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Short communication

Novel agmatine and agmatine-like peptidomimetic inhibitors of the West Nile virus NS2B/NS3 serine protease

Huichang Annie Lim, Joma Joy, Jeffrey Hill, Cheng San Brian Chia*

*Experimental Therapeutics Centre, Agency for Science, Technology and Research (A*STAR), 31 Biopolis Way, Nanos #03-01, Singapore 138669, Singapore*

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ABSTRACT

This communication reports the synthesis and inhibitory activities of novel non-covalent peptidomimetic inhibitors of the West Nile virus NS2B/NS3 protease containing a decarboxylated P1 arginine (agmatine; 4-aminobutylguanidine) and related analogues. One agmatine peptidomimetic (4-phenylphenacetyl-Lys-Lys-agmatine; compound **2**) was shown to be a competitive inhibitor with a binding affinity of K_i 2.05 ± 0.13 μM and was inactive against thrombin (IC_{50} > 100 μM). Our results suggest that peptidomimetics with agmatine at the P1 position could potentially be employed as starting tools in the design of non-covalent competitive protease inhibitors due to their relative stability and ease of chemical synthesis compared to inhibitors containing reactive electrophilic warheads.

1. Introduction

The West Nile virus (WNV) is a mosquito-borne virus of the Flaviviridae family. Originating from Africa, it has spread to humans in different continents, including North America [1,2]. Between 1999 and 2009, the United States Center for Disease Control reported more than 20,000 people in the United States have been infected, resulting in 1163 fatalities [3]. Symptoms of infection include fever, headaches, chills, diaphoresis and lymphadenopathy which can lead to high fever known as the 'West Nile Fever'. Some of these cases progress to meningitis, coma and death [4].

The WNV has a single-strand, positive-sense, 11-kb RNA genome which serves as a messenger RNA for protein synthesis as well as a template for RNA replication in a mammalian host cell. The viral genome encodes seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [5] and one attractive target for antiviral drug development is NS3 [6–8]. In the cytoplasm of a host cell, NS3 complexes to its NS2B cofactor to form a functional trypsin-like serine protease which cleaves polyprotein precursors into mature viral proteins [9]. The NS2B/NS3 complex recognizes and selectively cleaves the C-terminal end of two consecutive, highly-conserved basic residues (Fig. 1) [10]. This unusual specificity is not shared by many mammalian proteases and could thus be exploited as an antiviral drug target [6,11].

A common strategy used for the inhibition of the NS2B/NS3 protease involved covalent inhibitors that compete with the substrate for the catalytic site. Such peptide-based covalent inhibitors have their C-terminal carboxyl group chemically modified into reactive electrophilic 'warheads' (see [11,12] for reviews). A popular warhead is the aldehyde functional group and peptide aldehydes have been shown to inhibit the WNV NS2B/NS3 protease at submicromolar potencies [13]. However, warhead peptidomimetics have several undesirable characteristics, including lack of selectivity over other trypsin-like proteases due to their high reactivity and low chemical stability, curtailing their potential for drug development [14]. In this report, we investigated if peptidomimetics without electrophilic warheads could inhibit the WNV NS2B/NS3 protease. These inhibitors do not bind covalently to the catalytic serine in the active site but instead employ hydrophobic and/or electrostatic interactions to compete with the natural substrate for the active site. Such inhibitors should be less reactive and hence more 'druggable' compared to warhead inhibitors.

Our strategy focused on peptidomimetics containing a P1 decarboxylated arginine (agmatine; 4-aminobutylguanidine) and structurally-related analogues. Agmatine-containing peptidomimetics have been reported to inhibit trypsin-like serine proteases like furin, a mammalian protease responsible for the cleavage of inactive protein precursors involved in many physiological pathways [14] and thrombin, a protease involved in blood-clotting [15,16]. These inhibitors contained agmatine and agmatine analogues at the P1 position and were found to be competitive inhibitors with micromolar to sub-micromolar potencies. On this

* Corresponding author. Tel.: +65 64070348; fax: +65 64788768.
E-mail address: cschia@etc.a-star.edu.sg (C. San Brian Chia).

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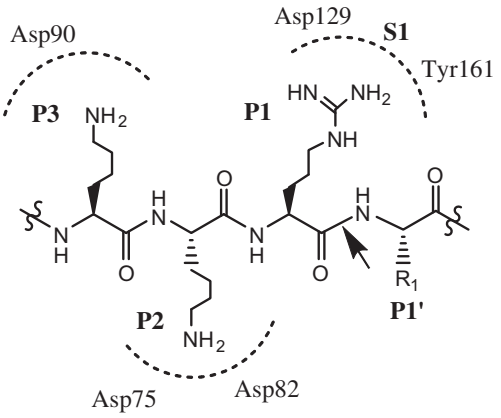

Fig. 1. Schematic diagram of a peptide substrate showing the position of the scissile bond (arrowed) and the basic residues: Lys (P3, P2) and Arg (P1). The WNV protease residues in the S1–S3 binding sites participating in substrate binding are numbered based on the crystal structure 3E90.pdb.

basis, we designed and synthesized ten novel compounds incorporating agmatine and its analogues at the P1 position of the WNV peptide substrate recognition sequence: Lys(P3)-Lys(P2)-Arg(P1) and tested them for inhibitory activities against WNV NS2B/NS3 serine protease (Fig. 2).

Lastly, we explored the enzyme inhibitory activity of our two most potent inhibitors against thrombin to gain an insight into their selectivity against trypsin-like serine proteases.

