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COMPARISON OF THE INHIBITORY EFFECT OF ISOTHIOUREA AND MERCAPTO-ALKYLGUANIDINE DERIVATIVES ON THE ALTERNATIVE PATHWAYS OF ARGININE METABOLISM IN MACROPHAGES

András Hrabák[1], Tamás Bajor[1], Garry J. Southan[2], Andrew L. Salzman[2] and Csaba Szabó[2]

[1]Department of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University of Medicine, Budapest, VIII. Puskin u. 9., H-1444 POB 260, Hungary, [2]Children's Hospital Medical Center, Division of Critical Care, 3333 Burnet Avenue, Cincinnatti, Ohio, 45229, USA

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Abstract. Novel, non-arginine based compounds have been identified as potent inhibitors of nitric oxide synthase (NOS). Members of the isothiourea and mercapto-alkylguanidine classes have generated much interest, as some members of these classes show selectivity towards the inducible isoform of NOS (iNOS), which plays a role in inflammation and shock. Here we compared the effect of a number of these compounds as well as L-arginine based NOS inhibitor reference compounds on macrophage-derived and liver arginase and macrophage iNOS activities. From the non-arginine based NOS inhibitors studied only S-aminoethyl-isothiourea (AETU) caused a slight inhibition of arginase activity. This inhibition was kinetically competitive and due to the rearrangement of AETU to mercapto-ethylguanidine (MEG). The weak inhibitory effect of non-arginine based iNOS inhibitors on arginase activity further supports the view that such compounds may be of practical use for inhibition of NO production in cells simultaneously expressing iNOS and arginase.

Key Words: nitric oxide, nitric oxide synthases, isothiourea, mercapto-alkylguanides, macrophages, arginase

Introduction

Nitric oxide (NO) is produced by the oxidation of L-arginine by a family of nitric oxide synthase isoenzymes (1-5). NO produced by the inducible isoform (iNOS) plays a role in the circulatory shock and inflammation (1-4) and the etiology of insulin dependent diabetes mellitus (6). For this reason selective inhibitors of iNOS are of considerable importance. The commonly used inhibitors of NOS are based on L-arginine. These include N^G-methyl-L-arginine (L-NMA), N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-amino-L-arginine (L-NAA). These L-arginine based NOS inhibitors, however, inhibit the constitutive isoforms of NOS preferentially (L-NAME) or with potencies equal to that

Correspondence: Csaba Szabó, Children's Hospital Medical Center, Division of Critical Care, 3333 Burnet Avenue, Cincinnatti, Ohio, 45229, USA, tel.: (513) 559-8714, fax: (513) 559-4892

for iNOS (L-NMA). (7,8). Recently aminoguanidine (9), some alkylisothioureas (10-12), amidines (13), mercaptoalkylguanidines (14) and isoselenourea compounds (14) have been shown to represent novel classes of NOS inhibitors, some of them with selectivity towards iNOS. The potential effect of these novel compounds on other enzymes, however has not been investigated. Here we compare the effects of isothiourea and mercapto-alkylguanidine NOS inhibitors on the activities of NOS and arginase both of which act on the same substrate, L-arginine as NOS.

Methods

Arginase determination: Bovine liver and macrophage arginase activities were measured as described (15,16) by the colorimetric determination of released urea. Briefly, 20 mM L-arginine (adjusted to pH9.7 by adding NaOH), 0.1 mM $MnCl_2$ and 10 mM compound tested for inhibition (final concentrations) were mixed in 125 µl volume and 125 µl arginase (10 µg enzyme per ml) was added. After 30 min incubation at 37°C, the reaction was stopped by 250 µl 1 N perchloric acid, centrifuged and 100 µl supernatant was used for urea determination by adding 1 ml diacetylmonoxime-thiosemicarbazide reagent (17). After 20 min heating at 100°C and cooling optical densities were read at 520 nm using urea standards.

Measurement of nitrite accumulation: Rat peritoneal macrophages (10^6 cells in 200 µl Dulbecco MEM (DMEM) supplemented by 2 mM Arg and containing 5 % fetal bovine serum, FBS) were cultured for 24 h in 96 well plates. Inducible NOS synthesis can be measured without in vitro induction under these conditions as we have shown earlier (18-20). NOS activity in the cell cultures was assessed by the measurement of nitrite formation, a stable end product of NO, using Griess' reagent (21). The concentration of both L-arginine substrate and NOS inhibitors were 2 mM.

Calculation of the values of structural properties: Molecules were considered to be rigid structures; therefore, individual molecular geometries were optimized by molecular mechanics to get reliable results. According to our previous findings the dynamic fitting of the molecules would lead to the same results as the rigid fitting of the most probable conformers (19,22). Molecular mechanics calculations were carried out by the Hyperchem (Autodesk Inc. 1992) package using MM+ parametrization and the Newton-Raphson optimization method.

