Project Title:

Inhibitors of the NS3 Proteinase of West Nile Virus

Grant Number:

MH077601

Screening Center Name:

University of Pittsburgh Molecular Screening Center

Principal Investigator of Screening Center:

John S. Lazo

Assay Submitter & Institution:

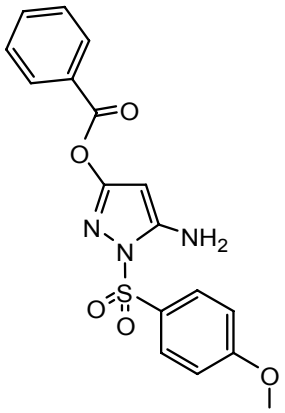
Alex Y. Strongin
Inflammatory and Infectious Disease center
Burnham Institute for Medical Research

Assay or Pathway Target:

West Nile Virus NS2B-NS3 Proteinase

Probe PubChem Compound Identifier (CID):

PubChem-CID 655490 (PubChem-SID 852843)



Specific Aim: Identify drug-like small molecule inhibitors of the NS3 West Nile virus protease by high throughput screening of chemical libraries.

Future plans include optimization the structure of the novel, low-molecular weight, synthetic inhibitors of the West Nile and Dengue virus NS3 proteinase and to validate the selectivity and potency of the selected compound leads in additional in vitro tests and assays. In addition, we will determine, at the atomic resolution level, the structure of the NS3 protease bound to lead antagonists. Recent exciting discoveries directly related to this particular project [1] and vast amounts of structure-activity data that will should flow from this project should facilitate creation of therapeutically important, anti-viral, drugs.

Significance: The purpose of this project was to identify small molecules that target the essential processing NS2b-NS3 serine proteinase of flaviviruses, a single proteinase encoded by the West Nile and Dengue viral genome. The presence of the functionally active, mature NS3 proteinase is absolutely essential for the virus life cycle, virus propagation and disease progression. The immediacy and intensity of our effort is directly responsive to the US government's decision that flaviviral infections caused by these viruses are a potential weapon of a bioterrorist. These viruses are categorized by NIAID as Category A-C priority pathogens. There are also hundreds of millions of cases of flaviviridae infections worldwide and, in addition, thousands of cases of West Nile virus in the US. Currently, there are no effective countermeasures against flaviviral infections. Despite the needs of Biodefense and a growing number of naturally-infected patients, there is currently no specific treatment or vaccine to cure or prevent West Nile and Dengue infections. Obviously, there is an urgent need for a potent and safe antiflavivirus therapy.

It is true that biological weapons have become a priority in international security plans. The offensive bioweapon programs are suspected to be covertly pursued in several countries from which the weaponized biomaterials may be secretly acquired by international terrorist organizations. These events have awakened the awareness of the US. Bioterrorist attacks are especially difficult to predict, detect, and prevent, and they are, sociologically, the most feared scenarios. Bioweapons, when compared with nuclear and chemical weapons, are relatively inexpensive and easy to manufacture in mass quantities, in addition to being potent and fearsome.

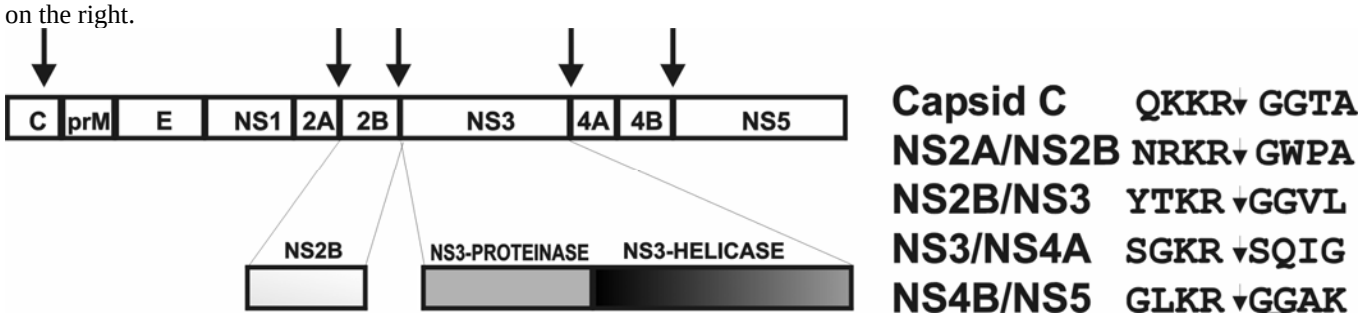
Currently, there are millions of cases of flaviviridae infections, especially Dengue throughout the world. In addition, Dengue haemorragic fever flaviviruses are of a special concern because of their potential use as bioweapons. Dengue virus has been rated by the NIAID as a Category A Priority Pathogen. West Nile virus is ranked as a Category B Priority Pathogen. In addition, West Nile virus is an emerging natural viral pathogen in the US.

The Assay Submitter believes that targeting the individual, unique NS3 processing protease, which is critical for the maturation of the viral proteins, could be a successful drug strategy for blocking the flaviviral infection and for treating patients with West Nile and Dengue worldwide. These novel inhibitors could also be valuable prophylactics in bioterror/battlefield situations and chemical probes for virologists and other biologists interested in these viruses. The primary objective of the project described here is to identify potent, synthetic inhibitors, which will inactivate the flaviviral NS3 serine proteinase. Based on the results of the initial inhibitory screens, the follow-on screens and the structure-activity relationships, we and others could perform reiterative design, chemical syntheses and in vitro analyses to develop the "mature", drug-like inhibitor compounds. We and others could synthesize, purify, and test the mature inhibitory leads in in vitro and cell-based assays. These studies cannot be successfully accomplished within a reasonable time frame without a complete understanding and knowledge of the precise fundamental structure-function relationship and the spatial structure of the NS3 proteinase. This knowledge is essential, not only for subsequent, expedient and rational, structure-based,lead optimization efforts, but it is also crucial for understanding the pathology of the disease. Reciprocally, understanding of the viral pathogenesis is required for finding alternative approaches and novel, currently uncharacterized, avenues for the anti-flavivirus therapies. The atomic resolution structure and the

unique enzyme characteristics of the NS3 proteinase can be used as templates for the design and the follow-on optimization of the specific, potent and safe anti-protease inhibitory drug leads.

