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COMPARISON OF SUBSTRATE AND INHIBITOR SPECIFICITY OF ARGINASE AND NITRIC OXIDE (NO) SYNTHASE FOR ARGININE ANALOGUES AND RELATED COMPOUNDS IN MURINE AND RAT MACROPHAGES

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Summary: Arginine utilizing enzymes in macrophages showed different specificities for various arginine analogues and derivatives as substrates and inhibitors.
Isolated arginase was strongly inhibited by L-canavanine(Can) and L-ornithine(Orn) but only slightly by L-homoarginine(Hom) and L-argininamide($ArgNH_2$). These effects were not or only weakly observed when released urea was measured in long term cell cultures. On the other hand, both L-canavanine and L-argininamide were substrates for arginase in long-term cultures. The known inhibitors of NO synthase were ineffective. The mechanisms of inhibition were different for L-canavanine and L-ornithine, but clear mechanisms could not be identified).
NO synthase was studied only in long term cell cultures without purification. Certain N-guanidino (NG)-substituted arginine derivatives caused a marked inhibition while inhibitors of arginase had only slight or no effect. L-homoarginine was also found to be the substrate of NO synthase.
The comparison of these effects of arginine analogues and derivatives made possible a computer-aided approximation for the fitting of active centers of these enzymes to their substrates.

L-arginine(Arg), a proteinogenic amino acid is the substrate of the enzymes arginase(E.C. 3.5.3.1.) and NO synthase(E.C.1.14.23.) [1,2]. NO has various roles in different cell types: it can cause vasodilation (endothelium- or platelet-derived), cytotoxic effect(macrophage or polymorphonuclear-derived) and it is a neurotransmitter(produced in the nervous system)[see ref. 3 for review]. Isoenzymes of NO synthase have been found: macrophages contain mainly the inducible, Ca^{2+}-independent enzyme[4] which is observed only after stimulating the cells by lipopolysaccharide(LPS) or interferon-γ (IFN- γ). This enzyme was isolated, characterized[5] and more recently cloned[6]. Arginase has been found together with NO synthase only in macrophages and related cells. Arginase and NO synthase differ in their affinity to the substrate: NO synthases have K_M values of 0.01-0.1 mM[7] while the K_M values of arginases were found above 1 mM[8].

The substrate specificities are also very different. NO synthase can also use Hom as a substrate which has a longer chain than Arg, but their stereochemical configurations are identical, while D-arginine is not a substrate[9, 10]. N^G-substituted arginine derivatives have been found to be effective inhibitors for NO synthase but their inhibitory effect was different on various NO synthase isoenzymes[3].

The utilization of these analogues in the regulation of various physiological processes may be very important in therapy. N^G-methyl-L-arginine(NMMA) is used as a drug decreasing the formation of

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vasodilatory NO[11]. Similar utilization of these inhibitors can be postulated in the regulation of immune defense where macrophages exert their function by producing cytotoxic NO.

In this study, we compare the actions of various arginine analogues and derivatives on arginase and NO synthase. We have found earlier a high arginase activity in murine and a high NO synthase activity in rat peritoneal macrophages without a previous stimulation by LPS or IFN-γ[12, 13]. For this reason we studied the effects of arginine related compounds on murine urea and rat NO production and on a partially purified macrophage arginase and on a commercially available arginase preparation for comparison. Based on our results, using a computer program we suggest an approximation for the groups of substrate essential for the substrate-enzyme fitting.

MATERIALS AND METHODS

Male CFLP mice(30-35 g) and Wistar rats(120-140 g) purchased from LATI(Gödöllő, Hungary) were injected i.p. with 2 % dephosphorylated casein as described earlier[12]. Peritoneal exudate cells were harvested in Ca^{2+}-Mg^{2+}-free Hanks medium after 96 hrs, centrifuged at 2000 g and suspended in Hanks medium containing Ca and Mg. Cells were then left to adhere in plastic dishes for 60 min at 37°C in 5 % CO_2 atmosphere and adhered cells were considered as macrophages.

Macrophages(10^6 cells in 96 well plastic plates or 5 * 10^6 cells per 35 mm petri dishes, Linbro) were cultured in Dulbecco Minimal Essential Medium(DMEM) free of phenol red and Arg for 24 hrs at 37°C in 5 % CO_2 atmosphere. Supernatants were tested for urea and nitrite production. When inhibitors were tested, Arg-free DMEM was supplemented by 5 mM Arg as substrate and 5 % foetal calf serum(FCS).

