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2-Substituted-2-amino-6-boronohexanoic acids as arginase inhibitors

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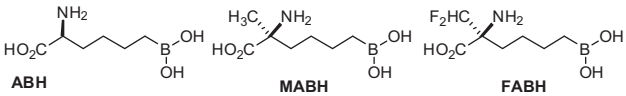
ABSTRACT

Substitution at the alpha center of the known human arginase inhibitor 2-amino-6-boronohexanoic acid (ABH) is acceptable in the active site pockets of both human arginase I and arginase II. In particular, substituents with a tertiary amine linked via a two carbon chain show improved inhibitory potency for both enzyme isoforms. This potency improvement can be rationalized by X-ray crystallography, which shows a water-mediated contact between the basic nitrogen and the carboxylic acid side chain of Asp200, which is situated at the mouth of the active site pocket of arginase II (Asp181 in arginase I). We believe that this is the first literature report of compounds with improved arginase inhibitory activity, relative to ABH, and represents a promising starting point for further optimization of in vitro potency and the identification of better tool molecules for in vivo investigations of the potential pathophysiological roles of arginases.

Arginase is a binuclear manganese metalloenzyme that catalyzes hydrolysis of L-arginine to urea and L-ornithine. Two isoforms are known, arginase I (Arg I) and arginase II (Arg II), which have different tissue distributions and biological functions. Arg I is expressed primarily in the liver, whereas Arg II is expressed in several extra-hepatic tissues, including the brain, spinal cord, kidney, small intestine, and mammary gland. The two isoforms are encoded by different genes, and have differing pI values, immunological reactivity, and subcellular locations, but share similar enzymatic reactivities.[1]

Human arginases are implicated in wide variety of diseases, including atherosclerosis, pulmonary hypertension, systemic hypertension, erectile dysfunction, asthma, wound healing, multiple sclerosis,[2] as well as several tropical diseases such as malaria and leishmaniasis.[3] Development of potent and selective arginase inhibitors would help to elucidate the pathophysio-logical roles that these enzymes play and potentially lead to novel therapies. Several compounds have already been reported, for example, 2(*S*)-amino-6-boronohexanoic acid (ABH),[4] which is the arginase inhibitor most widely used and reported in the literature.[5]

Recently, Ilies et al. have reported the synthesis and characterization of the quaternary amino acids 2-amino-6-borono-2-methyl-hexanoic acid (MABH) and 2-amino-6-borono-2-(difluoromethyl)-hexanoic acid (FABH), both of which showed decreased inhibitory potency, relative to ABH, against human Arg I.[6]



ABH racemic - our data: Arg1 1.4 uM (n=25) optically active ref 4: Arg1 0.8 uM;

MABH

FABH

Independently, we have developed a similar approach and present here the first steps in our research effort that, in contrast, resulted in significant improvements in arginase inhibitory potency compared to ABH.[7]

Our analysis of the binding mode of ABH to arginase I (PDB: 1D3V) revealed that while the whole molecule is tightly embedded in the active site pocket, its alpha-hydrogen projects out towards various surface residues of the enzyme and towards bulk solvent. Therefore, we reasoned that addition of appropriate functionality

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at this position might create new interactions and increase potency activity.

Most compounds were prepared by successive alkylations of the glycine equivalent **2**, using methodology developed by O'Donnell[8] (Scheme 1) or a variation thereof, that is, by alkylation of benzophenone protected amino acids (e.g., **1d** or **1j**).

