Discovery, Synthesis, and in vitro Evaluation of West Nile Virus Protease Inhibitors Based on the 9,10-Dihydro-3H,4aH-1,3,9,10a-tetraazaphenanthren-4-one Scaffold

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West Nile virus (WNV), a member of the Flaviviridae family, is a mosquito-borne pathogen that causes a great number of human infections each year. Neither vaccines nor antiviral therapies are currently available for human use. In this study, a WNV NS2B–NS3 protease inhibitor with a 9,10-dihydro-3H,4aH-1,3,9,10a-tetraazaphenanthren-4-one scaffold was identified by screening a small library of non-peptidic compounds. This initial hit was optimized by solution-phase synthesis and screening of a focused library of compounds bearing this scaffold. This led to the identification of a novel, uncompetitive inhibitor (1a40, IC_{50} = 5.41 ± 0.45 μM) of WNV NS2B–NS3 protease. Molecular docking of this chiral compound onto the WNV protease indicates that the S enantiomer of 1a40 appears to interfere with the productive interactions between the NS2B cofactor and the NS3 protease domain; (S)-1a40 is a preferred isomer for inhibition of WNV NS3 protease.

Introduction

The West Nile virus (WNV), a neurotropic flavivirus, is a mosquito-borne reemerging pathogen causing outbreaks worldwide.[1] While a larger proportion of infected people suffer from milder symptoms such as West Nile fever, there has been an observed increase in cases of severe neuroinvasive diseases such as meningitis, flaccid paralysis, and encephalitis over the past decade.[2] Furthermore, close follow-ups with WNV-infected people have shown that patients who manifest symptomatic infections often suffer lifetime consequences such as long-term cognitive and neurological impairment.[3] Despite the growing public health concern associated with WNV infection, to date there are no successful antiviral therapies or vaccines for human use against WNV.

The West Nile virion contains a 10.8-kb single-stranded positive-sense RNA genome 11 000–12 000 nucleotides long.[4] The RNA genome encodes three structural proteins (C, capsid; M, membrane; E, envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5),[5] which form the machinery involved in viral synthesis and replication.[4,6] Site-directed mutagenesis studies have shown that mutation of essential catalytic residues at NS3 protease cleavage sites lead to a halt in viral infectivity, indicating its importance in the WNV life cycle.[7] In addition, the NS2B protein has been found to be an essential cofactor that increases NS3 protease activity significantly.[8] The WNV NS2B–NS3 protease cleaves peptide substrates at their C termini; substrate recognition is brought about by a pair of basic amino acids (R and K) at the P1 and P2 positions. This mode of substrate recognition is conserved among other flaviviral proteases, but not in host cellular enzymes,[9] indicating the possibility of designing inhibitors that are selective for flaviviral proteases without adversely affecting normal cellular functions. Thus, the biological importance of the NS2B–NS3 complex in activation of viral proteins coupled with unique substrate preference make it an attractive target for selective viral inhibition.

Efforts toward the development of inhibitors against the WNV NS2B–NS3 protease have thus far focused mainly on natural or modified peptidic compounds,[10] and only a few non-peptidic compounds have been reported.[11] To date, various peptides containing highly charged moieties have been shown to have good inhibitory activity against the WNV NS2B–NS3 protease in vitro,[10a,b] underscoring the role of electrostatic interactions. However, a major disadvantage of peptide compounds is their short physiological half-life, as peptides are easily cleaved and inactivated in the body. Furthermore, high drug clearance rates in vivo lead to diminished therapeutic effects. For non-peptidic inhibitors, scaffolds reported earlier include compounds with isothiourea (IC_{50} = 183 μM)[11a] or guanidino[11b] moieties (K_{IC} = 13 ± 1 μM), sulfonylpyrazolyl (IC_{50} = 0.105 μM),[11c] 8-hydroxyquinoline (K_{IC} = 3.2 ± 0.3 μM)[11d] and isoquinoline (IC_{50} = 30 μM)[11e] derivatives. The most potent non-peptidic inhibitors known are several sulfonylpyrazolyl derivatives, which were identified by high-throughput screening of more than 65 000 compounds at the National Institutes of Health.[11c] However, these compounds were unstable under physiological conditions and degraded within one hour in aqueous buffer. Attempts to improve their stability without compromising biological activity proved unsatisfactory.[12]

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Hence, there is a need to examine other compounds for their activities and suitability as WNV protease inhibitors. Herein we describe the identification of a new scaffold with promising inhibitory activity against WNV NS2B–NS3 protease, the library synthesis and biological evaluation of 72 analogues of the lead compound, and the structure–activity relationship (SAR) data obtained.