Arginase was partially purified from the supernatants of peritoneal macrophage culture after adherence by a heat treatment(60°C for 30 min, 1 mM Mn^{2+})followed by ammonium sulfate precipitation[14].

Arginine hydrolysis by arginase was detected by cation exchange thin layer chromatography (TLC)[15]. Urea production was determined by a colorimetric method[16]. In the presence of Cit another photometric method was used to avoid the interference caused by the ureido group of Cit[17]. NO was measured as nitrite by a modified Griess-Ilosvay reaction [18]. Cell-free controls containing amino acids were measured both for urea and nitrite to detect any spontaneous transformation but it was negligible.

TLC: 10^6 macrophages were cultured in 200 µl Hanks medium containing 5 mM Arg or derivatives for 3 hrs at 37°C. Then the medium was removed, cells were washed two times and 50 µl water was added to disrupt the cells. 25 µl extraxt was chromatographed on Fixion 50x8 ion exchange TLC plates in a citrate buffer pH4.3 containing 0.4 M Na^+[15]. This method is suitable to separate basic amino acids.

Computer studies: The separated compounds were optimized at molecular mechanic level and the calculations were carried out by HyperChem(Autodesk Inc. 1992) package using MM+, with Newton-Raphson optimizer. To find the minimum energy conformers of the previously optimized molecules we used 300 K simulation temperature, run time 0.05 ps, time step 0.001 ps and steps to refresh 1 parametrized molecular dynamic calculation with conserved classical total energy, then we used the highest kinetic energy conformers to start the final geometry optimization. The geometry descriptor parameters, such as dihedral angles and molecular surface data were derived from the final geometries.

RESULTS

The effect of arginine analogues and derivatives on urea production in cultured murine macrophages

In these experiments, several arginine analogues were tested as substrates(5 mM) for urea production in 24 hrs cultures of murine macrophages cultured a: in Arg-free DMEM without FCS or b: in the presence of 5 mM Arg and 5 mM various analogues to test the inhibitory effect of these compounds on urea production characteristic for long-term arginase activity. The compounds studied had no effect on urea formation except Orn which caused a 40 % inhibition (Fig. 1.). Analogues and derivatives were also not substrates for urea production by intracellular arginase except $ArgNH_2$ and Can, but only to a small extent(Table 1).

The effect of arginine analogues and derivatives on NO(nitrite) production in cultured rat macrophages

In these experiments arginine analogues and derivatives were tested as substrates for NO production at 5 mM final concentration in 24 hrs cultures of rat macrophages cultured a: in Arg-free DMEM or b: in the

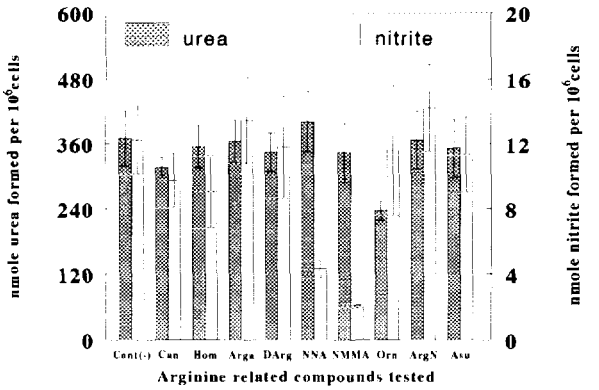

Fig. 1. Urea and nitrite production in murine and rat peritoneal macrophages, respectively, during 24 hrs. 10^6 murine or rat peritoneal macrophages were cultured for 24 hrs in DMEM containing 5 mM Arg and 5 mM of the indicated compounds(and 5% FCS when nitrite production was tested). S.E.M. values were calculated from 5-9 independent experiments.
Cont(-) = control samples without tested analogues or derivatives.

presence of 5 mM Arg and 5 mM analogues and derivatives as inhibitors in media containing 5 % FCS. We found that NMMA and N^G-nitro-L-arginine (NNA), the known inhibitors of NO synthase caused 65-85 % inhibition, while Can and Hom decreased NO_2^- production by approximately 30 %. Hom was also found to be a good substrate(75% activity) in good agreement with other authors [3]. (Fig. 1. and Table 1.)