Rationale: West Nile virus, a member of the Flaviviridae family, was first isolated in 1937 in the West Nile district of Uganda [2]. West Nile virus is transmitted to animals including humans, through mosquito bites. Mosquitoes become infected when they feed on infected birds [3]. In 2003, West Nile virus was detected in as many as 46 of the United States. The virus infected as many as 10,000 people and was the cause of approximately 300 deaths. The data for 2004 are even more troubling because the virus has spread and intensified throughout the US. West Nile virus is also a potential bioterrorism weapon. West Nile virus, Dengue and Yellow Fever flaviviruses are Priority Pathogens according to the classification issued by the NIAID. This project targets the NS3 proteinase of West Nile virus and the structurally and functionally similar NS3 proteinase from mosquito-born Dengue type 2 virus. Similar to other flaviviruses, West Nile has an icosahedral core (30- to 35-nm in size) comprising multiple copies of a 12-kDa capsid protein. The capsid encloses a single-stranded RNA with a single reading frame encoding a polypeptide precursor of approximately 3,400 amino acid residues (Fig. 1). There are three structural proteins (C, prM and E) and seven non-structural proteins encoded by the West Nile virus genome (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). Both furin of the host and the virus-encoded NS3 serine proteinase are required to process the polyprotein precursor into the individual functional proteins [4-25].

Figure 1. Organization of the WNV polyprotein precursor, showing cleavage sites by the NS2B-NS3 protease (arrows) and the parameters of the full-length NS2B-NS3 proteinase-helicase protein. The sequence of the cleavage sites is shown on the right.



The full-length NS3 peptide sequence in West Nile and Dengue viruses represents a multifunctional protein. The N-terminal 184 amino acid-long fragment of NS3 represents the NS3 proteinase. The C-terminal portion of the NS3 protein encodes a nucleotide triphosphatase, an RNA triphosphatase and a helicase. The NS3 proteinase is required for the maturation of the virus. The NS3 proteinase is responsible for cleaving the NS2a/NS2b, NS2b/NS3, NS3/NS4a and NS4b/NS5 junction regions [9, 15, 26]. This proteinase is also responsible for the cleavage at the C-terminal region of the C protein. As is the case with a number of flaviviruses, the NS2b protein, that is located in the polypeptide precursor upstream of the NS3 proteinase, functions as a cofactor and promotes the proteolytic activity of the NS3 enzyme. The cofactor activity of the 40 amino acid long central portion of the NS2b is roughly equivalent to that of the entire NS2b sequence. Most importantly, inactivating mutations of the NS3 cleavage sites in the polyprotein precursor abolish virus infectivity. The Assay Submitter hypothesizes that the processing NS3 proteinase, which is an essential component of the virus life cycle, is the most promising drug target for anti-flaviviral inhibitors, from which novel, anti-viral therapies will emerge.

Assay Implementation and Screening

PubChem Bioassay Names:

1. Primary Screen: HTS to identify Inhibitors of West Nile Virus NS2BNS3 Proteinase MH077601 **AID 577**
2. Concentration Response Confirmation Assay: West Nile Virus NS2BNS3 Proteinase Inhibitor Dose Response Confirmation **AID 653**

Primary Assay Description as defined in PubChem:

The primary objective of the HTS described here is to identify small molecule inhibitors that will inactivate the flaviviral NS3 serine proteinase. A homogenous, mix-and-measure, fluorescence peptide cleavage assay was proposed as the primary screening assay format. The cDNA fragment of the West Nile genome encoding the NS2b-NS3 proteinase were cloned from cDNA fragments provided by Drs. Richard Kinney, CDC, Fort Collins, CO, and Michael Diamond, Washington University, St. Louis, MO. The wild-type NS2b-NS3 proteinase construct was expressed in E. coli and pilot-scale quantities of the homogeneous material were purified by Dr. Strongin and his colleagues at the Burnham Institute. Autolysis of the NS2b-NS3 precursor was used to generate the soluble, mature and homogenous NS3 proteinase. The cleavage assay employs the proteolytic enzyme, purified NS3 proteinase of West Nile virus provided by Dr Strongin of the Burnham Institute, and a commercially available fluorescence substrate Pyr-RTKR-AMC. To reduce reagent requirements and to increase throughput, the PMLSC miniaturized a 96-well plate assay used in the Burnham's NS2B-NS3pro studies from a 100 μL total volume to a 15 μL assay in low volume 384-well microtiter plates (Greiner BioOne). An EP3 equipped with a 384-well transfer head was used to transfer tested compounds (5 μL; 10 μM final concentration), then NS2B-NS3pro (5 μL ; 10 ng/well) and lastly the Pyr-RTKR-AMC substrate (5 μL; 60 μM final concentration) Bachem (King of Prussia, PA). In the HTS, the enzyme was added to the compounds in the wells of the assay plate and pre-incubated for 30 min prior to the initiation of the reaction by the addition of substrate. The final concentration of the buffer was: 10 mM Tris-HCl (pH 8.0) including 20% glycerol, 0.005% Brij-35 and 1% DMSO. In the primary screen, the reactions were terminated after a 90 min incubation at ambient temperature by adding a NS2B-NS3pro inhibitor (5 μL of 4 μM aprotinin; Ki= 25 nM) to the individual wells. The resulting fluorescence was measured at λex=360 nm and λem=460 nm using a SpectraMax M5 fluorescence spectrophotometer (Molecular Devices, Sunnyvale, CA).

NS2B-NS3pro HTS Assay Conditions

1) Dilute compound plates in 20% glycerol (10 to 100 μM final, 1% DMSO).
2) Transfer 5 μL of diluted compounds and plate controls in 20% glycerol to wells of 384-well black low-volume 384-well assay plate, Greiner # 784076.
3) Transfer 5 μL/well 2 μg/mL NS2b-NS3 enzyme solution (3X assay buffer) to all wells.
4) Transfer 5 μL/well of180 μM Pyr-RTKR-AMC substrate to all wells.
5) Centrifuge plate at 50xg for 30 secs-1 min.
6) Incubate assay plate at ambient temperature with shaking for 90 min. Do not stack plates!
7) Stop assay by transfer of 5 μL/well of 4 μM aprotinin to assay plate.
8) Read fluorescence intensity at Ex 360 nm, Em 460 nm on the M5 plate reader.

The West Nile Virus NS2bNS3 HTS conducted at the PMLSC utilized a Z score statistical scoring method to identify active compounds (Brideau et al., 2003 J. Biomolecular Screening 8: p634-637).

The Z score for a compound is computed on a plate-by-plate basis. The Z score for the raw fluorescent intensity value Xi is defined as Zi = (Xi-Xm)/Sm, where Xm is the mean of all the raw fluorescent intensity

values of the compounds on a plate (n=320), and Sm is the standard deviation of all these values. A cut off Z score of -3 was selected as the active criterion for the West Nile Virus NS2bNS3 HTS.