Results and Discussion

Identification of the initial hit and synthesis of its analogues

In the preliminary WNV NS2B–NS3 protease inhibition assay, a collection of 110 compounds with various scaffolds were screened at 100 μM to filter for potential inhibitors. This initial screen provided a rapid identification of compounds that show the potential to inhibit WNV NS2B–NS3 protease. One of the hit compounds identified, compound 1a1 (Figure 1), was of particular interest, because to our knowledge, this is the first zwitterion-type inhibitor identified, and we reasoned that such an entity, together with its surrounding amino groups, could function like highly charged peptidyl-recognition components to provide favorable binding interactions. To provide access to structurally diverse analogues of compound 1a1 for biological evaluation of their inhibitory activities, a focused library of 1a1 analogues was synthesized by modifying a published procedure.[13]

The general strategy for the synthesis of compound 1a is shown in Scheme 1. A warm, aqueous solution of chloral hydrate and sodium sulfate was first reacted with an acidic solution of the respective aniline 3 followed by aqueous hydroxylamine at 90 °C to give isonitrosoacetanilide[14] 4, which precipitated upon cooling from the reaction mixture in 80–95% yield. Polychronopoulos et al.[15] reported the cyclization of 4 with ~68 equiv of concentrated sulfuric acid. However, in our hands, this large amount of concentrated acid hindered the precipitation for isatin 5. We explored the reaction by using a lower amount of concentrated sulfuric acid and found that at 46 equiv H2SO4, the cyclization reaction proceeded equally readily; the crude 5, which precipitated easily, could be used in the next step of the reaction without purification. Compound 5 was subsequently allowed to react at reflux with thiosemicarbazide in 10% aqueous potassium hydroxide[13a] to yield intermediate 6, which was precipitated from the reaction mixture at pH 5 as a yellow solid. Intermediate 6 was treated with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) at room temperature[16] to the corresponding compound 2a.

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with R² as a phenyl group (Table 1, compound 1a9), the inhibitory activity was also much better than that of compound 1a1. As having a phenyl group also made the compound more drug-like,¹⁴ we carried out further SAR studies of this novel scaffold type. Aromatic rings carrying an electron-donating or electron-withdrawing group at different positions of the phenyl ring were synthesized (Table 1, compounds 1a13–1a32), and the screening results showed that the presence of a p-chloro, p-bromo, or p-thiomethyl substituent on the phenyl ring (Table 1, compounds 1a21–1a23) significantly amplified the compound’s inhibitory activity.

We next proceeded to tune the R¹ group of the two strongest lead compounds—1a21 and 1a23—by first replacing H (=R⁰) with an electron-withdrawing group, such as a halide. This did not provide any improvement in the inhibitory activities of the two compounds (Table 1, compounds 1a33–1a39). However, if R¹ was changed to an electron-donating methyl group (Table 1, compounds 1a40–1a47), a slight enhancement in the inhibition of WNV NS2B–NS3 protease was observed in one of the two compounds (Table 1, compound 1a40). However, increasing the size of the alkyl group from methyl to ethyl resulted in a complete loss of inhibitory activity for the two compounds (Table 1, compounds 1a48–1a49).

To explore the effect of the Ar group on the biological activity of the compound, we replaced the phenyl ring with a phenyl group containing either an electron-donating or electron-withdrawing substituent (Table 1, compounds 1a50–1a67). The best inhibition was observed with compound 1a53, which contains a 4-methoxyphenyl group at the Ar position and a 4-(methylthio)phenyl moiety at R². In addition, the inhibitory activity of compound 1a52 is similar to that of 1a21. Finally, we synthesized compounds 1a68 and 1a69 to analyze the combined effects of the preferred substituents at R¹, R², and Ar. However, the inhibitory activities of compounds 1a68 and 1a69 were weaker than those of compounds 1a23, 1a40, or 1a53, the three strongest candidates identified from our earlier SAR studies.

