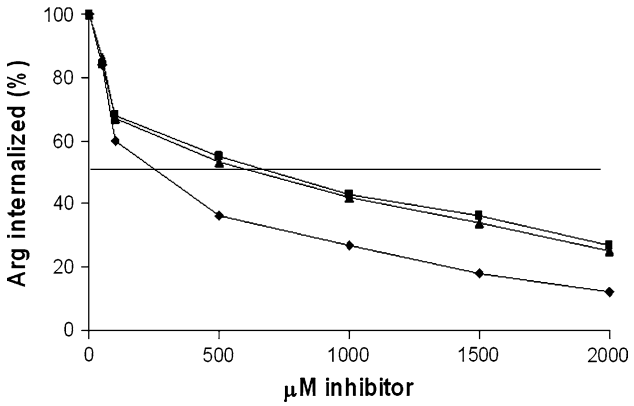

Fig. 2 Determination of the IC_{50} values of the inhibitors. 10^6 peritoneal cells were adhered onto 96-well plates and were tested for inhibition in 200 µl HBSS for 2 min. For other details, see "Materials and methods". The concentration dependence of three efficient inhibitors is shown: N^G-methyl-L-arginine (*filled diamond*), 1 mM *N*-iminoethyl-L-lysine (*filled square*) and 2 mM L-canavanine (*filled circle*). The IC_{50} value of each tested compound was determined in this manner

and CAT-1 transfected cells: the latter cell line internalized almost four times more L-arginine than U373 cells (3.94 vs. 1.02 pmoles per µg cellular protein); supposing that the endogenous transporters are present in 1:1 proportion, 90% of the total uptake may be due to the CAT-1 transporter in transfected cells. Therefore, the arginine uptake of CAT-1 cells can be considered as CAT-1 specific. The effects of the tested compounds were similar to those on macrophages, with smaller differences. The most efficient inhibitors were again *N*-iminoethyl-L-lysine, *N*-iminoethyl-L-ornithine, L-homoarginine, N^G-methyl-L-arginine, L-canavanine, L-ornithine, L-lysine and, in addition, putrescine; their IC_{50} values varied from 0.1 to 2 mM, markedly lower than in macrophages. The next group (IC_{50} between 3 and 10 mM) consisted of 23 compounds, mainly neutral apolar amino acids and arginine analogues, for example L-histidine, L-citrulline, L-glutamine, tryptamine, L-norvaline, L-norleucine, L-methionine, L-valine, L-leucine and isoleucine—the same compounds as in macrophages, but their IC_{50} values were slightly higher. Most of the tested amino acids and derivatives (n = 48) were considered inefficient, showing IC_{50} values exceeding 10 mM. A weak inhibitory effect of the D-analogs of certain amino acids has been observed, too.

Analysis of the characteristic properties of efficient compounds

The efficient compounds displayed an almost competitive inhibition with a weakly mixed character. None of them was found to be a non-competitive inhibitor. These results prompted us to perform a comparative analysis to define the most characteristic structural features for the efficient inhibition.

The datasets were analysed with our in-house developed software, 3DNET4 W (Vichem Ltd., 2002). This program was designed to automatically select the descriptors needed for the optimal structure-property (or structure–biological activity) model. The MBS's were used to build the model and the EVS's to validate the obtained model. In the cyclic-iterative model optimization process, the MBS's were further split randomly and repeatedly (three times) in two halves yielding the training (TS) and monitoring (MS) sets. The former was used to fit the actual model and the latter to control the predictive ability of that model.

We used the artificial neural network (ANN) method to build the models. Automatic variable subset selection was performed with a genetic algorithm. The aim of the optimization was to improve repeated evaluation statistics (cross-validated correlation coefficient, Q^2) of the predictions of the MS and effective descriptor scoring functions were used to facilitate quick generation of the final ANN models. The optimizations were stopped when any change in the descriptor set of the given model decreased the average Q^2 of the cross validations for that model. This way, the program selected an ANN model with 15 variables and 3 hidden neurons for the macrophage assay. The 15 descriptors are vdw_vol, logP, L, PKA_N, similarity, BASE, C_NUM, C_LEN, AAA, ALIF, AAM, NEUTR, CARBOX, PKA_O, and GUA_MOD, listed according to their weights in the model.