Direct substrate and inhibitory test of various arginine analogues and derivatives on isolated arginase enzymes

In these experiments, partially purified murine macrophage arginase and commercially available bovine liver arginase(Serva) were investigated. Urea formation during 30 min was used to measure the activities [12]. Arginine analogues were used as substrates at 25 mM concentration instead of Arg, but they cannot replace L-Arg in this function at all(Table 1). Can and $ArgNH_2$ were also tested for 24 hrs, because they proved to be weak substrates in cell cultures. Moreover, 25 mM Arg and 10 mM Arg-analogues were used

Table 1. Arg analogues and derivatives as substrates for arginase and NO synthase

Compound	nmoles per 10^6 cells		Arginase
	Urea released	Nitrite released	mU/ml
-	18.14	1.14	0
L-arginine	456.00	13.51	35.45
D-arginine	20.67	1.35	0
L-homoarginine	14.50	8.49	0
L-canavanine	108.04	1.79	0.86
L-canavanine$^+$			16.81
L-citrulline	66.50	5.70	0
L-argininic acid	8.98	1.79	0
L-argininamide	83.57	6.05	1.19
L-argininamide$^+$			15.35
Agmatine	47.34	1.00	0

Urea and nitrite were determined in the supernatant of cultured macrophages(24 hrs,Arg-free DMEM, completed with the Arg-derivatives). Arginase activities are expressed in nmole released urea per min(mU) per ml enzyme.
Concentrations(final): 5 mM in cell cultures, 25 mM for isolated arginase. Results are the mean values of two independent experiments.
$^+$Arginase activity was measured using a 24 h incubation instead of 30 min.

Table 2. Inhibitory effect of urea cycle intermediates and Arg analogues and derivatives on arginase preparations

Compound added	arginase	
	Macrophage	Commercial
Control	73.12	71.38
D-arginine	67.06	78.69
L-homoarginine	51.64	58.76
L-canavanine	4.82	17.84
L-ornithine	17.85	37.08
L-citrulline	62.85	70.52
L-argininosuccinate	74.58	71.03
L-argininic acid	62.88	68.52
L-argininamide	49.17	76.43
Agmatine	69.46	68.86
N^G-methyl-L-arginine	66.02	79.94
N^G-nitro-L-arginine	64.81	67.16
N^G-tosyl-L-arginine	67.05	78.11
N-α-acetyl-L-arginine	66.15	77.89
N-α-benzoyl-L-arginine	63.67	79.80

Arginase activities are expressed in nmole released urea per ml enzyme per min. Commercial arginase was diluted to give a similar activity as macrophage arginase. Arg concentration 25 mM, inhibitor concentration 10 mM, except Asu(2 mM). Results are the mean values of two independent experiments.

together to test their inhibitory activity. Can caused a very strong inhibition and the inhibiting effect of Orn was also marked. A slight inhibitory effect was found using Hom, $ArgNH_2$ and Cit(Table 2.). Increasing the Arg concentration, the effect of Can could be suspended suggesting a competitive inhibition, while the effect of Orn could not be eliminated by this treatment. Nevertheless, clear mechanisms could not be identified because of a marked inhibition on arginase without inhibitor by the excess substrate(Arg) over 25 mM.

Table 3. Structural data of studied compounds calculated by a computer program

Compound	Total surface A^2	Chain length nm	Dihedral angle degree	Gua surface A^2
L-arginine	201	0.627	114.01	99
D-arginine	204	0.626	112.21	99
L-homoarginine	235	0.749	163.25	99
L-argininic acid	202	0.630	145.45	99
L-canavanine	201	0.505	66.77	96
L-argininamide	209	0.628	120.93	99
L-citrulline	199	0.626	120.93	96
L-argininosuccinate	288	0.614	143.22	184
N^G-methylarginine	227	0.628	110.07	122
N^G-nitroarginine	230	0.620	100.82	124
N^G-tosylarginine	336	0.640	90.99	235
N-α-acetylArg	242	0.635	105.09	99
N-α-benzoylArg	293	0.630	164.50	99
N-iminoethyl-Orn*	217	0.602	119.01	90
N^G-cyclopropyl-Arg*	254	0.624	139.11	127
N^G-allyl-Arg *	262	0.626	137.83	133

Chain length is the carbon chain length between the α-C and N-atoms connected to the 5-C-atom of the arginine chain. Dihedral angle is the angle formed by the triangle bondings of the three substituents located on the α-C and guanidino C-atoms projected onto the plane perpendicularly situated on the axis of the two C-atoms mentioned above(Fig. 2.). Gua surface is the surface of the guanidino group together with its substituents.

*These inhibitors were not tested in our experiments; however,their effect was proved[31].