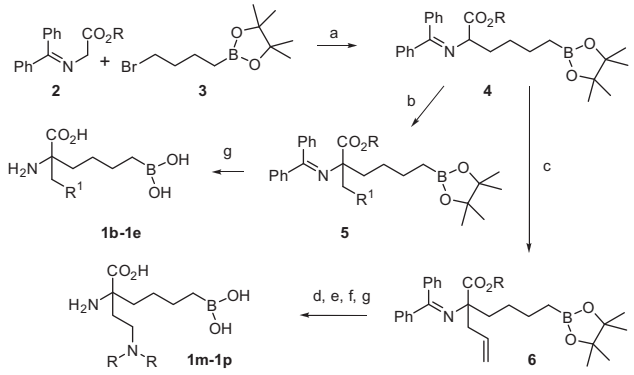
Alkylation with the commercially available bromide **3** allowed installation of the protected *n*-butyl boronic acid fragment in one easy step. The second alkylation (**4** → **5**) required more aggressive reaction conditions or the use of more active alkylating agents, which limited the choice of potential side chains. The alkylated products **5** were deprotected under acidic conditions to provide the desired final products **1b**–**1e** as racemates. Alternatively, alkylation with allyl bromide allowed us to prepare compound **6**. Ozonolysis of **6** followed by reductive amination and deprotection provided the desired products **1m**–**1p**. Final compounds obtained after acidic deprotection were very polar and were purified using reverse phase HPLC and lyophilized.

Compounds were tested for inhibition of human Arg I and Arg II using a colorimetric assay based upon the published method.[9,10] The inhibition of arginases by boronic acid inhibitors is strongly pH dependent.[11] Although the pH optimum for arginase is 9.0–9.5,[12] we decided to perform our assay at pH 7.4 which is more physiologically relevant. Our observed inhibitory potency for ABH for Arg I is very similar to the value reported in the literature (IC_{50} = 800 nM; optically active,[4] vs 1450 nM for racemic compound/our data).

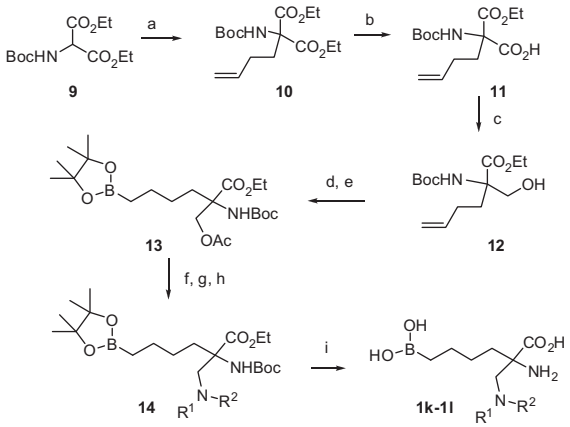
2,3-Diaminopropionic acid derivatives **1k** and **1l** were prepared starting from diethyl (Boc-amino)malonate (Scheme 2). Although the key step (the partial hydrolysis of the diester **10** to mono acid **11**) was achieved in non-selective way, it does provided a potential entry into enantioselective synthesis (e.g., via chemoenzymatic desymmetrization).[13] Acidic hydrolysis of intermediate **13** provided compound **1f**.

The initial compounds with alkyl and benzyl substituents at the alpha-position (**1b**–**1e**) had similar or lower potencies to the unsubstituted compound ABH (**1a**), but these findings confirmed that there is enough room to accommodate a larger alpha-substituent. Analogs with one to three carbon atom aliphatic chain-linked alcohols and ethers were synthesized (e.g., **1f**–**1i**) and tested in the hope of identifying new interactions at the opening of the active site pocket of Arg I, for example, Thr136, Ser137, Asp181 Asp183 (Ser155, Ser156, Asp200, Asp202, respectively in Arg II). Most of these compounds in this class showed little or no improvement in potency compared to ABH. An X-ray crystal structure of compound **1h** bound to Arg II measured at 2.2 Å resolution on a laboratory X-ray source,[14,15] revealed a contact distance of around 2.9 Å between the terminal hydroxyl group and a single oxygen atom of Asp202 (Fig. 1). The corresponding structure of **1h** bound to Arg I (2.0 Å resolution) revealed an almost identical binding mode, with the terminal hydroxyl group oriented toward Thr136 at distance 3.2 Å. In both Arg I and Arg II complexes, all other enzyme inhibitor interactions remained essentially unchanged compared to the respective ABH–enzyme complexes (data not shown).