Test compounds: L-NMA monoacetate salt was obtained from Calbiochem (San Diego, CA), S-methyl-mercaptoethylguanidine hydrochloride (S-MEG), guanidino-ethyldisulfide hydrochloride (GED), mercaptoethylguanidine bromide (MEG) and aminoethyl-isoselenourea bromide (AE-SeU) were prepared as previously described (14,23). Bovine liver arginase was from Serva (Heidelberg, Germany). All other compounds were from Sigma/Aldrich (St. Louis, MO).

Results and Discussion

Recent reports demonstrate the induction of arginase activity in macrophages (17,19,24,25) in response to immunostimulation. Since arginase induction is concurrent to NOS induction and may diminish NO production by decreasing the concentration of their common substrate, it is of practical importance to select NOS inhibitors that do not interfere with the activity of arginase. Our previous study (19) demonstrated a selectivity

of the L-arginine based NOS inhibitors due to their different size; e.g. N^G-substituted arginine derivatives exerted a slight or no inhibitory effect on arginase vs. their strong effect on iNOS. However, the novel, promising iNOS selective sulfur-containing inhibitors have not been tested yet on arginase.

Most of the tested inhibitors were found to be potent inhibitors of NO synthesis in rat peritoneal macrophages derived from inflammatory sources without in vitro cytokine activation. The potency of the compounds was thiourea < L-NAME < aminoguanidine < L-NAA < aminothiazoline (ATZ) < guanidinoethyldisulfide (GED) < aminoethyl-isoselenourea (AE-SeU) < S-methyl-isothiourea (SMT) < AETU (Table I) which is similar to previous reports (7-14).

Among the various non-L-arginine based compounds only AETU produced an approximately 25 % inhibition on macrophage arginase activity (Table I). A similar effect was observed with L-NAME from the L-arginine based NOS inhibitors tested. The inhibition was kinetically competitive, the K_M of arginase was changed from 5 to 45 mM in the presence of 10 mM NAME (plot not shown). The degree of inhibition by the various compounds was similar for macrophage and bovine liver arginases (not shown). Since AETU undergoes intramolecular rearrangement yielding ATZ or MEG depending on pH values (14), and ATZ does not inhibit arginase activity, we conclude that the inhibition of arginase is due to MEG. This conclusion is further supported by the fact that arginase activity is measured at a basic pH optimum (pH 9.7) which favors the rearrangement of AETU to MEG. The dimer of MEG, GED does not inhibit arginase probably due to its too large size.

To directly confirm our conclusion we performed kinetic studies with MEG on arginase. The mechanism of inhibition was kinetically competitive (Fig. 1A). The K_I of the inhibitor was surprisingly high (12 mM, which is over the K_M of the arginase) indicating that MEG is a low affinity competitive inhibitor of arginase (Fig. 1B). Previously we postulated five potential binding sites in the substrate binding area of arginase and compounds should have at least three structural features making them effective inhibitors (22). MEG contains only two such features: the 6-guanidino-N-atom (group bound to the N-capturing site) and the apolar chain which is similar to L-arginine. Nevertheless, the existence of a complete guanidino group and the slightly polar character of the sulfhydryl group may explain this binding and the inhibitory effect on arginase. MEG tended to be more potent than AETU on MPAse and BLAse activity at 10 mM inhibitor concentration (Table I). This difference may be consistent with the rearrangement of AETU to MEG as well as some amount of ATZ (14) since the latter is ineffective as an inhibitor of arginase (Table I). AETU contains a sulfur atom for the N-capturing site and a basic amino group instead of slightly polar sulfhydryl group which causes a worse fitting to the enzyme. The product of the rearrangement at acidic pH, ATZ is a cyclic compound lacking the carbon chain, which cannot bind to arginase at all. The comparison of the structures of L-arginine and the three inhibitors is given in Scheme 1.

The explanation of the excellent inhibitory effect of structurally different sulfur-containing compounds on NO synthase may lie in their sulfur atom which has a high potency to form complexes with heavy metals, i.e. with the iron atom of porphyrin ring at the active site of NOS. Therefore, the structural similarity of compounds to the substrate L-arginine is required for the inhibition of arginase, but not for the inhibition of NO

synthase. The excellent and highly selective inhibitory effect of isothiourea and mercaptoalkyl-guanidino compounds for NO synthase vs. arginase may be explained by their action on the heme iron. The theoretical explanation of the specificity of these compounds for iNOS compared to constitutive NO synthases (14,23) requires further investigations.