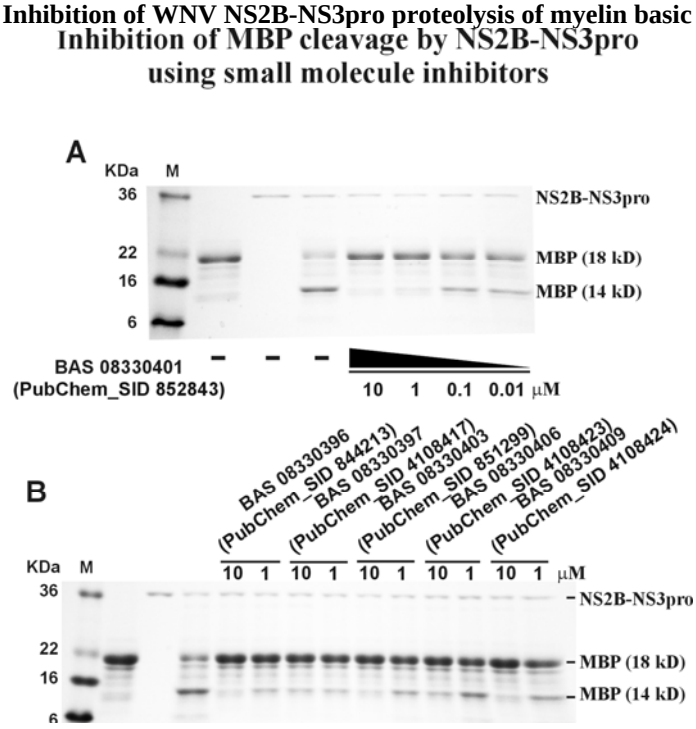
West Nile Virus NS2bNS3 Activity scoring rules:
PUBCHEM_ACTIVITY_OUTCOME
1 - Substance is considered inactive when Z-score is > -3
2 - Substance is considered active when Z-score is </= -3
3 - Substance activity outcome is inconclusive

Center Summary of the Primary Screening Assays:

- Primary HTS - 65,239 compounds were screened at 10 μM, **AID 577**
  - 119 (0.18%) compounds exhibited a Z-score < -3
  - A cherry pick order for 126 compounds was submitted to Biofocus-DPI for compounds that exhibited a Z-score < -3 in the primary screen
- 10-point concentration response confirmations, **AID 653**
  - 15 (11.9 %) compounds exhibited IC_{50}s < 50 μM; 4 exhibited IC_{50}s < 1.0 μM, 6 exhibited $IC5_{50}$s in the 1.0 to 10 μM range, and 5 exhibited IC_{50}s in the 10 to 50 μM range
  - Compound structures that exhibited IC_{50}s < 50 μM were analyzed using the Leadscope Enterprise 2.4.6-1 software and subjected to structure based clustering and classification techniques based on recursive partitioning
    - 5 singletons and one cluster of 10 structurally related compounds

Summary Secondary Assays Performed by Assay Provider:

Figure 2. Inhibition of WNV NS2B-NS3pro proteolysis of myelin basic protein



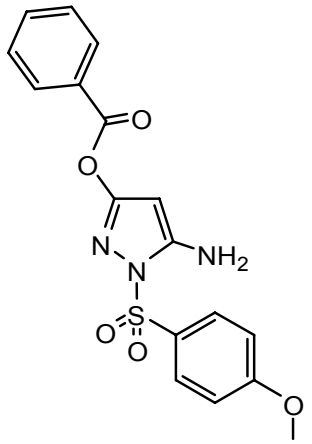
In addition to blocking the proteolysis of the low molecular weight Pyr-RTKR-AMC peptide substrate, the 5-amino-1-(phenyl)sulfonyl-pyrazol-3-yl inhibitors efficiently inhibited the WNV NS2B-NS3pro proteolysis of myelin basic protein (MBP). The inhibitory effect of the most promising inhibitor we identified (SID 852843) was observed at the concentration as low as 10 nM (Fig. 2). **Fig. 2:** MBP (4 μg; 11 μM) was co-incubated with NS2B-NS3pro (0.36 μg; 0.5 μM; an enzyme/substrate ratio - 1:22) for 60 min at 37°C. Where indicated, inhibitors (SID 852843, SID 844213, SID 4108417, SID 851299, SID 4108423, SID 4108424) (0.01–10 μM) were each added to the reactions. Inhibitors were preincubated with NS2B-NS3pro for 60 min. The cleavage was stopped by adding a 5xSDS sample buffer. The digested samples were analyzed by SDS/PAGE using 4–20% polyacrylamide gels.

In contrast, this class of WNV NS2B-NS3pro inhibitors was inactive against the structurally-similar NS2B-NS3 proteinase of Dengue type 2 virus and the NS4A-NS3 proteinase of hepatitis C virus.

The inhibitors are especially interesting because they do not directly bind and inhibit the enzyme's active site, but bind instead to a different ("allosteric") surface that is required to generate the "productive" conformation of the enzyme. This class of inhibitors provides exciting leads for antiviral therapeutics, because they do not recognize human enzymes that share a similar active site but not the allosteric site. This greatly increases the probability of finding viral inhibitors with high specificity and few side-effects.

Probe Optimization:

WNV NS2B-NS3pro Probe



PubChem-SID 852843
PubChem-CID 655490
IC50 183 nM

Chemical name:
Benzoic acid 5-amino-1-(4-methoxy-benzenesulfonyl)-1H-pyrazol-3-yl ester.

(IUPAC: [5-amino-1-(4-methoxyphenyl)sulfonylpyrazol-3-yl] benzoate).