Compounds **1f**–**1i** are representative examples of alpha-linked C1–C3 chains with non-basic hydrogen bond donor or acceptor groups. Several other aliphatic chain linked ureas, amides and sulfonamides were synthesized and tested with similar results (IC_{50} potencies in 1–5 μM range). Based on the improved potency seen for the histidine analog **1j**, compared to alpha-benzyl analog **1e**, efforts were focused on increasing the interaction of the



Scheme 1. Synthesis of inhibitors **1b**–**1e**, **1g**–**1j** and **1m**–**1p**. Reagents and conditions: (a) LiHMDS, THF, −78 °C to +50 °C (64%); (b) LiHMDS, R^1CH_2I, THF, −78 °C to rt; (c) LiHMDS, allyl bromide 3 equiv, THF, −78 °C to rt, 16 h (59%); (d) Et_2O–1 N HCl, rt then Boc_2O, EtOAc–$NaHCO_3$ sat (80%); (e) O_3, DCM, −78 °C then Ph_3P −78 °C to rt (93%); (f) R_2NH, 1,2-DCE, $NaBH(OAc)_3$, rt, 16 h (70–90% yield); (g) 6 N HCl, 95 °C, 16 h (50–90% yield).



Scheme 2. Synthesis of inhibitors **1k** and **1l**. Reagents and conditions: (a) 4-bromobut-1-ene, NaH, DMF, 90 °C (90%); (b) KOH, EtOH, 0 °C to rt, 19 h, (82%); (c) $ClCO_2Et$, Et_3N, then $NaBH_4$ −40 °C, 1 h, (78%); (d) Ac_2O, DMAP 1 equiv, DCM, rt, 16 h, (80%); (e) pinacol borane, chloro(1,5-cyclooctadiene)iridium(I) dimer, 1,2-bis(diphenyl-phosphino)ethane, DCM, rt, 10 h, (54%); (f) K_2CO_3, EtOH, rt, 2 h, (78%); (g) $(COCl)_2$, DMSO, Et_3N, DCM, −78 °C, 30 min, (57%); (h) R_2NH, 1,2-DCE, $NaBH(OAc)_3$, AcOH, 60 °C, 2 h (30–50% yield); (i) 6 N HCl, 95 °C, 16 h (50–80% yield).

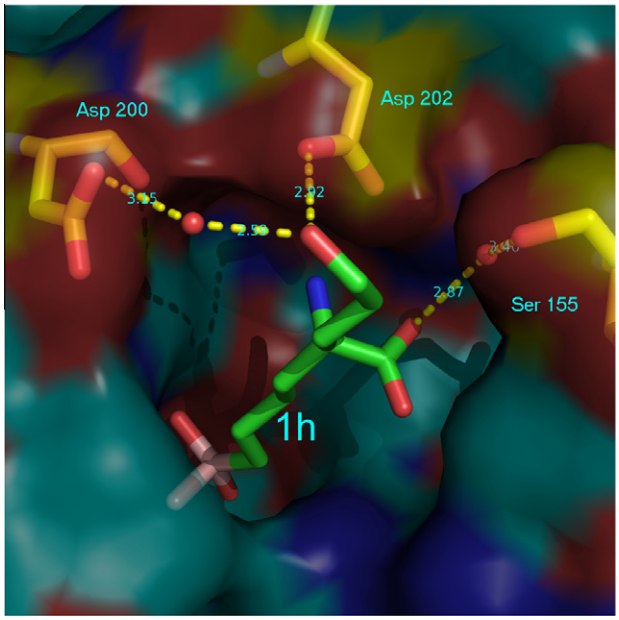


Figure 1. X-ray structure of compound **1h** in human Arg II.