The same method was used for CAT-1 cells. In this case, an ANN model with 10 variables and 12 hidden neurons

Table 3 IC_{50} values for tested efficient amino acids and derivatives

Inhibitor	Arg uptake IC_{50} mM—macrophage	Arg uptake IC_{50} mM—CAT-1	$IC_{50} > 10$ mM for both cell types
N-iminoethyl-L-lysine	0.72	0.32	N^G-amino-L-arginine
N-iminoethyl-L-ornithine	2.84	1.05	N^G-nitro-L-arginine
N^G-methyl-L-arginine	0.36	0.32	α-hydroxyvaleric acid
L-canavanine	0.68	0.75	*N*-acetyl-L-arginine
L-homoarginine	0.36	0.48	N^G-tosyl-L-arginine
L-ornithine	0.84	1.25	*N*-methyl-L-leucine
L-lysine	1.8	0.88	Indole
Putrescine	>10	1.84	Glycine
L-glutamine	6.8	8.6	L-glutamic acid
L-leucine	4.4	8.3	L-threonine
L-isoleucine	4.3	6.8	L-proline
L-valine	7.3	8.9	L-phenylalanine
L-valinol	7.5	> 10	L-tyrosine
L-methionine	3.2	> 10	L-aspartic acid
2-amino-L-butyric acid	4.8	8.4	L-cysteic acid
L-norvaline	3.8	6.8	*N*-benzoyl-L-valine
L-norleucine	4.2	7.4	γ-guanidinobutyric acid
L-cysteine	6.5	9.2	β-guanidinopropionic acid
L-homocystein	8.4	5.3	Taurine
L-histidine	5.5	3.6	β-alanine
D-arginine	9.6	8.6	γ-aminobutyric acid
L-alanine	7.2	10.0	δ-aminovaleric acid
D-lysine	9.8	9.4	ε-aminocaproic acid
L-asparagine	10.0	7.5	Imidazole
L-tryptophan	8.4	8.4	Imidazole-3-lactic acid
Phenyl-ethyl-amine	9.4	>10	Histamine
L-citrulline	8.4	>10	L-histidinol
Tryptamine	3.5	3.5	L-homophenylalanine
Serotonin	9.3	4.8	AETU
L-argininic acid	>10	9.8	*S*-methyl-isothiourea
Agmatine	4.8	4.8	*S*-ethyl-isothiourea
5-hydroxy-tryptophan	10.0	10.0	Creatine
D-leucine	9.6	>10	Thiourea
L-argininamide	9.6	9.6	L-thiocitrulline
D-ornithine	>10	9.6	Guanidine
L-serine	>10	7.3	Aminoguanidine
			Methylguanidine
			Nitroguanidine
			Hydroxyguanidine
			L-tryptophol
			DOPA
			Tyramine
			Indole-3-lactic acid

were selected. The majority of the ten descriptors were identical with that of macrophages and listed here according to their weights in the model: vdw_vol, logP, C_LEN, pKA_SIDE, L, AAA, similarity, BASE, TERM_AM, and ACID. Three descriptors, pKA_SIDE and two of the lowest significance (TERM_AM, ACID) were not

selected for macrophages, while seven descriptors of macrophages (pKA_N and the six least significant ones) were not selected for CAT-1.

When their values were calculated, the highest significances between strong efficient compounds and others were found for the pK_a value of the side chain, the number of carbon atoms in the side chain and the similarity. The logP and van der Waals volume values of ineffective compounds were also significantly different (Table 4). Among the non-numerical descriptors, the L-configuration, the basicity of the whole compound and the presence of α-amino groups were also essential for a strong binding. Interestingly, certain non-essential descriptors or their absence were also characteristic of the strong binding. The absence of D-configuration and of α-hydroxyl groups belongs to these descriptors. In addition, a significant difference was detected between the strong binding and medium and/or ineffective binding for the clogP and slogP and the whole volume of the molecule (Table 5).