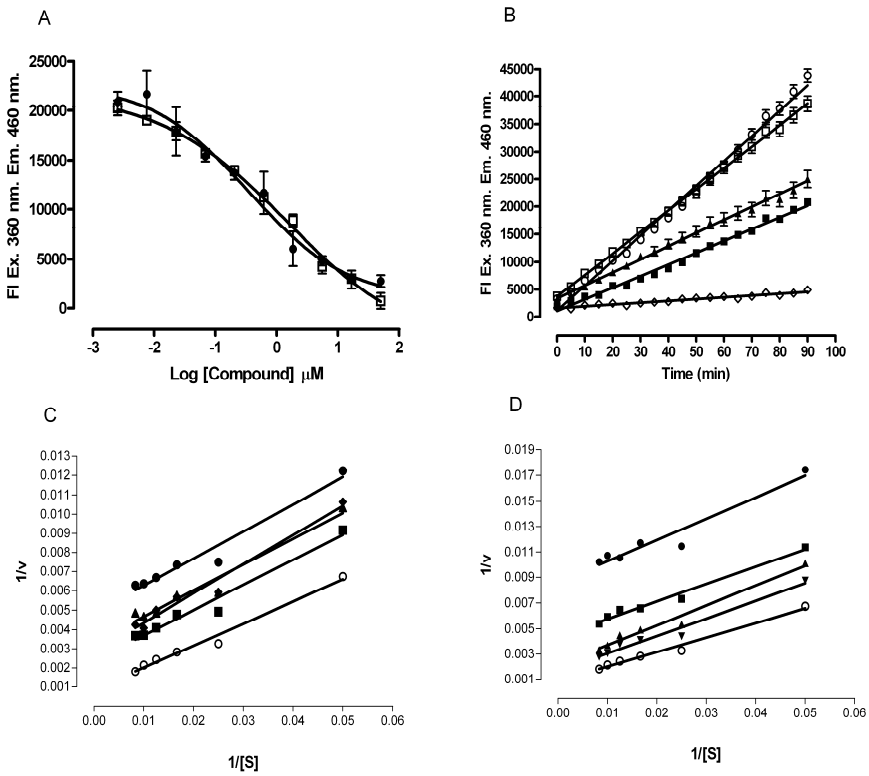
Molecular Formula:
$C_{17}H_{15}N_3O_5S$

Molecular Weight:
373.3831

Describe mode of action for biological activity of probe:
The inhibitory parameters of the two most efficient 5-amino-1-(phenyl)sulfonyl-pyrazol-3-yl compounds were analyzed in more detail (Fig. 3.). In good agreement with the concentration dependence confirmation assays, PubChem-SIDs 852843 and 4245669 produced IC_{50} values of 183 nM and 187 nM, respectively, in 10-point 3-fold dilution series IC_{50} assays conducted in triplicate (Fig 3A). The inhibition was reversible, since pre-incubation of 500 ng of the NS2B-NS3pro enzyme with 1.0 μM of 852843 or 4245669 for 30 min at ambient temperature followed by a 1:500 dilution of the enzyme-compound mixture and the addition of 60 μM substrate to 1 ng/well of enzyme in the presence of 2.0 nM inhibitor failed to inhibit enzyme activity (Fig 3B). Indeed, pre-incubation of the NS2B-NS3pro enzyme with 852843 or 4245669 appears to enhance enzyme activity post dilution (Fig 3B). To investigate the mechanism of inhibition of NS2B-NS3pro by these compounds we measured the initial velocity of the enzyme as a function of substrate concentration at fixed concentrations of the inhibitors; 0, 22.8, 68.6, 205.8 and 617.3 nM 852843 or 4245669 respectively (Fig 3C and 3D). In these experiments, the compounds were added to the enzyme and pre-incubated for 30 min prior to the initiation of the reaction by the addition of substrate. The raw kinetic fluorescence intensity data was acquired every 5 min on the M5 reader for 90 min and was imported into GraphPad Prism to perform linear regression analysis to obtain the initial velocities of the enzyme at the substrate and inhibitor concentrations indicated (data not shown). The data were then fit to the Michaelis-Menten equation using nonlinear regression to calculate Km and Vmax in the presence and absence of inhibitor (data not shown). Consistent with uncompetitive inhibition,

both the Vmax and apparent Km were decreased in the presence of inhibitor (data not shown). To illustrate the uncompetitive inhibition we created the double reciprocal plots of the data (Fig. 3C and 3D). A table was created of substrate concentration (X) versus velocity (Y) for each of the different inhibitor concentrations. Both the X and Y data sets were transformed to 1/X and 1/Y respectively, and the data were subjected to linear regression analysis. The parallel lines for the control data and the plus inhibitor data in the double reciprocal plots of $1/V_o$ vs 1/[S] at each inhibitor concentration were consistent with an uncompetitive mechanism of inhibition of the WNV NS2B-NS3 proteinase (Fig 3C and 3D). Our data suggest that the 5-amino-1-(phenyl)sulfonyl-pyrazol-3-yl class of NS2B-NS3pro inhibitor compounds perform as slow-binding, reversible, uncompetitive inhibitors (Johnston, P. A. et al, Assay and Drug Development Technologies, In press).

Figure 3. Characterization of WNV NS2B-NS3pro Inhibitors 852843 and 4245669.



(3A) Concentration dependent inhibition of WNV NS2B-NS3pro by 852843 and 4245669. Recombinant NS2B-NS3pro enzyme (10 ng/well) was incubated with 60 µM Pyr-RTKR-AMC and the indicated concentrations of 853843 or 4245669. The mean fluorescent intensity ± SD (n=3) values produced at each compound concentration after the 90 min incubation are plotted together with the resulting 4 parametric non-linear regression curves produced by the GraphPad Prism 3.03 analysis software. (●) 856843 and (□) 4245669. **(3B)** Inhibition of WNV NS2BNS3pro by 852843 and 4245669 is reversible. Compounds 852843 and 4245669 were pre-incubated at 1.0 µM with 500 ng of the NS2B-NS3pro enzyme for 30 min at ambient temperature and then the enzyme-compound mixture was diluted 1:500 in assay buffer, 60 µM Pyr-RTKR-AMC substrate was added to 1ng/well of enzyme, and the reaction was followed for 90 min.