The uptake and hydrolysis of Arg analogues and derivatives detected by TLC [15]

Both Arg(R_f= 0.11) and Can (R_f= 0.21) entered the cells. After incubating in the presence of trypsin or macrophage supernatants, $ArgNH_2$(R_f= 0) was partly hydrolyzed and as free Arg could be detected by TLC.

Computer-aided approximation of the fitting of the active centers of arginase and NO synthase to the substrate

At the molecular mechanical level of the approximation we calculated the structure of the minimal energy conformers of tested compounds. The characteristics of the molecular geometry are summarized in Table 3

As seen there are no significant differences in the molecular shapes of such closely related compounds like Arg, D-Arg, L-argininic acid(Arga), Can, Cit and $ArgNH_2$. On the contrary, there are significant differences in the substrate and inhibitor properties of these compounds indicating strong geometric and chemical restrictions in the molecular binding properties with respect to both enzymes. A slight qualitative correlation was found between the activity or inhibitory effect and the optimal dihedral angle of the compounds with a non substituted guanidino residue; this can contribute to the differences in their biological activities. A correlation between the size and the binding of certain molecules may also be observed mainly at the guanidino region(Table 3.). The lack of the basic α-amino and the acidic α-carboxyl group makes the binding of the substrate or inhibitor to both enzyme impossible as seen for Arga , agmatine(Agm) or $ArgNH_2$ in short term experiments where the amide group is intact.

DISCUSSION

The understanding of the inhibitory effects on NO production is essential for applying the inhibitors in the therapy. NMMA, the most known inhibitor has been already tested[19-21]. The inhibitory effect of Can on the relaxation of rat aorta cannot be due unambiguously to its effect on NO synthesis[22-24].

In macrophages, NO has a cytotoxic effect[3,5,6,9,]. The influence of macrophage NO synthesis by various drugs may modulate the role of these cells in the immune defense. The arginase and NO synthase pathways are essential in the regulation of the progression or the rejection of the tumor[25]. Considering the simultaneous presence of arginase and NO synthase, mainly in murine macrophages, we tested the inhibitory effect of Arg-analogues and derivatives on urea formation and the activity of isolated arginase.

Based on the experiments, we tried to approximate and compare the binding groups of the arginine substrate for arginase and NO synthase because this may have some importance in the selection of their specific inhibitors. It can be assumed that the active centers are rather similar in arginase and NO synthase in a number of aspects. The α-amino group should be fitted to the binding sites in both enzymes. If N-α-substituted derivatives are applied (N-acetyl-L-arginine and N-benzoyl-L-arginine, abbreviated as AcArg and BenzArg, respectively) the sterical fitting is not possible(Table 3). The essential role of the free α-amino group is supported further by the observation, that Arga, which contains a hydroxyl group instead of the basic α-NH_2 group is neither a substrate nor an inhibitor for these enzymes indicating the importance of the basic character of α-NH_2 group. DArg, having the opposite enantiomeric configuration is also neither a substrate nor an inhibitor for the investigated two enzymes(Fig. 1, Tables 1.,2, ref.[3.]). The α -COOH group may also have a great importance in the binding. Agm, the decarboxylated derivative of Arg is ineffective. $ArgNH_2$, which has a slightly bigger size, causes only a small inhibition in isolated arginase. This compound is not an inhibitor if enzymes were studied in cell cultures (Fig. 1.) but it was a weak substrate of both enzymes under the same conditions. For isolated arginase (Table 1) $ArgNH_2$ is not a substrate unless it