TABLE I.
Comparison of various structural properties and biological effects of sulfur containing NOS inhibitors.

Compound	NPS	WAS	Area^G	Thick^G	Biological activity (%) on NItrite	MPAse	BLAse
L-arginine	70	204	5.78	2.37	100	100	100
L-NMA	108	220	8.83	2.38	29.2± 4.6**	97.0±10.1	93.9± 3.6
L-NAME	112	265	9.59	2.31	72.7±15.1*	71.2±18.1*	66.6± 9.6*
L-NAA	66	136	8.38	2.36	53.8± 9.4**	94.7± 3.1	84.6±12.0
SMT	61	114	9.80	2.28	4.9± 2.2**	109.3± 9.8	112.4±19.4
AETU	66	146	7.37	2.69	4.2± 3.5**	74.3± 5.0*	73.6± 6.9*
MEG	78	156	5.31	2.27	8.4 ± 5.3**	54.3±16.0**	45.1±26.5*
SMEG	100	162	5.31	2.26	39.2± 2.0**	111.9± 8.0	108.9±10.5
ATZ	72	119	6.73	2.53	11.5± 4.6**	129.2±26.6	144.5±21.8
GED	120	237	8.28	2.02	4.2± 3.5**	106.7± 7.1	113.1±12.7
AESeU	70	147	7.84	2.75	7.4± 2.8**	103.8±13.1	103.7±13.1
AGua	0	93	8.47	2.34	54.9±10.2**	84.1± 9.6	102.2±10.3
Thiourea	0	88	6.18	2.35	74.8±20.6	99.1±10.0	92.5± 4.8

NPS, non-polar saturated water accessible surface of the hydrocarbon chain, WAS, total water accessible surface, Area^G, the surface area of the guanidino analogue part, given in A^2; Thick^G, thickness, i.e. the lowest distance between the heavy atoms of the guanidino analogue part, given in A. Nitrite, the nitrite production % of the control, control value is 19.0±1.9 nmoles per 10^6 macrophages, MPAse, macrophage arginase activity %, control value 553±84 nmole urea per mg protein per min BLAse, bovine liver arginase activity %, control value 762± 69 nmole urea per mg protein per min. Data are means ± S.E.M. of three independent experiments performed in duplicate. Student's t test was used to compare means of groups; **($p<0.01$) and *($p<0.05$).

It is conceivable that an inhibitory effect of a NOS inhibitor on arginase activity would diminish the inhibitory potency of the agent as a competitive NOS inhibitor, because it would raise the intracellular L-arginine concentration. Inasmuch we did not observe a marked inhibitory effect of mercaptoalkylguanidines on arginase activity in the current study, our data further support the view that these compounds can be of use for potent inhibition of NO production in cells simultaneously expressing iNOS and arginase.

