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Exploring the binding of peptidic West Nile virus NS2B–NS3 protease inhibitors by NMR

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ABSTRACT

West Nile virus (WNV) NS2B–NS3 protease is an important drug target since it is an essential protein for the replication of the virus. In order to determine the minimum pharmacophore for protease inhibition, a series of dipeptide aldehydes were synthesized. The 50% inhibitory concentration (IC_{50}) measurements revealed that a simple acetyl-KR-aldehyde was only threefold less active than 4-phenyl-phenylacetyl-KKR-aldehyde (**1**) (Stoermer et al., 2008) that was used as the reference compound. The ligand efficiency of 0.40 kcal/mol/HA (HA = heavy atom) for acetyl-KR-aldehyde is much improved compared to the reference compound **1** (0.23 kcal/mol/HA). The binding of the inhibitors was examined using ^{1}H-^{15}N-HSQC experiments and differential chemical shifts were used to map the ligand binding sites. The biophysical studies show that the conformational mobility of WNV protease has a major impact on the design of novel inhibitors, since the protein conformation changes profoundly depending on the structure of the bound ligand.

1. Introduction

West Nile virus (WNV), a member of the flavivirus genus, is an arthropod-borne human pathogen first isolated from a febrile patient in the West Nile district of Uganda in 1937, and recognized in the mid 1950s to be the cause of severe meningitis and encephalitis in elderly patients in Israel. The virus was first introduced to the USA in 1999 and has seen an astounding proliferation to most of the states causing thousands of human infections (Gubler, 2007). Since neither a safe human vaccine nor a drug is available, the treatment of WNV infections is symptomatic, aimed at preventing clinical complications and reducing patient discomfort.

WNV is a small enveloped virus with a single-stranded, 11 kb RNA genome encoding a polyprotein processed by host and viral proteases (Robin et al., 2009). This polyprotein is cleaved into three structural proteins and seven nonstructural (NS) proteins including NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Lindenbach et al., 2007). The multifunctional NS3 protein that contains the protease activity needed for polyprotein processing is one of the most promising targets for drug discovery against flaviviral infections because of its important role in the replication process (Chambers et al., 1990). In addition the successful development of inhibitors against Hepatitis C virus protease (Perni et al., 2006), a member of the *flaviviridae* family, has further encouraged the search for protease inhibitors of WNV and dengue virus (DENV) serotypes.

The proteases of WNV and DENV have proven to be very challenging for drug discovery, due to the nature of the binding site that consists of shallow grooves on the protein surface. Although a number of potent peptide inhibitors have been described (Stoermer et al., 2008), all attempts to progress these chemical starting points towards high affinity peptidomimetics with acceptable pharmaceutical characteristics for oral delivery have not been successful. Similarly, the optimization of small molecular weight hits from high throughput screening (e.g. Su et al., 2009a,b; Ezgimen et al., 2012) has so far not provided any compounds with adequate potency for *in vivo* studies.

The active protease of WNV is formed by the interaction of NS3 and NS2B, both proteins contributing residues to the active site of the enzyme (Erbel et al., 2006; Chappell et al., 2008). In order to produce an active, stable protein for inhibition studies, 40 residues of NS2B have been tethered to the protease domain of NS3 by a flexible linker (WNV NS2B–NS3pro) (Nall et al., 2004). This artificial

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WNV protease construct has proven to be a satisfactory mimic of the natural protein and has become the *de facto* standard for drug discovery (Nall et al., 2004). X-ray crystal structures have shown that this protein exhibits a very different protein fold in the apo-form than in complex with a peptide inhibitor (Benzoyl-Nle-KRR-aldehyde) (Erbel et al., 2006). NMR studies have confirmed the existence of an open and closed conformation and it has been shown that low molecular weight inhibitors shift the conformational exchange equilibrium towards the closed conformation (Su et al., 2009a,b). Only the closed conformation of NS2B–NS3pro is catalytically competent while in the open conformation the C-terminal region of NS2B detaches from NS3.

One of the reasons for the slow progress towards clinically useful WNV protease inhibitors is the lack of detailed structural information for a structure-guided drug discovery campaign. Although crystal structures are available with peptide aldehyde inhibitors (Erbel et al., 2006; Robin et al., 2009) and with aprotinin (Aleshin et al., 2007), attempts to use crystallography for the development and rationalization of structure activity relationships (SAR), as demonstrated during the discovery of HCV and HIV protease inhibitors (Tsantrizos, 2008), have encountered difficulties because the WNV NS2B–NS3pro is not amenable to high throughput crystallization.

We have been searching for an alternative to X-ray crystallography in order to study the interaction of inhibitors with WNV protease and here we demonstrate the use of NMR spectroscopy to gain insight into the SAR of such compounds.