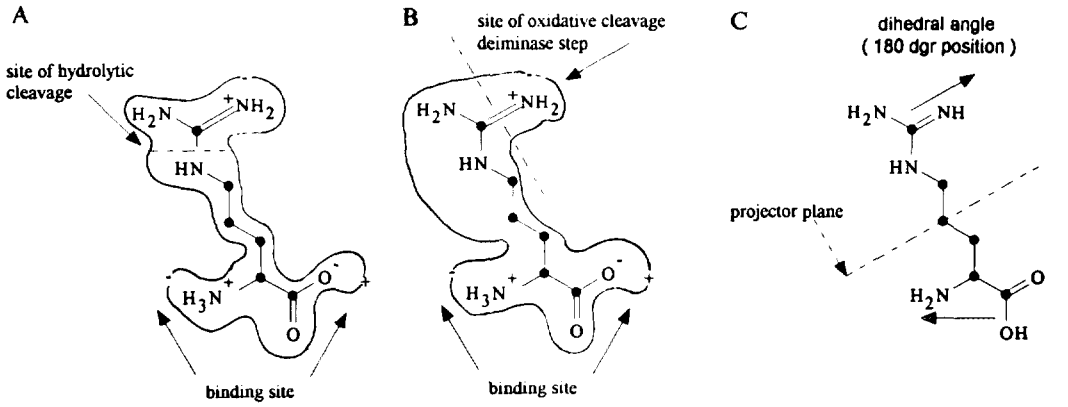

Fig. 2. Hypothetic fitting of arginase(A) and NO synthase(B) onto their substrates. The principle of the formation of dihedral angle is shown on L-arginine(C).

is incubated for a long term due to the presence of proteases in cell cultures splitting the amide from Arg .

The guanidino group of Arg should be accessible for the catalytic sites of both enzymes. In this aspect, the two enzymes have different fitting properties. Can is a strong, probably competitive inhibitor for isolated arginase(Table 2) and a weaker inhibitor of NO synthase (Fig. 1.). N^G-substituted Arg-derivatives as NMMA and NNA are potent inhibitors of NO synthase in accordance with other authors[3] but have no such effect on arginase. N-tosyl-L-arginine(NTA) and argininosuccinic acid(Asu) were not inhibitors at all. This difference may be explained by the extremely big size of the guanidino region of the latter derivatives which makes impossible their fitting to the catalytic site. On the basis of these experiments, the surface of this binding region should be below 180 A^2 while the total surface must not exceed 280 A^2 . Hom(total surface 235 A^2) is a good substrate and weak inhibitor of NO synthase but a very weak inhibitor and no substrate for arginase (Fig. 1., Tables 1, 2).

Considering the sizes of these molecules calculated by our computer program, we suppose that the fitting of the compounds in the guanidino region is much looser in the case of NO synthase. This enzyme can tolerate a longer carbon chain in the substrates(Hom, refs. [23,26]) or a not extremely big substituted form of guanidino group in the inhibitors although the latter ones cause the change of the basicity and they are only inhibitors, but not substrates. On the other hand, arginase should require a tight fitting: both the longer carbon chain(Hom) and even the relatively smaller guanidino substituents prevent or decrease the effect of these compounds. The only analogue which meets the requirements is Can: its only difference compared to Arg is an oxygen replacing a $-CH_2-$ group. This should explain the observation that Can is a "weak substrate" and a strong inhibitor of arginase(Fig. 1., Table 2.). The differences of its effect on isolated enzyme and cell culture can be explained by the difference of the incubation periods(Table 1). The lack of its effect was not due to the difficulties in its transport as evidenced by TLC. It is noteworthy that Cit[27] and $ArgNH_2$ are precursors of Arg in cell cultures and this explains their "weak substrate" property (Table 1.)

Summarizing our results and the data from the available literature the hypothetic structural fitting of the substrate to the active sites of macrophage arginase and NO synthase may be imagined as shown in Fig. 2. Both the basic and acidic characters and the sizes of the substituents of α-carbon atoms are essential including their stereochemical configuration. Computer-aided approximation will be improved so as to give further details to a theoretically supported explanation for the different behavior of various compounds.

These conclusions are valid only for macrophage enzymes. Although, arginases from different sources are similar in their enzymatic properties, however, cloned NO synthases from macrophages or from cerebellum show only a limited homology(51 %) and they are homologous to cytochrome P450 reductase as well[9].

Finally, this computer-aided approximation of the enzyme-substrate binding makes possible the prediction of the behavior of certain arginine analogues or derivatives which is important in pharmacological trials. N-iminoethyl-L-ornithine(NIO), N^G-cyclo-propyl -L-arginine and N^G-allyl-L-arginine should be able to bind strongly to NO synthase(Table 3,*marked compounds) based on their structural data. Indeed, all of them have been found to be NO synthase inhibitors[28]. On the other hand, it is not likely that these latter N^G-substituted Arg-derivatives can act on arginase. The case of NIO is doubtful: its size, surface and dihedral angle should allow the binding, but the decreased base character of its "pseudo-guanidino" group may prevent it. This compound is surely not a substrate of arginase but the inhibitory effect cannot be excluded.

The inhibitory effect of Orn on arginase activity(Fig. 1., Table 2, ref.[29]) suggests that other amino acids not closely related to Arg may also influence the activity of enzymes utilizing L-arginine. The study of the effect of these amino acids is in progress.

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