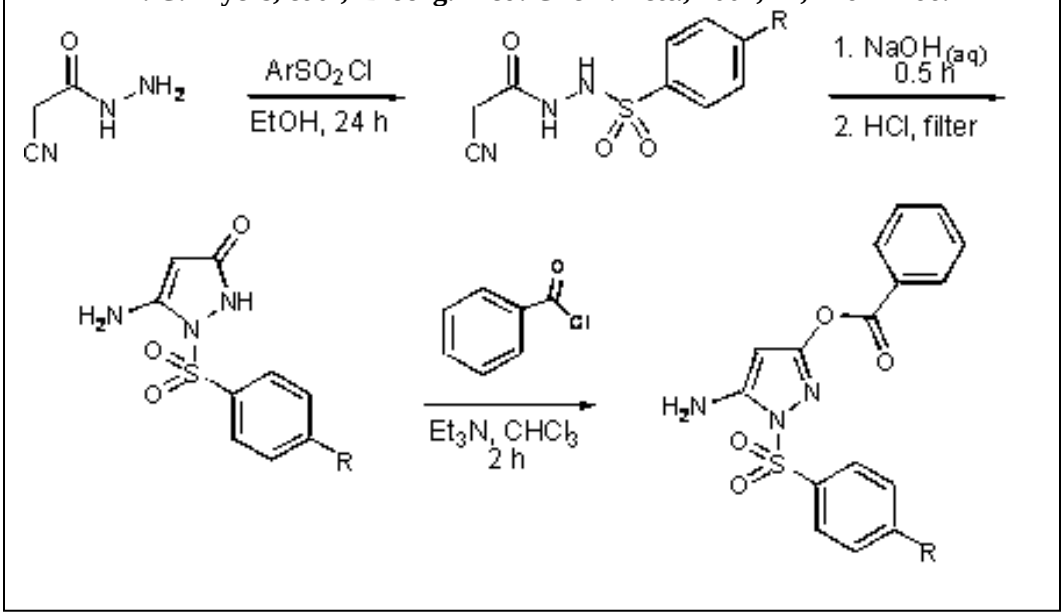
For comparison, NS2B-NS3pro enzyme preparations pre-incubated in assay buffer, or not pre-incubated at all, were tested on the same plate. The mean fluorescent intensity ± SD (n=50) values produced at each 5 min time point are plotted together with the resulting linear regression line produced using GraphPad Prism 3.03 analysis software. (□) NS2b-NS3 pre-incubated for 30 min with 1 µM 856843, (○) NS2b-NS3 pre-incubated for 30 min with 1 µM 4245669, (■) NS2b-NS3 pre-incubated for 30 min in assay buffer, (▲) NS2b-NS3 not pre-incubated prior to the initiation of the assay by substrate addition, and (◊) NS2b-NS3 not pre-incubated prior to the initiation of the assay by substrate addition but inhibited by 1.0 µM Aprotinin. Uncompetitive mechanism of inhibition of WNV NS2B-NS3pro by 852843 **(3C)** and 4245669 **(3D)**. The initial velocity of 10 ng/well NS2b-NS3 enzyme in triplicate wells of a 384-well plate was acquired over time at the indicated Pyr-RTKR-AMC substrate concentrations at fixed concentrations of the 852843 **(3C)** or 4245669 **(3D)** inhibitors respectively; (○) no inhibitor, (■) 22.8 nM, (◆) 68.6 nM, (●) 205.8 nM and (▲) 617.3 nM. Double Reciprocal Plots $1/V_o$ vs 1/[S] for 852843 (3C) and 4245669 (3D) at each inhibitor concentration were plotted using the Enzyme Kinetics template of the GraphPad Prism 3.03 analysis software.

Has the compound been provided to the MLSMR:
A sample of this compound is present in the MLSMR.

Center comments on chemistry strategy leading to probe identification:
This compound was identified in the primary screen, and is a constituent of the NIH MLSMR. A number of related analogs are also present, and provided preliminary SAR. Instability of this series of compounds was noted and occurs through reaction at the ester group, and subsequent hydrolysis to give the pyrazolone (Fig. 5. Appendices below). The reaction is particularly fast in the presence of nucleophiles (such as DTT, cysteine,). Several analogs in which the ester group was modified to non-reactive and non-hydrolyzeable moieties were prepared, but were devoid of activity (Fig. 6. Appendices below [27]).

Detailed synthetic pathway for making probe:

M. C. Myers, et al, Bioorg. Med. Chem. Lett., 2007, 17, 4761-4766.



Center Summary of Probe Properties:
From PubChem:
Hydrogen Bond Donor Count: 1; Hydrogen Bond Acceptor Count: 8;Rotatable Bond Count: 6; Tautomer Count: 3; Topological Polar Surface Area: 114; XLogP: 3.7;Exact Mass: 373.073241; MonoIsotopic Mass: 373.073241; Heavy Atom Count: 26; Charge: 0; Complexity: 580; Isotope Atom Count: 0; Defined Atom StereoCenter Count: 0; Undefined Atom StereoCenter Count: 0; Defined Bond StereoCenter Count: 0; Undefined Bond StereoCenter Count: 0; Covalently-Bonded Unit Count: 1

Leadscope Properties:

Structure ID	Parent Molecular Weight	ALogP	Hydrogen Bond Acceptors	Hydrogen Bond Donors	Lipinski Score	Molecular Weight	Parent Atom Count	Polar Surface Area	Rotatable Bonds
852843	373.3815	1.93	4	1	0	373.3815	26	113.51	6

Appendices:

I. Comparative data on probe, similar compound structures and prior probes

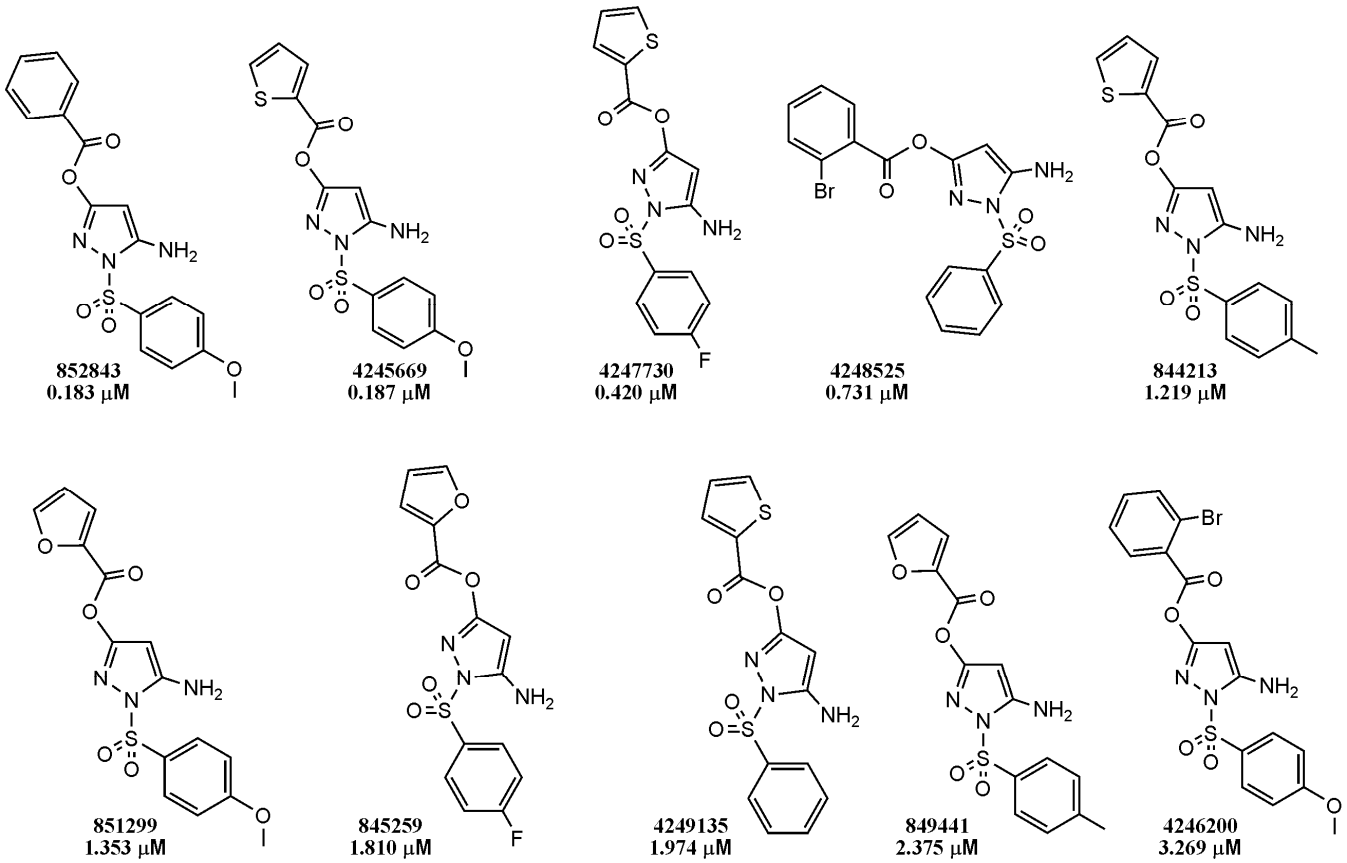
Currently, there are no inhibitors of flaviviral proteinases which can be transformed into promising drug leads and then into therapeutics. Aprotinin, a potent inhibitor of trypsin-like enzymes and also of flaviviral proteinases, is highly useful in the in vitro studies, but, because of the presence of the multiple additional targets in humans and because of its inability to penetrate efficiently into the cellular compartment, has an exceedingly low pharmacological value as an anti-viral therapeutic.

II. Structure Activity Data on the Inhibition of NS2B-NS3pro by Related 5-amino-1-(phenyl)sulfonyl-pyrazol-3-yl Compounds

Compounds were tested 10-point 3-fold dilution series concentration response curves run in triplicate starting at a maximum concentration of 50 μM (Fig. 4.).

Figure 4. Inhibition of NS2B-NS3pro by Related 5-amino-1-(phenyl) sulfonyl-pyrazol-3-yl Compounds.

5-amino-1H-pyrazol-3-yl SAR



III. Cross Target Query of PubChem Database

A cross target query of the PubChem database revealed that the confirmed WNV NS2B-NS3pro inhibitors were fairly selective in that they had been tested in between 83 and 124 bioassays (Table 1), but had only been flagged as active in 1-4 of those screens. The 5-amino-1-(phenyl)sulfonyl-pyrazol-3-yl compounds have exhibited activity against other protease targets, most notably the cysteine proteases Cathepsins B and G, and the serine proteases Factor XIa and Factor XIIa (Table 1).

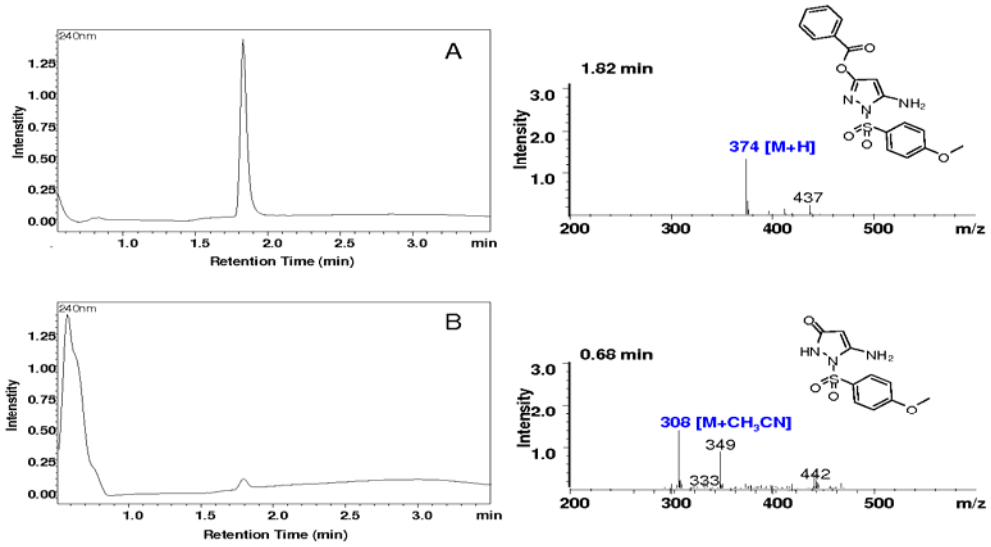
Table 1. WNV NS2B-NS3pro Inhibitor Confirmation and Cross Target Query Data.

WNV NS2B-NS3pro IC_{50} Confirmation and Cross target Query						
	HTS	**IC_{50} Confirmation**		**Cross-target Query of PubChem**		
PubChem SID	**Z-score**	**% Inhib at 50 μM**	**IC_{50} μM**	**# Assays**	**# Active Flags**	**Comments**
852843	-6.4	115.6	0.105	109	4	NS2bNS3, Cathepsins B & G, Factor XIa
4245669	-5.6	116.3	0.110	83	3	NS2bNS3, Cathepsins B & G
4246200	-4.2	112.5	0.297	91	1	NS2bNS3
851299	-7.7	99.3	0.553	109	3	NS2bNS3, Cathepsin B & Factor XIa
4247730	-5.3	99.1	1.161	91	2	NS2bNS3, Cathepsin B
4248525	-6.2	100.8	1.263	98	1	NS2bNS3
3717586	-11.4	105.9	1.353	110	6	NS2bNS3, CathepsinG, Factor XIa & XIIa, C1S, SIP3 Antag
845259	-11.2	107.7	1.651	96	3	NS2bNS3, Cathepsin B, Factor XIa
4249135	-8.1	101.4	2.376	102	5	NS2bNS3, Cathepsins B & G, Factor XIa & D1-Receptor
844213	-6.4	86.8	2.405	111	4	NS2bNS3, Cathepsins B & G, Factor XIa
842717	-4.5	98.2	10.068	99	16	NS2bNS3, HIV RNase H, Bfl-1, Hsp90, HPDE-C7& C7K cytox. Etc.
849441	-5.4	96.6	14.391	124	4	NS2bNS3, Cathepsin B, Factor XIa & XIIa
7976321	-4.2	56.0	34.090	94	2	NS2bNS3, SF-1
3712504	-3.1	51.6	38.573	110	4	NS2bNS3, BclB, ER-alpha, ER-beta
4257662	-3.0	68.8	47.708	119	7	NS2bNS3, HIV RNase H, MKP-1, Cdc25B, HPDE-C7K cytox., FAK, ER-alpha