Considering that the compounds tested thus far had been racemates, we proceeded to investigate the influence of stereochemistry on the compounds’ biological activities. However, attempts to separate the R and S enantiomers of 1a23, 1a40, and 1a53 proved futile, as a single peak was constantly observed in chiral HPLC analysis. In addition, removal of the chiral...
center by DDQ oxidation gave a new scaffold 2a, the inhibitors from which were completely inactive toward WNV NS2B–NS3 protease. (Table 1, compounds 2a1–2a3). Thus compounds 1a23, 1a40, and 1a53 were used for subsequent in vitro evaluations.

The stabilities of compounds 1a23, 1a40, and 1a53 in the assay buffer (pH 8) were determined by analyzing the amount of compound remaining over time by LC–MS–IT-TOF detection. Quantification of the compounds was determined by LCsolution software (Shimadzu). Compounds 1a23, 1a40, and 1a53 showed excellent stability in pH 8 buffer.

We next evaluated the cytotoxicities of compounds 1a23, 1a40, and 1a53 against baby hamster kidney fibroblasts (BHK21 cells) for incubation times of 48 and 72 h. Dimethyl sulfoxide (DMSO) was also included to serve as vehicle control, as the test compounds were dissolved in DMSO. The amount of DMSO used in the assay was kept constant at 0.2% of the total volume; this is to ensure that DMSO does not result in cytotoxicity toward the cells. From the results obtained (Figure 2), compounds 1a23, 1a40, and 1a53 were shown to be non-cytotoxic to BHK21 cells. To determine the mode of inhibition of compound 1a40, we followed the kinetics of inhibition with respect to substrate, with a $K_i$ value of 4.47 ± 0.37 μM.

**Docking analysis**

The crystal structure of the WNV NS2B–NS3 protease in complex with peptide Bz-Nle-Lys-Arg-Arg-H (PDB ID: 2FP7) was reported earlier. To identify the potential binding site of compound 1a40 in NS2B–NS3 protease, in silico docking studies were performed by using these crystal structure coordinates. As 1a40 is an uncompetitive inhibitor of WNV NS2B–NS3 protease, we focused our analysis on regions beyond the substrate binding sites. Earlier studies have shown that integration of residues 78–87 of NS2B into the protease–cofactor complex affected the formation of the catalytic active sites. Deletion or mutagenesis of these residues produced an inert enzyme. Therefore, the region on NS3 protease where key interactions with the NS2B cofactor occur (residues 78–87) is important for NS2B–NS3 protease activity, and particular attention was paid to this region during our docking studies.

Because compound 1a40 contains a chiral center, the $R$ and $S$ enantiomers were separately analyzed for their interactions with the WNV NS2B–NS3 protease. Our docking analysis predicted that the binding energies for both enantiomers with the protease are similar [(S)-1a40: −8.10 kcal mol$^{-1}$; (R)-1a40: −8.22 kcal mol$^{-1}$]. Amino acid residues that are in close proximity (<5 Å) to the atoms of the $S$ enantiomer include Arg78, Leu79, and Phe85 of NS2B and Ser71, Lys73, Glu74, Arg76, Lys88, Pro119, Glu120, Ile123, Ala164, Ile165, and Gln167 of NS3 protease. Residues in close proximity (<5 Å) to the atoms of the $R$ enantiomer are Val77, Arg78, and Leu79 of NS2B and Lys88, Thr118, Glu120, Ile123, and Gln167 of NS3 protease. The guanidine group in Arg78 of the NS2B cofactor binds strongly with the Ar group of the $S$ enantiomer via $\pi$–cation-type interactions. A strong $\pi$–cation interaction was also observed between the 4-chlorophenyl moiety of (S)-1a40 and Lys73 on the NS3 protease (Figure 4a). On the other hand, (R)-1a40 could potentially form a hydrogen bond with Lys73 on the NS3 protease as well as a $\pi$–cation interaction with the same residue. However, we did not observe any strong interaction between the $R$ enantiomer and key residues from the NS2B binding cavity (Figure 4b). This indicates that the $S$ enantiomer of 1a40 interferes more strongly with the productive interactions between the NS2B cofactor and the NS3 protease domain; it is therefore the preferred isomer for inhibition of WNV NS2B–NS3 protease.
system with a Phenomenex C18 column (50 mm x 3.0 mm, 5 µm). Purity of the compounds was determined at λ 254 nm, and integration was carried out with Shimadzu LCsolution software. 1H and 13C NMR spectra were recorded using CDCl3, [D]acetone, or [D]DMSO as solvents and tetramethylsilane (TMS) as internal reference on a Bruker AMX500 or a Bruker ACF 300 Fourier-transform spectrometer. The following abbreviations are used to denote multiplicities: s (singlet), d (doublet), t (triplet), dd (doublet of doublets), q (quartet), and m (multiplet). The number of protons (n) for a given resonance is indicated as nH. Mass spectrometry was performed on a Finnigan MAT LCQ mass spectrometer using electron spray (ESI) ionization.