2. Methods and materials

2.1. Protein expression

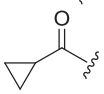
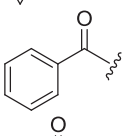
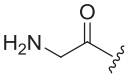
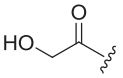
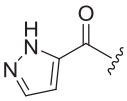
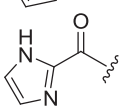
The cDNA encoding the two-component WNV protease that contains 47 residues from NS2B, a G_4SG_4 linker and 184 residues from NS3 was cloned into the pET21d vector to generate pET21-WNV (Supplementary Fig. 1). pET21-WNV was chemically transformed into *Escherichia coli* BL21 (DE3) RILP codon plus competent cells and the cells were grown on a LB agar plate containing kanamycin (30 μg/ml). Two to three colonies were incubated in 30 ml of M9 medium with 30 μg/ml kanamycin. The overnight culture was further transferred into 1 L of M9 medium containing the antibiotic. When the OD_{600} reached 0.6–0.8 induction was performed by adding β-D-1-thiogalactopyranoside (IPTG) to 1 mM final concentration, followed by incubation for 2 h at 25 °C. *E. coli* cells were harvested by centrifugation at 8000*g* for 10 min at 4 °C. The cell pellet was resuspended in a lysis buffer containing 20 mM sodium phosphate, pH 7.8, 500 mM NaCl, and 2 mM β-mercaptoethanol. Cells were lysed by sonication in an ice bath and the cell lysate was cleared by centrifugation at 40,000*g* for 20 min. The supernatant was passed though a gravity column containing Ni^{2+}-NTA resin. Resin was washed with at least 10 column volumes of washing buffer containing 20 mM sodium phosphate, pH 7.8, 1 M NaCl, 10 mM imidazole and 2 mM β-mercaptoethanol to remove nonspecific binding proteins from the resin. Protein was eluted with an elution buffer containing 500 mM imidazole, pH 6.5, 500 mM NaCl and 2 mM β-mercaptoethanol. The purified protein was buffer exchanged to one containing 20 mM Tris–HCl, pH 7.8, 10 mM NaCl, 1 mM dithiothreitol (DTT) using a PD10 column. The protein was further purified using ion-exchange chromatography and then concentrated to 0.2–0.5 mM in an NMR buffer consisting of 20 mM HEPES, pH 7.3, 1 mM DTT and 10% D_2O.

2.2. NMR experiments

For NMR studies, protein samples in an NMR buffer were transferred into 3 mm or 5 mm NMR tubes. All NMR spectra were acquired at 298 K on a Bruker Avance II 700 MHz spectrometer equipped with a triple-resonance cryoprobe. The spectra were processed with Topspin 2.1 (Bruker) and NMRPipe (Delaglio et al., 1995), and visualized with NMRView (Johnson, 2004) or Sparky (http://www.cgl.ucsf.edu/home/sparky/). For backbone assignment of WNV, protease inhibitor **1** was added to a final concentration of 2 mM, by adding a 30 mM stock solution prepared in NMR buffer or d_6-DMSO to 0.8 mM triple-labeled WNV protease. The backbone ^{1}HN, ^{15}N and ^{13}Cα resonances were assigned using two dimensional (2D) and Transverse Relaxation Optimized Spectroscopy (TROSY) (Pervushin et al., 1997)-based three dimensional (3D) experiments including ^{1}H-^{15}N-HSQC (heteronuclear single quantum coherence), 3D-HNCACB, CBCA(CO)NH, and HNCA experiments. All the pulse programs were from the Bruker standard library. Protein secondary structure was analyzed using Talos+ (Shen et al., 2009) and chemical shift index analysis was performed by comparing the deviation of the Ca values from the random coil values (Wishart et al., 1992).

To obtain distance restraints between WNV protease and inhibitor **1**, 3D ^{15}N(F1)-edited/^{15}N(F3)-filtered NOESY was acquired. A 0.5 mM ^{15}N/^{13}C/^{2}H-labeled WNV protease solution in NMR buffer was mixed with a 2 mM solution of inhibitor **1** in NMR buffer and data was acquired with a mixing time of 100 ms (Iwahara et al., 2001). The unambiguous NOEs between protease and inhibitor **1** were used to analyze the models from a docking study. To compare the chemical shift difference from different inhibitors, ^{1}H-^{15}N-HSQC spectra of 0.2 mM WNV protease in the presence and absence of 1 mM inhibitors were collected and superimposed. All the inhibitors were dissolved in the NMR buffer, except for some inhibitors with lower solubility were dissolved in d_6-DMSO.

Table 1
Inhibition of WNV protease by X-KR-aldehyde inhibitors.

Inhibitor	X	IC_{50} (μM)[a]	Ligand efficiency (LE)[b,c]
2		0.17 ± 0.04	0.40
3		0.34 ± 0.10	0.37
4		0.43 ± 0.09	0.35
5		1.84 ± 0.18	0.28
6		1.53 ± 0.08	0.31
7		5.84 ± 0.76	0.30
8		0.25 ± 0.02	0.38
9		0.22 ± 0.02	0.34
10		0.12 ± 0.01	0.35

[a] Average of three independent determinations.
[b] Calculated as described in Hopkins et al., 2004.
[c] kcal/mol/HA (HA = heavy atom).