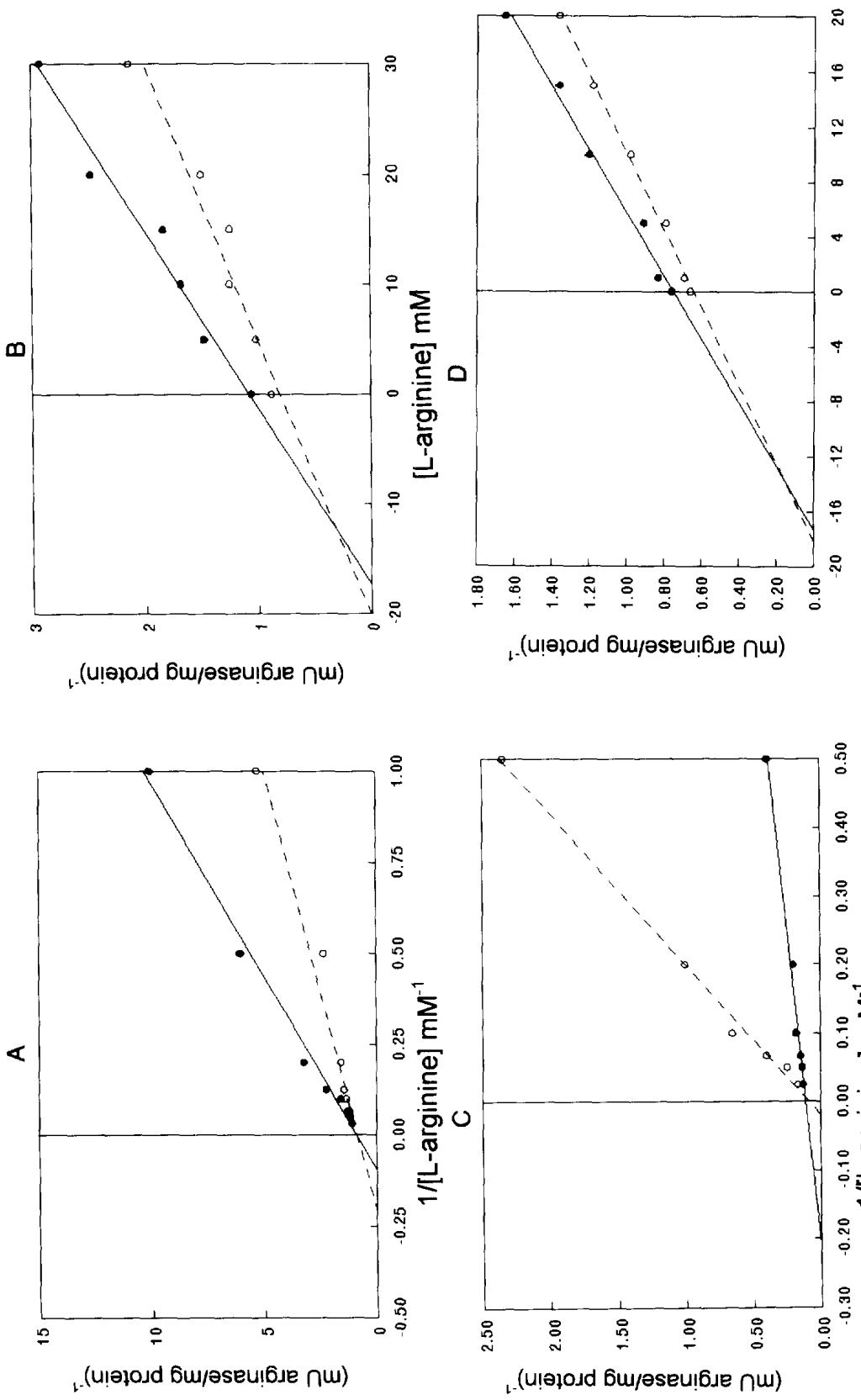
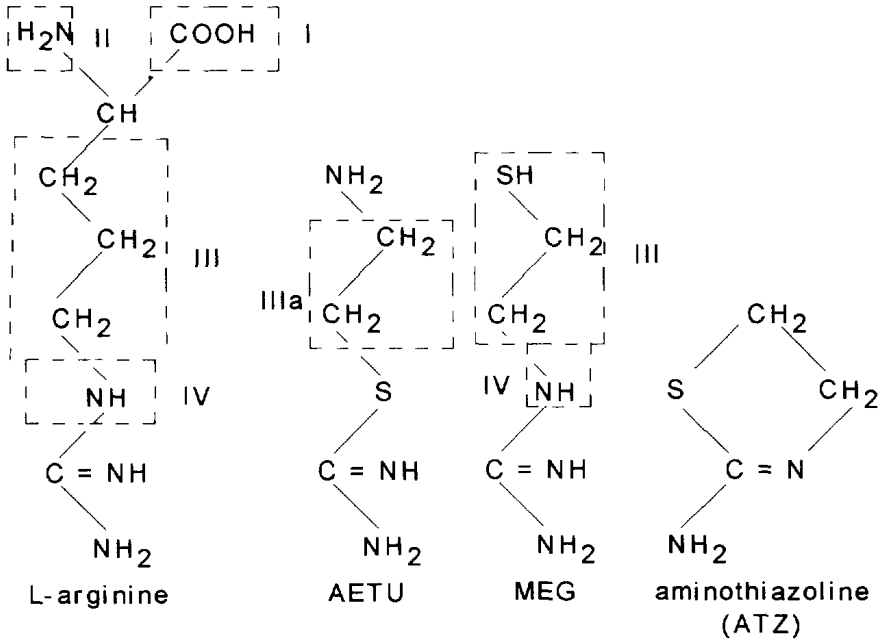


FIG. 1.
The kinetic analysis of the effect of MEG and L-NAME on arginase. Double reciprocal plots show a competitive inhibition caused by MEG (A) and L-NAME (B). Dixon plots show a K_i of 12 mM for MEG (C) and of 14 mM for L-NAME (D). Without inhibition (O), with inhibitor (●).



SCHEME 1.

The comparison of the structures of L-arginine substrate and three NOS inhibitors. Important binding groups are framed by dashed line. It can be seen that MEG is the most similar to the substrate lacking only the amino-carboxyl end (I and II) of the molecule. Both AETU and ATZ contain sulfur atom at the site of the 6-N of L-arginine which serves for the N-capturing site (IV) of arginase suggested by our computer-based studies (19). MEG has the same carbon chain as the substrate (III) while that of AETU is shorter (IIIa).

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