General procedure for the synthesis of 4: C10H7(OH)2 (3.84 g, 23.2 mmol) and Na2SO4 (60 g, 422 mmol) were dissolved in H2O (20 mL) in a 500 mL beaker and warmed to 35 °C. A warm solution of the commercially available aniline or its derivatives 3 (21.5 mmol) in H2O (6 mL) and an aqueous solution of concentrated HCl (1.83 mL, 22 mmol) were added (a white precipitate of the aniline salt was formed) followed by a warm solution of NH2OH·HCl (4.72 g, 67.94 mmol) in H2O (8.25 mL). The mixture was heated at 90 °C for 2.5 h and then allowed to cool to 50 °C before filtering. The pale-cream solid obtained was washed by stirring with H2O (100 mL), filtered, and dried overnight in a vacuum oven at 50 °C to give the corresponding isonitrosocarbazanilide 4 in 80–95% yield.

General procedure for the synthesis of 5: Concentrated H2SO4 (18 mL, 337.7 mmol) was heated in a 250 mL beaker at 60 °C. A warm solution of the respective dried compound 4 (7.3 mmol) was added portionwise with stirring over 30 min so that the temperature did not exceed 65 °C. The mixture was then heated at 80 °C for 15 min, allowed to cool to 70 °C, and cooled on ice-cold H2O. The solution was then poured very slowly into ice and allowed to stand until a reddish precipitate of isatin 5 was formed. The crude solid 5 was filtered and used in the next step without further purification.

General procedure for the synthesis of 6: The respective isatin 5 (10.2 mmol) was dissolved in 10% aqueous KOH (10 mL, 45.5 mmol) and then treated with thiosemicarbazide (985 mg, 10.7 mmol). The reaction mixture was heated at 115 °C for 1 h, cooled to room temperature, and then poured over ice. Acidification of the reaction mixture to pH 5 with glacial acetic acid provided a yellow fluffy precipitate, which was filtered, washed copiously with H2O, dried in air and then dried in a vacuum oven (at 50 °C) to afford a yellow solid 6 in 60–78% yield. Purification of compound 6 by column chromatography gave pure product at 52–70%.

General procedure for the synthesis of 7: The respective crude compound 6 (6 mmol) was dissolved in a mixture of EtOH (12 mL) and 10% aqueous KOH (18 mL, 27.3 mmol), then treated with a solution of the respective benzyl bromide (6 mmol) and allowed to stir overnight. Thereafter, the reaction mixture was diluted with Et2O (100 mL) and H2O (70 mL). The ether layer was separated, and the aqueous layer was washed with Et2O (3 x 100 mL) and then poured over ice. Upon careful dropwise addition of glacial acetic acid with vigorous shaking at 0–4 °C, a yellow precipitate formed. This precipitate was filtered, washed with H2O (3 x 30 mL), and dried in a vacuum oven (at 50 °C) to provide compound 7 in 80–90% yield.

General procedure for the synthesis of 1a: To a suspension of the respective compound 7 (0.32 mmol) in anhydrous EtOH (6 mL) was added glacial acetic acid (25 μL, 0.42 mmol) followed by the respective aldehyde (0.42 mmol, 1.3 equiv). The reaction mixture was
held at reflux for 10–15 min, resulting in a dark-red solution. Upon cooling to room temperature, the orange–yellow solid 1a precipitated from solution. Sometimes the hot reaction mixture contained insoluble particles, which were removed by filtering the reaction mixture under hot conditions. The filtrate was then cooled to room temperature to provide the crude solid compound 1a. The crude product was recrystallized with EtOH, filtered, and washed with cold EtOH (2 × 3 mL). In a few cases, the product was purified by column chromatography and then dried in a vacuum oven at 50 °C. Compound 1a was obtained in 50–90% yield.

