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Rational design of novel irreversible inhibitors for human arginase

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ABSTRACT

Parasites have developed a variety of strategies for invading hosts and escaping their immune response. A common mechanism by which parasites escape nitric oxide (NO) toxicity is the activation of host arginase. This activation leads to a depletion of L-arginine, which is the substrate for NO synthase, resulting in lower levels of NO and increased production of polyamines that are necessary for parasite growth and differentiation. For this reason, small molecule inhibitors for arginase show promise as new anti-parasitic chemotherapeutics. However, few arginase inhibitors have been reported. Here, we describe the discovery of novel irreversible arginase inhibitors, and their characterization using biochemical, kinetic, and structural studies. Importantly, we determined the site on human arginase that is labeled by one of the small molecule inhibitors. The tandem mass spectra data show that the inhibitor occupies the enzyme active site and forms a covalent bond with Thr135 of arginase. These findings pave the way for the development of more potent and selective irreversible arginase inhibitors.

1. Introduction

Arginase is a manganese metalloenzyme that catalyzes the hydrolysis of arginine to form ornithine and urea.[1] In the liver, this reaction constitutes the final step of urea biogenesis.[2] In other non-hepatic tissues such as red blood cells, mammary glands and kidneys, the main function of arginase is thought to be the production of ornithine, which serves as a biosynthetic precursor to proline and polyamines.[3] Recent studies suggest that arginase also plays an important role in modulating the immune response since it regulates arginine levels, and arginine is the substrate for nitric oxide synthases (NOS) (Fig. 1).[4,5] When the level of extra-hepatic arginase is elevated, arginine availability as a substrate for NOS is limited due to the enhanced consumption of arginine by arginase. Thus, arginase and NOS appear to be reciprocally regulated under some conditions. Up-regulation of arginase has been observed in several diseases including Chagas disease and sleeping sickness.[6,7] These diseases are caused by parasitic protozoa of the genus *Trypanosoma*. These parasites evade the human immune response and escape NO toxicity by activating the host's arginase.[8–10] The activation of arginase leads to (1) depletion of arginine leading to decreased NO production; (2) increased production of polyamines, which are necessary for parasite growth and differentiation; and (3) enhancement of the polyamine biosynthetic precursor for trypanothione, a molecule that is essential for parasites to maintain their intracellular redox system.

Recently, arginase and polyamine biosynthetic pathways have been being targeted for the treatment of African sleeping sickness and Chagas disease.[11,12] Potent arginase inhibitors are valuable chemical tools for deciphering the complex immune response mediated by arginase, and for testing biological and therapeutic hypotheses associated with parasitic diseases. However, only a few arginase inhibitors have been reported to date (Fig. 2).[13–15] The boronic acid analogs of L-arginine (Fig. 2, ABH and BEC) have been widely used as reversible arginase inhibitors to study the biological function of arginase. Both inhibitors contain a boronic acid in place of the guanidinium group in arginine. The inhibitors bind to the active site of arginase, and react with a Mn^{+2} bound hydroxide ion to give a tetrahedral boronate species. These boronic acid inhibitors often have high toxicity, and relatively poor pharmacokinetic profiles, selectivities and bioavailabilities.[16,17] Thus, their potential applications as chemotherapeutic reagents are limited. The development of novel arginase inhibitors is an urgent need.