Finally, the predictive capability of the obtained models was tested by predicting the biological data of the external validation set molecules. Table 6 shows that the models predicted the biological data quite acceptably for both cell types.

In the case of macrophages, three compounds of 19-member EVS were only predicted incorrectly. The inefficient L-serine and L-threonine were considered medium-efficient, while medium-efficient phenylethylamine was regarded to be inefficient. As an explanation, the size of the two hydroxyl amino acids is very similar to medium-efficient aliphatic amino acids and their IC_{50} values exceed the 10-mM efficiency limit only slightly (12 mM for Ser and 10.8 mM for Thr). The unfavorable effect of the presence of hydroxyl groups has also been found for l-argininic acid, α-hydroxyvalerate, or indole-3-lactate. Phenylethylamine had also an IC_{50} value near the limit for inefficiency as a possible reason for the difference between calculated and measured values.

In the case of CAT-1 cells the prediction was similarly good. Three compounds were predicted incorrectly: threonine (as for macrophages), indole and L-norleucine. For norleucine, a possible explanation is the higher significance of the basic pK_a of the side chain in CAT-1 cells compared to macrophages: NorLeu contains carbon atoms in terminal position instead of N-containing groups.

Table 4 List of the important descriptors selected by the ANN method

Descriptors	Strong effect	Medium effect	Ineffective
pKA_SIDE*** (C)	11.59±0.84	18.02±0.54	14.48±0.70
pKA_N** (M)	9.41±0.13	10.38±0.54	14.29±0.80
pKA_O* (M)	2.44±0.05	6.33±1.43	9.81±1.26
C_NUM*** (M)	4.43±0.20	2.81±0.21	2.13±0.23
C_LEN***	4.43±0.20	2.63±0.20	2.09±0.23
Similarity**	0.79±0.04	0.50±0.04	0.47±0.04
vdW_vol*	225±12	191±8	173±10
logP (o/w)*	−2.99±0.15	−1.60±0.39	−1.24±0.31
L-config	7/7	20/25 (23)	23/47 (48)
D-config	0/7	5/25 (23)	0/47 (48)
BASE	7/7 (8)	12/25 (23)	16/47 (48)
NEUTR (M)	0/7	17/25	21/47
ACID (C)	0/7	0/(23)	6/(48)
AAA	7/7 (8)	20/25 (23)	15/47 (48)
AAM (M)	7/7	16/25	26/47
ALIF (M)	7/7	18/25	34/47
CARBOX (M)	7/7	21/25	28/47
GUA_MOD (M)	4/7	1/25	13/47
TERM_AM (C)	2/7 (8)	8/(23)	13/48

Mean values ± SEM are shown where they could be calculated. For other descriptors the number of positive cases per total number is shown

C characteristic descriptor only in CAT-1 cells

M characteristic descriptor only in macrophages

Strong: $n = 7$ (8 for CAT-1), medium, $n = 25$ (23), ineffective, $n = 47$ (48)

pKA_SIDE values were considered as 20 if not applicable (i.e. no side chain)

vdW values are given in $Å^3$

Bonferroni post-tests show significant differences between the following pairs

pKA_SIDE: strong-medium ($P < 0.01$) and strong-ineffective ($P < 0.01$)

C_NUM: strong-medium ($P < 0.01$) and strong-ineffective ($P < 0.01$)

Similarity: strong-medium ($P < 0.05$) and strong-ineffective ($P < 0.01$)

logP: medium-ineffective ($P < 0,05$)

vdW_vol: strong-ineffective ($P < 0.05$)

Discussion

The uptake of arginine is an important step to provide the substrate for the arginine-utilizing systems in mammalian cells. These systems, the arginase and NO synthase metabolic routes and the protein synthesis, being together in the same cell, may establish an equilibrated system, which can be termed as a metabolom. The arginine demand of macrophages is high, due to their intensive arginine metabolism and this amino acid must be taken up from the environment of the cell (Baydoun et al. 1994; Hrabák et al. 1994a, b; Closs et al. 2000; Nicholson et al. 2001). Similar results were obtained for the NOS II-produced NO in astrocytes (Manner et al. 2003). Recently, the role of Th1 and Th2 cytokines in the induction of CAT-2 system in various cells has also been observed (Flodstrom et al. 1999; Martin et al.