**General procedure for the synthesis of 2a:** The respective compound 1a (0.16 mmol) was dissolved in CH₂Cl₂ (10 mL), and the reaction mixture was kept at 0 °C followed by slow addition of DDQ (0.2 mmol). After the addition of DDQ, the temperature of the reaction mixture was allowed to rise to room temperature and was kept at this temperature for 4 h. During this time, the reaction was monitored by TLC. Upon completion, the reaction mixture was extracted with a mixture of CH₂Cl₂/H₂O (20:30). The organic layer was concentrated, purified by column chromatography (EtOAc/hexane 1:4→1:1) and dried under vacuum at 50°C to yield compound 2a in 70–85% yield.

**Biology**

All compounds used in biological assays were ≥98% pure. Compounds were tested against WNV NS2B–NS3 protease using the Sensolyte 440 WNV Protease Assay Kit (Cat. #72079) and active recombinant WNV protease (Cat. #72081), which were purchased from AnaSpec (USA). This protease assay kit uses the fluorogenic peptide Pyr-RTKR-AMC as substrate. Eight different substrate concentrations ranging from 1 to 80 μM were incubated in 96-well plates with 0.3 μM mL⁻¹ recombinant WNV protease at 37°C in the buffer provided. The increase in fluorescence intensity was monitored with an Infinite F200 microplate reader (Tecan, Switzerland) at an excitation wavelength of 354 ± 10 nm and an emission wavelength of 442 ± 15 nm. The initial velocity was determined from the linear portion of the progress curve, and the value of Km (3.45 ± 0.41 μM) was determined by the Michaelis–Menten equation: \(v = \frac{v_{\max} [S]}{[S] + K_M}\). Triplicate measurements were taken at each data point, and the data are reported as mean ± SE. In the preliminary WNV NS2B–NS3 protease inhibition assay, compounds 1a and 2a were screened at a fixed concentration of 100 μM to highlight potential inhibitors. Compounds effecting > 50% inhibition were further investigated for their IC₅₀ determination.

**Determination of IC₅₀:** For IC₅₀ calculations, recombinant WNV protease at a concentration of 0.3 μg mL⁻¹ and seven different concentrations of the inhibitor ranging from 10 nM to 100 μM were used. For each experiment, the protease was pre-incubated with the inhibitor at 37°C for 15 min in separate wells, and enzyme reactions were initiated by adding the Pyr-RTKR-AMC substrate at a final concentration of 16.7 μM. The increase in fluorescence intensity was monitored continuously with an Infinite F200 microplate reader at an excitation wavelength of 354 nm and emission wavelength of 442 nm. Fluorescence values obtained from the positive control (no inhibitor) were considered as 100% complex formation, and those values obtained in the presence of inhibitors were calculated as the percentage of inhibition relative to control. Triplicate measurements were obtained at each data point. The IC₅₀ values were calculated using a sigmoidal dose–response curve with the GraphPad Prism 3.0 software package (San Diego, CA, USA). Triplicate measurements were recorded as the mean ± SE.

**Determination of inhibition mechanism:** Two different inhibitor concentrations and a no-inhibitor control (0–25 μM) were each assayed at five substrate concentrations ranging from 3.3 to 83.3 μM. In each assay, the enzyme and inhibitor were incubated at 37°C for 15 min, followed by the addition of the substrate to start the kinetic measurements. The rate of substrate cleavage (v) was monitored using the F200 microplate reader. To illustrate the inhibition mechanism, 1/V versus 1/[S] was plotted for each inhibitor concentration using OriginPro 8.

**In silico studies**

Molecular docking simulations were performed with the AutoDock4 program. Both enantiomers of compound 1a were constructed in ChemBio 3D Ultra 11.0 by energy minimization. The active site of WNV protease was prepared with the crystal structure of WNV NS2B–NS3 protease in complex with peptide Bz-Nle-Lys-Arg-Arg-H (PDB ID: 2FP7). The structure was stripped of all water molecules and bound ligand. Hydrogen atoms were added to all polar atoms of the protein, followed by addition of Gasteiger–Marshall charges. AutoDock simulations were performed using the Lamarckian Genetic Algorithms (GA) subroutine at default settings for GA population size, crossover rate, mutation rate, and starting with fully randomized ligand position, orientation, and conformation; 50 GA runs were performed for the inhibitor–enzyme pair, and results were analyzed using Discovery Studio 3.1 client.

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