A cross target query of the PubChem database revealed that the confirmed WNV NS2B-NS3pro inhibitors were fairly selective in that they had been tested in between 83 and 124 bioassays (Table 1), but had only been flagged as active in 1-4 of those screens. The 5-amino-1-(phenyl)sulfonyl-pyrazol-3-yl compounds have exhibited activity against other protease targets, most notably the cysteine proteases Cathepsins B and G, and the serine proteases Factor XIa and Factor XIIa (Table 1). Structurally related 3-substituted pyrazol-esters exhibit characteristics of reversible competitive inhibitors of Cathepsin B, and 4249135 was shown to be

unstable in the presence of DTT and appears to be a substrate for Cathepsin B [27]. To test whether 852843 and 4249135 were hydrolyzed under our assay conditions, we incubated the inhibitors at ambient temperature in 10 mM Tris-HCl, pH 8.0, containing 20% glycerol, 0.005% Brij-35 and 1% DMSO and then performed the LC-MS analysis of the inhibitor samples (Fig. 5.). The analysis confirmed that the ester bond in 852843 was hydrolyzed resulting in 5-amino-1-[(4-methoxyphenyl)sulfonyl]pyrazol-3-ol and benzoic acid (Fig. 5.), both of which failed to inhibit WNV NS2B-NS3pro (data not shown). The LS-MS data indicated that both 852843 and 4245669 exhibit a half-life of 1-2 hours in aqueous solutions, and that both compounds were quantitatively hydrolyzed in the course of an 18 hr incubation.

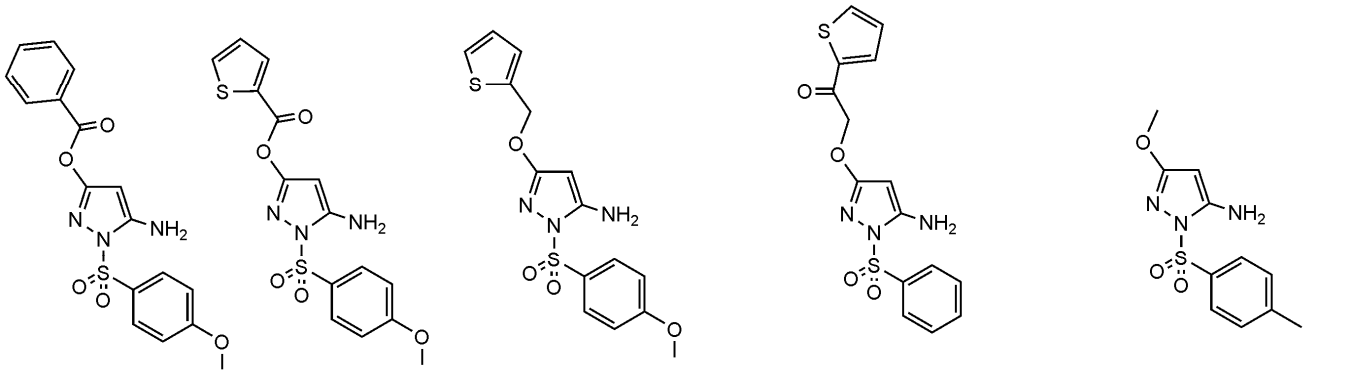
Fig. 5. LC-MS of 852843. 853843 was analyzed before (A) and after an 18 h incubation (B) in 10 mM Tris-HCl buffer, pH 8.0, containing 20% glycerol, 0.005% Brij-35 and 1% DMSO at ambient temperature. The LC and MS profiles and the structure of the initial inhibitor and the identified degradation product are shown.



Six of the ten substituted pyrazole ester WNV NS2B-NS3pro inhibitors identified in the screen have previously been characterized as reversible competitive inhibitors of the cysteine protease Cathepsin B, with IC50 values ranging from 0.25 to 1.26 μM [27]; PubChem-SIDs 4249135, 4247730, 4245669, 844213, 849441 and 845259. The stability of 4249135 in aqueous buffers was examined, and after a 1 hour of incubation in the presence of 2 mM DTT 4249135 was shown to undergo 72% conversion to a pyrazolone, compared with only 5% conversion in the absence of DTT [27]. However, after only a 15 min incubation in the presence of Cathepsin B, 100% of 4249135 was converted to the pyrazalone, prompting the authors to conclude that the 3-substituted pyrazolyl esters behaved as alternate Cathepsin B substrates rather than as inhibitors [27]. We have also observed that this class of compounds are unstable in aqueous buffers (Fig. 5.), but our assay buffer did not contain DTT, or any other reducing agent, and the 5-amino-1-(phenyl)sulfonyl-pyrazol-3-yl class of compounds behaved as uncompetitive inhibitors of the WNV serine NS2B-NS3 protease (Fig. 3.). However, based on our modeling studies (Fig. 7. below) we believe that the planar conformation provided by the ester is required for the observed inhibition of the WNV NS2B-NS3pro.

The ester group of the 3-subtituted pyrazolyl ester compounds was identified as the functional moiety important for activity against the cysteine protease Cathepsin B [27]. To examine the importance of the ester group for WNV NS2B-NS3pro inhibition, a series of analogs of 4245669 prepared by the Penn Center for Molecular Discovery (PCMD, Philadelphia, PA) chemistry core were tested and found to be inactive against NS2B-NS3pro (Fig.6.).

Figure. 6. Analogs of 4245669 with a modified pyrazol-3-yl ester linkage are inactive against WNV NS2B-NS3pro.
5-amino-1-(phenyl)sulfonyl-pyrazol-3-yl Analogs



852843
0.183 μM

4245669
0.187 μM

PCMD-CC-MM-I-80-126
> 50.0 μM

PCMD-CC-MM-I-195-289
> 50.0 μM

PCMD-CC-MM-I-20-41
> 50.0 μM

Three analog compounds of 4245669, in which the pyrazol-3-yl ester linkage was modified were obtained form the Penn Center for Molecular Discovery (PCMD) chemistry core; PCMD-CC-MM-I-20-41, PCMD-CC-MM-I-195-289, and PCMD-CC-MM-I-80-126. The three analog compounds were directly compared to the WNV NS2B-NS3pro inhibitors 853843 and 4245669 in 10-point concentration response cleavage assays run in triplicate. The PubChem substance identity numbers (PubChem-SID) or PCMD chemistry core identity numbers (PCMD-CC), NS2B-NS3pro IC50s (in μM) and chemical structures are presented.