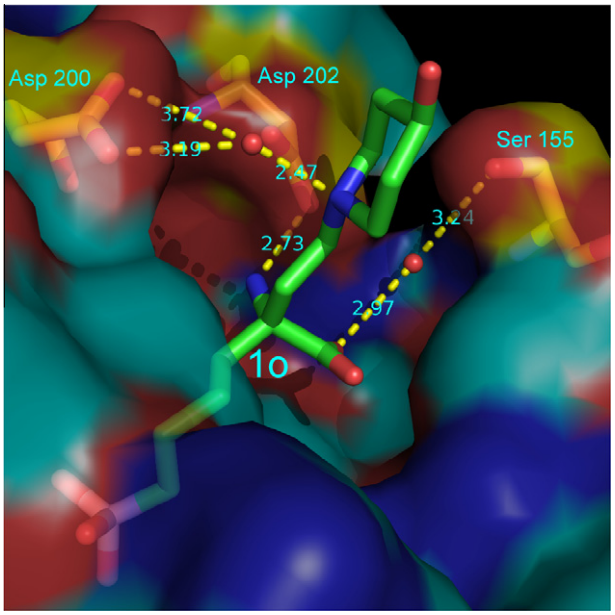


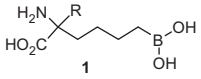
Figure 2. X-ray structure of compound **1o** in human Arg II.

alpha-substituent with the acidic side chains of Asp200 and/or Asp202 (Arg II numbering). Although the 2,3-diaminopropionic acid analog **1k** had a similar potency to the simple ethyl analog **1c**, we found that potency increases could be achieved by extending the spacer to two carbon atoms (compounds **1m**–**1p**). Interestingly, the morpholine compound **1m** and the related piperazine and thiomorpholine analogs (not shown) exhibited substantially lower activity (IC_{50}s in the 2–4 μM range). Simple ethylene-linked tertiary amines (represented by compound **1n**–**1p**) demonstrated a clear improvement (5- to 10-fold) in potency compared to compounds lacking the amine and provided a promising starting point for further optimization of in vitro activity. This improvement in potency was rationalized by a co-crystal structure obtained at 2.35 Å in a laboratory X-ray source of **1o** with Arg II, which revealed a water-mediated interaction between the tertiary amine of the inhibitor side chain and the carboxylic acid side chain of Asp200, (Fig. 2), rather than with Asp202, as in the complex with the hydroxypropyl compound **1h** (Fig. 1). The close proximity of the water molecule to the amine nitrogen (2.47 Å) and the near symmetrical interactions of the water with both carboxylic acid oxygens (3.19 and 3.72 Å) might suggest that these functional groups are present as ammonium and carboxylate ions, respectively. Such an interaction is not possible with the hydroxyl group in **1h** which may, at least in part, explain the difference in potency.

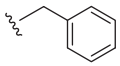
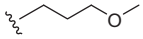
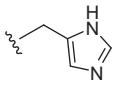
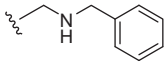
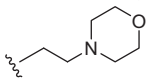
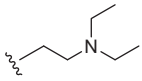
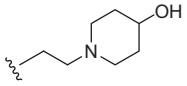
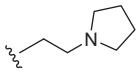
It is apparent from the structure–activity data (Table 1) that the compounds presented here show little evidence of in vitro selectivity between Arg I and Arg II which ultimately may be desirable for a therapeutic. Given the close homology between Arg I and Arg II in the active site region, this is not surprising and may represent a significant challenge in the future.

In conclusion, we have shown that, relative to the literature benchmark compound ABH, improvements in arginase inhibitory potency can be achieved by targeting new interactions with the amino acid residues of the enzyme at the mouth of the active site pocket, particularly Ser155 and Asp200 in the case of Arg II. Further optimization of these novel compounds, including synthesis of optically active analogs, further improvement of in vitro activity as well as pharmacokinetic data and in vivo POC for myocardial ischemia, will be reported elsewhere.

Table 1
SAR for α,α-disubstituted ABH analogues[a]



1

Entry	R	Arg I IC_{50} (nM)	Arg II IC_{50} (nM)	Arg I/Arg II ratio
1a (ABH)		1450	1920	0.75
1b		3430	5070	0.68
1c		4100	4600	0.89
1d		11,240	15,500	0.72
1e		10,500	17,000	0.62
1f		2250	4280	0.53
1g[b]		19,000	57,000	0.33
1h		NT[c]	4500	NA
1i		5120	6440	0.80
1j		2280	3720	0.61
1k		2070	1350	1.53
1l		3260	5980	0.55
1m		5380	4020	1.34
1n		520	934	0.56
1o		370	980	0.38
1p		320	620	0.52

[a] All compounds were tested as their racemates.
[b] Cyclizes to the lactone under the assay condition.
[c] Not tested.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.02.024.