Table 5 List of other descriptors

Descriptors	Strong effect	Medium effect	Ineffective
SlogP*	−0.75±0.24	−0.04±0.20	−0.60±0.13
ClogP	−2.83±0.17	−1.79±0.27	−2.27±0.21
vol	167±8	141±6	129±7
AOH	0/7	1/27	3/45
GUA	1/7	1/27	7/45

Mean values ± SEM are shown where they could be calculated. For other descriptors the number of positive cases per total number is shown

Strong: $n = 7$, medium, $n = 27$, ineffective, $n = 45$

pKA values were considered as 20 if not applicable (i.e. no side chain)

vol values are given in $Å^3$

Symbols: $*P < 0.05$; $P < 0.01$; $P < 0.001$ by ANOVA

Bonferroni post-tests show significant differences between the following pairs

pKN: strong-medium ($P < 0.05$) and strong-ineffective ($P < 0.01$)

pKO: strong-ineffective ($P < 0.05$)

C_LEN: strong-medium ($P < 0.01$) and strong-ineffective ($P < 0.01$), medium-ineffective ($P < 0.05$)

Table 6 The validation of the obtained model using the external validation set

	Experimental results		
	0	1	2
For macrophages			
Calculation			
0	9	1	0
1	2	5	0
2	0	0	2
For CAT-1 cells			
Calculation			
0	9	1	0
1	2	5	0
2	0	0	2

2006; Yeramian et al. 2006). Therefore, the arginine transporter is an essential element of this complex arginine-utilizing system.

The amino acid transporters are membrane-bound proteins with a limited specificity, that is they can transport amino acids, although their affinity for various amino acids is different. Arginine, together with other basic amino acids, is transported mainly by the system y^+, or the cationic amino acid transporters. The most important properties of y^+-transporter in mammalian cells were summarized in the paper of White (1985). After the discovery of NO synthesis in various mammalian cells, these transporters were tested more deeply in endothelial cells and macrophages (Closs et al. 2000). Three distinct CAT genes were identified earlier in mammalian cells (Closs 1996; MacLeod and Kakuda 1996), which can code four proteins (CAT-1, CAT-2A and B, CAT3, Closs et al. 2004) and the preferential expression of the CAT-3 system in human peripheral tissues has been described (Vekony et al. 2001). The definitive role of the CAT-2 transporter in arginine transport was proved in macrophages (Kakuda et al. 1999). Kinetic studies revealed that the K_t value of CAT systems is 100–200 μM, and the transporter can bind and consequently is inhibited by some NOS inhibitors such as *N*-methyl- L-arginine or L-canavanine (Baydoun and Mann 1994; Closs et al. 1997). The arginine transport is also inhibited by some neutral amino acids in a non-competitive way; this may partly be due to the involvement of other transporters in the uptake of arginine (White 1985). Baydoun et al. (1994) found that the transport of L-arginine and L-citrulline into macrophages is mediated by different transporters.

Both basic and other inhibitory amino acids showed an almost completely competitive inhibition, although the inhibitory effect of cationic compounds was more profound. Based on these experiments, we launched a systematic study for the identification of the essential binding properties of various amino acids and derivatives. Their inhibitory effect on the L-arginine transport is considered as the biological activity. The database containing the IC_{50} values of 79 different amino acids and derivatives has been analysed by a QSAR method using numerous descriptors.

The QSAR study helped us to select the most definitive structural properties in arginine binding. Characteristic descriptors of strong inhibitors of the transporter were the following: L-configuration, overall basicity, the presence of α-amino groups, and the optimal values of pK_a of the α-amino group (~9.4) and the pK_a (~11.6) and the number of carbon atoms in the side chain (4 or 5), the similarity to L-arginine (>0.79), logP values (−2.99) and van der Waals volume (225 $Å^3$). The hydrophobicity and the size of the molecule were also calculated by other methods, but logP (o/w) and van der Waals volume were chosen as the best descriptors that fit into the model built by ANN method. Lipophilicity can be expressed as logP, which is defined as the negative logarithm of the distribution coefficient of a compound between an organic solvent and water; thus, a high logP denotes a high level of lipophilicity. Interestingly, the SlogP and 3DNET3W_logP values were not changed continuously from the strong to inefficient compounds: a maximal value was found for the medium-efficient group (−0.04 ± 0.20, Table 5). This finding is in good accordance with the observation of Hansch et al. (1987): there is no linear relationship between the central effect of a compound and log P; instead, this relationship is parabolic. For this