2. Results and discussion

2.1. Chemistry

The general synthetic procedure for compounds **1**–**10** is shown in Scheme 1: the appropriate diamine was guanylated with *N*,*N*-di-(*t*-butoxycarbonyl)-*S*-methylisothiourea based on the method by Verdini et al. [17] to give the Boc-protected agmatine and its analogues. These were then coupled with Fmoc-Lys(Boc)-Lys(Boc)-OH using O-benzotriazole-N,N,N′,N'tetramethyluroniumhexafluoro phosphate (HBTU) as coupling reagent. After Fmoc removal by 1,8-diazabicyclo[5.4.0] undec-7-ene (DBU), the deprotected peptidomimetics were N-capped with acetic anhydride or 4-biphenylacetic acid. Finally, the Boc protecting groups were removed by TFA treatment and the pseudopeptides were purified (HPLC) and characterized (LCMS).

2.2. Biological activity

Compound **1** showed an inhibitory activity of IC_{50} = 18.2 ± 4.2 μM against WNV NS2B/NS3 protease (Table 1), proving that agmatine-containing peptidomimetics could inhibit the WNV NS2B/NS3 serine protease.

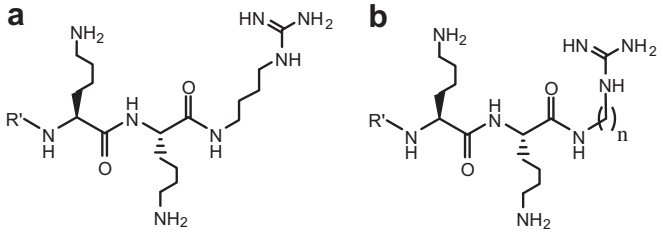

Fig. 2. Structures of compounds (a) **1** and **2**; (b) **3**–**5**. Agmatine is shown in the hashed box. Structures of compounds **6**–**10** can be found in Table 1.

Encouraged by this, we synthesized compound **2** where the N-terminus was capped with a 4-phenyl-phenacetyl (4-Ph-Phac) group. This group has been reported to be the most potent N-capping group in a panel of tripeptide aldehyde WNV inhibitors (IC_{50} = 32 nM for 4-Ph-Phac-KKR-H) and computer docking studies using simulated annealing showed that the 4-Ph-Phac group could squeeze into the S4 WNV protease binding site [13]. Expectedly, the potency of **2** improved approximately 4-fold compared with **1**, with an IC_{50} of 4.7 ± 1.2 μM (Table 1). A Lineweaver-Burk plot of inhibitor **2** showed that it acted as a competitive inhibitor with a K_i of 2.05 ± 0.13 μM (Fig. 3).

We next tested a panel of compounds containing agmatine homologues with different alkyl chain lengths (compounds **3**–**5**). Using the recent crystal structure of the tripeptide aldehyde inhibitor Naphthoyl-KKR-H complexed to WNV NS2B/NS3 protease (3E90 pdb [18];), the P1 arginine's guanidino group was observed to take part in electrostatic attraction with the side-chain carboxylate anion of Asp129 of NS3 within a distance of 3.2–4.3 Å in the S1 binding site. Altering the length of the agmatine alkyl chain should alter the guanidino-carboxylate distance and affect binding affinity. As expected, compounds **3**–**5** failed to inhibit the NS2B/NS3 enzyme (IC_{50} > 100 μM; Table 1). An intriguing observation was that the addition or removal of just one methylene group (compounds **3** and **4** respectively) abrogated all binding affinity, suggesting that a 4-carbon alkyl chain between the amide and guanidino group was optimal for binding. Indeed, molecular modelling of compound **3** using 3E90 pdb as a template showed that lengthening the alkyl chain by one carbon caused the guanidino group of **3** to clash with Asp129 side-chain. Removing one methylene from the alkyl chain (compound **4**) increased the distance between the guanidino group and the carboxyl side-chain of Asp129 to approximately 4.1–5.0 Å, possibly disrupting the salt bridge between them. With this knowledge, we synthesized compound **6** in which the presence of a *trans*-(β-γ) double bond rigidifies the 4-carbon alkyl chain. Interestingly, the unsaturated analogue **6** was approximately 13-fold less potent than its saturated counterpart **2** (IC_{50} 61.2 ± 8.6 and 4.7 ± 1.2 μM respectively), suggesting that a flexible 4-carbon alkyl chain was needed for optimal positioning of the guanidino group relative to Asp129 and Tyr161 in the S1 binding site. Based on these results, we could conclude that a saturated 4-carbon chain was needed for optimal binding.

A final series of inhibitors containing conformationally restrained agmatine analogues **7**–**10** (Table 1) were synthesized using inhibitor **2** as a template to test the effect of increasing the agmatine *n*-butyl chain rigidity on enzyme S1 site binding affinity. Hence, 5 and 6-membered cyclic agmatine mimetics **7**–**10** were synthesized and assayed. Experimental results revealed that 6-membered analogues **7** and **8** did not possess any inhibitory activities (IC_{50} > 100 μM), suggesting that the rigid 6-membered rings may have oriented the guanidino group away from its optimal position in the S1 binding site. This was supported by molecular modelling studies using the crystal structure 3E90 pdb adopted by the tripeptide aldehyde inhibitor KKR-H when complexed to the WNV protease. Our models revealed that replacing the flexible agamatine *n*-butyl chain in compound **2** with 6-membered rings steered the guanidino group away from the S1 binding site which possibly abrogated all binding affinities (Fig. 4A and B).

The assay results for agmatine mimetics containing 5-membered pyrrolidine rings (compounds **9** and **10**) were more interesting; compound **9** showed no inhibitory activity (IC_{50} > 100 μM) while **10** exhibited moderate activity (IC_{50} 20.0 ± 5.8 μM). Our molecular models suggested that the stereogenic carbon in the pyrrolidine ring played an important role in orienting the guanidino group either