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9. Arg I converts L-arginine to L-ornithine and urea, and relative Arg I activity is determined by measuring urea levels in a colorimetric assay according to the published method.[10] We used purified recombinant full length human Arg I protein to develop a 96 well plate Arg I assay. Tested compounds were dissolved in DMSO. Both Arg I and compounds were further diluted in the assay buffer (0.1 M $NaPO_4$, 130 mM NaCl, 1 mg/mL ovalbumin, pH 7.4) prior to addition to well. L-Arginine (Sigma) was dissolved in a solution containing glycine and $MnSO_4$. Final concentration of $MnSO_4$ and glycine was 0.3, and 13.8 mM, respectively, in each well. Each compound was tested at the final concentration of 0.005, 0.01, 0.04, 0.12, 0.37, 1.11, 3.33, and 10 μM. Compounds, Arg I (67 ng/mL–1.34 nM), and L-arginine (8.3 mM) were added to each well and the plate was incubated at 37 °C for 1 h. For experimental determination of the K_i, inhibition was determined using six concentrations of compound and eight concentrations of substrate. Duplicate wells were done for each condition. The amount of urea in each well was measured by addition of a chromogenic substrate which binds to urea. The absorbance was measured at wavelength 530 nm using a spectroMAX plate reader. The absorbance in each well is directly proportional to the amount of urea produced by arginase-1. The urea production in the absence of any compound was considered the maximum arginase-1 activity. The absorbance in the absence of L-arginine (background) was subtracted from all the values. The % inhibition of Arg I activity was calculated in the presence of each compound and the Prism application was used to generate an IC_{50} curve and to determine the IC_{50} values for each compound ($n = 3$).
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14. Crystals of the human Arg I/inhibitor complexes were obtained by soaking crystals of the native enzyme with the inhibitor. Crystals of the native enzyme Arg I were obtained by vapor diffusion method at 4 °C. Drops containing 4.0 μL of protein at 5 mg/mL in 10 mM Tris·HCl pH 7.5, 10 mM NaCl, 1 mM β-mercaptoethanol, 0.2 mM MnSO4 and 4 μL of precipitant solution [100 mM malonic acid, imidazol, boric system pH 5, 25% polyethylene glycol (PEG) 1500] were equilibrated against a reservoir with precipitant solution. Then the native crystals of Arg I were soaked with 15 mM of the inhibitor during 1 week. They were cryo-protected by the precipitant solution containing 30% ethylene glycol prior to flash cooling in liquid nitrogen.
    Crystals of human Arg II/inhibitor complexes were obtained by co-crystallization with 2 mM of inhibitor. Crystals of complexes were obtained by vapor diffusion method at 4 °C. Protein was concentrated at 5 mg/mL in 10 mM Tris·HCl pH 8.0, 10 mM NaCl, 1 mM β-mercaptoethanol, 0.2 mM $MnSO_4$. Silver Bullets screen (Hampton Research) allowed to obtained crystals. They were cryo-protected by the reservoir solution containing 30% ethylene glycol prior to flash cooling in liquid nitrogen.
15. Structural refinement and structure analysis: the atomic coordinates of rat arginase protein complexed with a boronic inhibitor (Protein Data Bank (PDB) code 1D3V) were used to solve the structure of all Arg I/II: inhibitor complexes. Crystallographic refinement was performed initially with the CCP4 suite and later with the program PHENIX. Amino acid side-chains were fitted into 2Fo – Fc and Fo – Fc electron density maps. The final Fo – Fc map indicated clear electron density for the different inhibitors. Water molecules were fitted into difference maps and in the final cycles riding H-atoms were introduced. The program Coot was used for fitting the models to the electron density. The Arg I complex was solved from a twin crystal and was refined according to '$-h,-k,l$' as twin law. The atomic coordinates have been deposited in the PDB (PDB codes: 4IE1, 4IE2 and 4IE3) and will be released upon publication. In order to study the inhibitor binding sites, Coot was used for analysis, while figures were built with the PyMOL Molecular Graphics System (Schrödinger LLC). Data collection and refinement statistics are shown in the Supplementary data.