reason, Hansch et al. (1987) defined the principle of "optimal hydrophobicity". However, the genetic algorithm selected logP (o/w) instead of these descriptors, which changed continuously between the group.

If the values of these descriptors are compared for the strong, medium and ineffective effectors, the most important properties are the following: L-configuration (the least frequent in inefficient group), the number of carbon atoms in the side chain, the van der Waals volume (highest in strong, lower in medium and the lowest in the inefficient group for both descriptors), and the presence of α-amino groups (the least frequent in the inefficient group).

The overall basicity, the pK_a value of the side chain, the similarity and the hydrophobicity were less important in the binding. This is the reason why neutral, aliphatic, hydrophobic amino acids and basic, but not arginine-related derivatives can also be bound to the cationic amino acid transporter even if by weaker linkages.

The differences between the macrophages and CAT-1 cells may be due to the different expression of the cationic transporters: macrophages express y + L transporter (Martin et al. 2006) and, in addition, the differential expression of CAT-1 and CAT-2B in the two cell types may also be responsible for the observed differences.

Comparing these properties to the binding to arginase (Hrabák et al. 1994a, b) and to NO synthase (Hrabák et al. 1996), numerous properties are identical or similar. The L-configuration, the size of the molecule, the presence of the α-amino and α-carboxyl groups were essential for the binding to both enzymes. Nevertheless, the lack of guanidino group for arginase binding and a slight tolerance of NOS II to the length of carbon side chain are different in the enzyme-binding sites, while these properties are characteristic of arginine transporters. For the overall basicity and pK_a of the side chain, most of strong inhibitors of the transporters are similar to that of NOS while medium inhibitors are similar to that of arginase. Similarity and various logP values were not calculated for the arginase and NOS inhibition. The similarity of the binding to NOS and arginine transporter(s) has also been observed by other investigators (Inoue et al. 1993).

Binding studies for the arginine-activating system in protein synthesis were also attempted without any significant result: there was no amino acid or derivative which could efficiently inhibit the incorporation of arginine into proteins. This inefficiency may be explained by the enormous specificity of the amino acid activation: K_M values of aminoacyl-tRNA synthetases are lower by a 10^4 factor for the corresponding amino acids compared to any other amino acid (Lehninger et al. 1993). This is necessary to prevent the erroneous amino acyl-tRNA formation which may not be corrected later resulting in false sequences in proteins.

We conclude that the transport of L-arginine into mouse macrophages may be inhibited by various amino acids and derivatives. The QSAR analysis of these inhibitors has revealed 15 and 10 important descriptors (for macrophages and for CAT-1 cells, respectively) characteristic of the structural properties involved in substrate and inhibitor specificity to the transporter. When these descriptors are compared, 7 + 1 common descriptors have been considered as the generally important binding characteristics of the cationic amino acid transporter: (1) the van der Waals volume of the amino acid; (2) the logP value; (3) the L-configuration; (4) the size of the carbon side chain; (5) the general similarity to L-arginine; (6) the presence of the α-amino group; (7) the general basicity of the molecule; (8) the pK_a value of the α-amino group (in the case of macrophages) and the side chain (in the case of CAT-1 cells).

Acknowledgments The authors wish to express their thanks to dr. Gergely Keszler for the helpful discussion, to Miss Judit Szabó and Mr. Antal Holly for their skillful technical assistance. The contribution of the laboratory of Prof. Ellen I. Closs (University of Mainz, Germany) by providing CAT-1 cells is also highly appreciated. The work was supported by the grants of the Hungarian Ministry of Welfare (ETT 556/2006) and of the National Foundation of Scientific Research (OTKA 043075).

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