Irreversible inhibitors typically contain a reactive functional group that reacts with amino acid side chains to form a covalent adduct. A higher level of selectivity can be achieved if the reactive functional group is only unmasked as a consequence of an enzyme-catalyzed

Abbreviations: AIBN, azobisisobutyronitrile; NBS, *N*-Bromosuccinimide; DMAP, 4-dimethylaminopyridine; TEA, Triethylamine; TFA, trifluoroacetic acid; DAST, diethylaminosulfur trifluoride; DCM, Dichloromethane; Tris, tris(hydroxymethyl)aminomethane; DTNB, 5,5-dithio-bis-(2-nitrobenzoic acid); TNB, 2-nitro-5-thiobenzoate; CID, collision induced dissociation; TLC, thin layer chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; HRMS, high-resolution mass spectrometry; ESI, electrospray ionization; NMR, nuclear magnetic resonance

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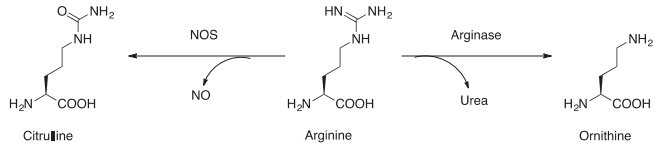


Fig. 1. Two enzymes that use arginine as a substrate.

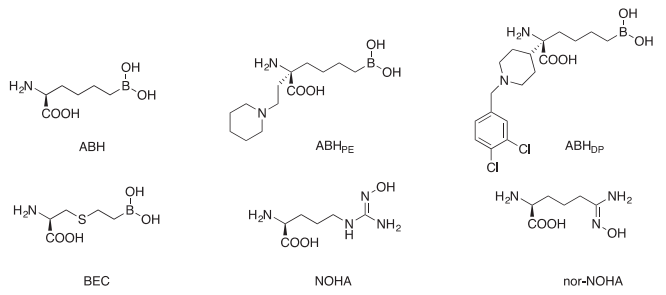


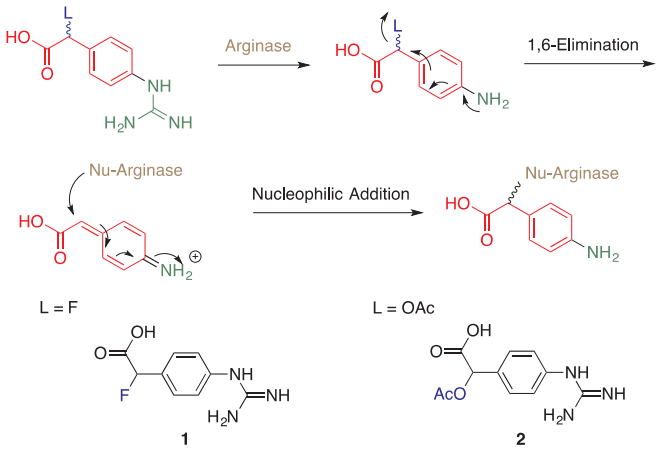
Fig. 2. Reversible arginase inhibitors.

reaction. In this way, such mechanism-based inactivators generate the reactive group in the active site of the target enzyme, and avoid non-specific reaction with other proteins. Irreversible inhibitors have been developed for a variety of enzyme families, including proteases,[18,19] kinases[20,21] and phosphatases.[22,23] In 2011, researchers estimated that 26 covalent drugs accounted for over $33 billion in annual worldwide sales.[24] Inspired by the natural structure of arginine, we designed and synthesized two mechanism-based irreversible arginase inhibitors. We characterized these compounds in biochemical and kinetics assays. Furthermore, we determined the labeling site on human arginase 1 that reacts with inhibitor **1**. Such small molecule inhibitors for human arginase may show therapeutic potential for the treatment of a range of pathological conditions, and have promise as new anti-parasitic drugs.

2. Results and discussion

2.1. Design

The inhibitors have three structural components: (1) the 4-guanidinobenzyl group that mimics the side chain of L-arginine; (2) a leaving group that triggers formation of a reactive electrophile in the active site[25–28]; and (3) a carboxyl group that anchors the inhibitor in the active site through hydrogen bonds with several amino acid side chains. The proposed mechanism is shown in Scheme 1. After binding to the active site of arginase, the guanidinium group on the inhibitor is first hydrolyzed by arginase to generate an aromatic amine. This intermediate undergoes rapid 1,6-elimination to give a *para*-azaquinone methide, which is a strong electrophile. The azaquinone methide reacts with a proximal nucleophilic residue in the active site of arginase, and results in irreversible covalent labeling of the enzyme. Two leaving groups, fluoride and acetate, were chosen due to their stability in aqueous solution and reasonable leaving group ability.