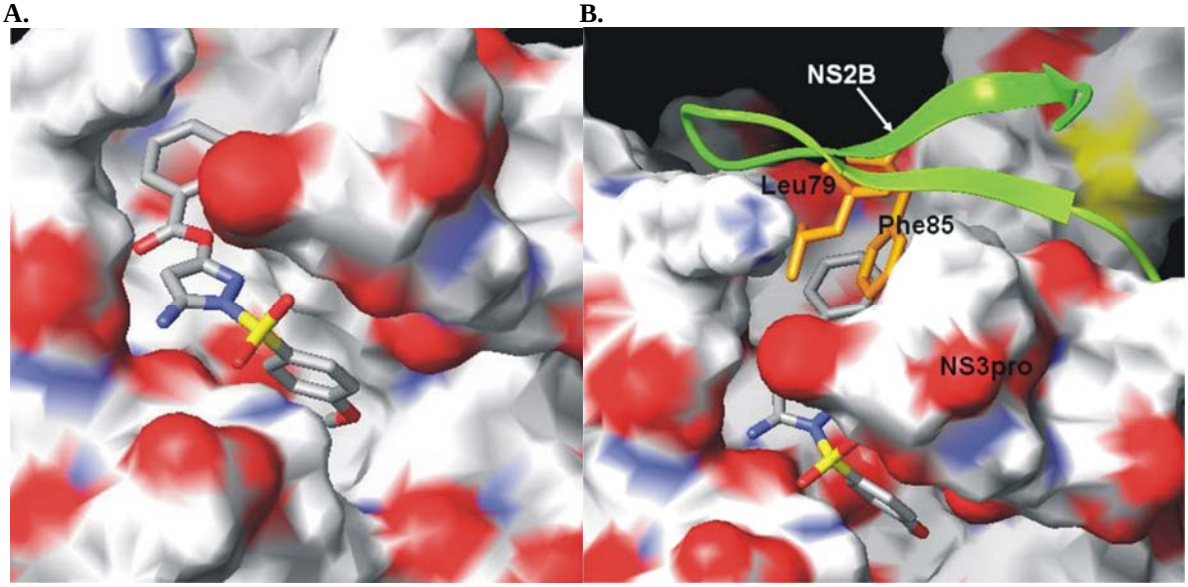
IV. *In silico* Docking Analysis

Recently, the crystal structure of the WNV NS2B-NS3pro-aprotinin complex was solved. We, therefore, examined the structure of WNV NS2B-NS3pro (PDB 2IJO) to identify potential cavities that might be the sites of interaction with the 5-amino-1-(phenyl)sulfonyl-pyrazol-3-yl inhibitors. Since the 5-amino-1-(phenyl)sulfonyl-pyrazol-3-yl molecules are uncompetitive inhibitors of WNV NS2B-NS3pro, we focused our analysis on regions beyond the substrate binding site. Intuitively, a region on the NS3pro structure where the key interactions of NS2B with NS3pro occur was identified as the most probable binding site for uncompetitive, allosteric inhibitors. A surface model of NS3pro is shown with 852843 docked into this extended binding region on NS3pro (Fig. 7A). We propose the binding of 852843 (sticks) may inhibit the proteolytic activity of the two-component NS2B-NS3pro enzyme by interfering with the normal interactions of NS3pro with the NS2B cofactor. The 2IJO structure shows that Leu-79 and Phe-95 (both shown as orange sticks) of NS2B (green ribbon) are the key residues important for the binding of NS2B to NS3pro (Fig. 7B). Docking of the 5-amino-1-(phenyl)sulfonyl-pyrazol-3-yl compounds suggested that the inhibitors bind to NS3pro with good shape and complementarity and that the bound inhibitors collided with Leu-79 and Phe-85 of the L79DDDGNF85 NS2B cofactor sequence and might thus interfere with and compete for the productive associations of NS2B with NS3pro (Fig. 7). Our additional results demonstrated that alanine mutagenesis of the DDD patch inactivated the catalytic activity of NS2B-NS3pro (Radichev et al., submitted for publication). These results provide

additional evidence that the normal position of the L79DDDGNF85 sequence is required for the productive interactions of NS2B with NS3pro and for the catalytic activity of the two-component NS2B-NS3pro viral enzyme.

Figure. 7. Inhibitor docking to WNV NS2bNS3pro.

A. **B.**



NS3pro is shown as a surface model generated using Schrödinger's Maestro 6.5. (7A) Docking of 852843 into the drug-able cavity of WNV NS2B-NS3pro (PDB 2IJO). (7B) The binding of 852843 (sticks) may inhibit the proteolytic activity of the two-component NS2B-NS3pro enzyme by interfering with the normal interactions of NS3pro with the NS2B cofactor The positions of Leu-79 and Phe-85 (both shown as orange sticks) of NS2B (green ribbon) are shown because these residues are important for the interactions of NS2B with NS3pro.

Our in silico docking analysis suggested that the 5-amino-1-(phenyl) sulfonyl-pyrazol-3-yl class of compounds interacted with the key NS2B binding cavity on NS3pro domain in the two-component WNV proteinase molecule (Fig. 7). We hypothesize that the binding of the inhibitory molecule interferes with the productive interactions of the cofactor with the NS3pro domain. These interactions are essential for the activity of NS2B-NS3pro. Both the deletion and the mutagenesis of the NS2B sequence that produce either the cofactor-free NS3pro or the abnormal interactions of NS2B with NS3pro, respectively, result in the inert enzyme. The identified 5-amino-1-(phenyl) sulfonyl-pyrazol-3-yl inhibitors, unfortunately, were unstable in water solutions and degraded in hours. However, we believe that the planar conformation provided by the ester may be required for binding to the NS2B binding cavity and the observed inhibition. Despite the fact that the identified inhibitors cannot themselves be used for viral therapy, we believe that these probes will provide valuable clues to find a chemical means for the design of stable, selective and potent inhibitors of NS2B-NS3pro. The structural basis for species selective allosteric inhibitors of flavivirus may emerge from this work, making the identified inhibitory scaffold a strong candidate for the development of new anti-flaviviral compounds.

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