Scheme 1. Structures and proposed mechanism of action of the irreversible inhibitors **1** and **2**.

We generated a computer-based model of inhibitor **1** docked into the active site of arginase using the crystal structure of the arginase-ABH complex (PDB 2AEB)[29] in conjunction with a flexible side chain docking strategy. The ABH molecule in the crystal structure was first removed, then inhibitor **1** was docked and energy minimized in the binding site. Fig. 3A illustrates that the guanidinium group of inhibitor **1** can hydrogen bond with the side chains of His126, Asp128, Asp232 and Asp234, while the carboxylate can hydrogen bond with Asn130, Ser137 and Asn139. An overlay of the crystal structure of ABH with the model of inhibitor **1** suggests that the two inhibitors can bind in a similar manner (Fig. 3B). Similar docking studies with inhibitor **2** (Supporting Information, Fig. S1) suggest that inhibitor **2** also fits into the binding site, but with less favorable interactions compared with inhibitor **1**.

2.2. Synthesis

Inhibitors **1** and **2** were synthesized as shown in Scheme 2. 2-(4-Nitrophenyl)acetic acid **3** was esterified to give methyl 2-(4-nitrophenyl)acetate **4**. Bromination followed by hydrolysis of the intermediate benzylic bromide **5** gave alcohol **6**. The nitro group was reduced using Pd/C and H_2, and the resulting aromatic amine **7** was reacted with 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea to form the Boc protected guanidine compound **8**. Inhibitor **1** was then prepared from **8** by converting the alcohol to a fluoride using DAST, and removing the methyl ester and Boc protecting groups with aqueous KOH and TFA, respectively. Inhibitor **2** was synthesized from **8** by saponifying the methyl ester, followed by acylating the benzylic alcohol with acetic anhydride to give compound **12**. TFA removed the Boc protecting groups to give inhibitor **2**.

2.3. Kinetic assays of irreversible inhibitors with arginase

We used the substrate thioarginine, in conjunction with the colorimetric reagent 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), to measure the activity of arginase.[30] After thioarginine is hydrolyzed by arginase, the resulting free thiol group undergoes thiol-disulfide interchange with DTNB to give 2-nitro-5-thiobenzoate (TNB). TNB has a yellow color and can be quantified by measuring its absorbance at 412 nm (Scheme 3). We used this substrate to measure K_m and V_{max} values for arginase 1 and 2 that are similar to the values reported in the literature (see Supporting Information, Table S1).[31,32] The minor differences between our experimental and literature values are likely due to minor differences in the assay conditions including temperature, pH, and the presence of additives.

Inhibitors **1** and **2** were assayed against arginase using thioarginine as the substrate (Fig. 4). Based upon our mechanistic proposal, we expected to observe time- and concentration-dependent irreversible inhibition. Arginase 1 and 2 were incubated with varying concentrations of the inhibitors, and at several time points aliquots were removed and assayed for remaining enzyme activity by measuring the initial hydrolysis rate of the substrate using a UV-Vis micro-plate reader. The apparent exponential decay rate constant (k_{app}) was plotted against the inhibitor concentration according to the equation $k_{app} = k_{inact} \times$ [inhibitor] / (K_I + [inhibitor]) to calculate inhibition constants K_I and inactivation rate constants k_{inact}. The full data set is shown in the Supporting Information (Figs. S4 and S5) and the inhibition results are summarized in Tables 1 and 2. The K_I values of both inhibitors are significantly lower than the K_m values for the thioarginine substrate (1.4 and 1.5 mM for arginase 1 and 2, respectively). This may be due to