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Cell-active carbazole derivatives as inhibitors of the Zika virus protease

Gerasimos Rassias,^{1,*,#} Vasiliki Zogali,^{1,#} Crystall M.D. Swarbrick,^{2,3,#} Kitti Wing Ki Chan,² Shu Ann Chan,² Chin Piau Gwee,² Sai Wang,² Entzy Kaplanai,¹ Aleksander Canko,¹ Dimitrios Kiouis,¹ Julien Lescar,⁴ Dahai Luo,⁵ Minos-Timotheos Matsoukas,⁶ Subhash G. Vasudevan.^{2,*}

Graphical Abstract

Rational Design of cell active ZIKV(pro) inhibitors

Cell-active carbazole derivatives as inhibitors of the Zika virus protease

Gerasimos Rassias,^{1,*,#} Vasiliki Zogali,^{1,#} Crystall M.D. Swarbrick,^{2,#} Kitti Wing Ki Chan,² Shu Ann Chan,² Chin Piau Gwee,² Sai Wang,² Entzy Kaplanai,¹ Aleksander Canko,¹ Dimitrios Kioussis,¹ Julien Lescar,³ Dahai Luo,⁴ Minos-Timotheos Matsoukas,⁵ Subhash G. Vasudevan.^{2,*}

¹ Department of Chemistry, University of Patras, Patra, 26504, Greece

² Program in Emerging Infectious Diseases, Duke-NUS Medical School, 8 College Road 169857, Singapore

³ School of Biological Sciences, Nanyang Technological University, Singapore

⁴ Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore

⁵ Department of Pharmacy, University of Patras, Patras 26504, Greece

* Correspondences: rassiasg@upatras.gr & subhash.vasudevan@duke-nus.edu.sg

Equal first author contribution

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Abstract

Zika virus (ZIKV) infection recently resulted in an international health emergency in the Americas in 2015-16 and despite its high profile there is still no approved treatment for ZIKV infection with millions of people being at risk. ZIKV is a member of Flaviviridae family which includes prominent members such as dengue virus (DENV) and West Nile virus (WNV). One of the best validated targets for developing anti-flaviviral treatment for DENV and WNV infection is the NS2B/NS3 protease. However the inhibitors reported to date have shown limited promise for further clinical development largely due to poor cellular activity. Prompted by the conserved nature of the viral NS2B/NS3 protease across flaviviruses, we envisaged that small molecule inhibitors of the ZIKVpro may be developed by applying rational design on previously reported scaffolds with demonstrated activity against other flaviviral proteases. Starting with an earlier WNVpro hit we performed a scaffold hopping exercise and discovered that certain carbazole derivatives bearing amidine groups possessed improved potency and significant cellular activity against ZIKV. We successfully addressed various issues with the synthesis of novel N-substituted carbazole-based amidines thus permitting a targeted SAR campaign. The *in vitro* biochemical (ZIKVpro IC₅₀ 0.5-0.8 µM) and cell-based (EC₅₀ 1-2 µM) inhibitory profiles exhibited by the novel lead molecules described in this work, are among the best reported to date. Furthermore, these molecules possess capacity for further optimization of pharmacokinetics and may evolve to broad spectrum flaviviral protease inhibitors.

Introduction

Flavivirus is a genus of viruses in the family *Flaviviridae* which includes West Nile virus (WNV), tick-borne encephalitis virus (TBEV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), dengue virus (DENV) and Zika virus (ZIKV) among others [1, 2]. ZIKV infection was highlighted as a global health emergency when the epidemic outbreak in the Americas during 2015-2016 was found to be associated with severe neurological disorders such as microcephaly in new born children due to the mother being infected in the first trimester during pregnancy [3], testis damage [4, 5], ocular damage [6], and also Guillain-Barré syndrome in adults [7, 8]. The potential that ZIKV could be transmitted sexually in addition to the spread by mosquitoes has led to calls for urgent countermeasures to be developed [9, 10]. Despite the high impact of ZIKV infections in global public health there are no relevant antivirals available [11, 12].

The flaviviral polyprotein is translated from its ~11kb RNA genome within an infected cell. The polyprotein has a common arrangement which comprises three structural proteins, capsid [C], premembrane [PrM], and envelope [E], followed by seven nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 of which only NS3 and NS5 possess enzymatic activities that are vital for virus life-cycle [13-16]. The host encoded signalase in the ER-lumen, furin in the trans-Golgi as well as virus encoded NS2B-NS3 protease are required for processing the polyprotein into individual mature proteins required for viral RNA genome replication and virion packaging [17].

The NS3pro domain of flaviviruses consists of N- and C- terminal β -barrels with flexible loops that juxtapose the catalytic triad residues to be adjacent to the NS2B cofactor region to permit substrate binding and nucleophilic cleavage of the recognition sequence that is characterised by the presence of basic amino acid residues in the P1 and P2 positions [18, 19]. Crystal structures of flaviviral proteases have revealed that the NS2B region supports the stability of the protease by contributing residues 51-57 of NS2B as a β -strand of the N-terminal β -barrel, while the C-terminal residues 58 – 97 of NS2B form a belt around the NS3pro assembling a β -hairpin that completes the substrate binding site. This is thought to be the active conformation and referred to as the “closed” form of NS2BNS3pro when occupied by peptide-based inhibitors [20]. Recently, focus has turned to ZIKV protease structures where a number of ZIKV NS2B/NS3(pro) structures have been resolved primarily in the closed conformation even in the absence of peptidic inhibitors, a feature that is also true for other flaviviruses as revealed by solution NMR studies [21-24]. The 3D structures of DENV, WNV and ZIKV proteases all appear to be quite similar in architecture with active sites that do not differ significantly when their “closed” forms are overlaid [25, 26]. Prompted by the conserved nature of the viral NS2B/NS3 protease across flaviviruses, we envisaged that small molecule inhibitors of the ZIKVpro may be developed by applying rational design on previously reported scaffolds with demonstrated activity against other flaviviral proteases [27, 28].

Initial peptidomimetic efforts to develop inhibitors for flaviviral proteases were based on the incorporation of the recognition KRR peptidic anchor capped with a serine trap moiety (such as a CF₃-ketone, an aldehyde, or a boronic acid) in order to mimic the substrate binding mode of the viral polyprotein to the protease and block its activity *via* a stabilized tetrahedral intermediate [29]. Despite the intensive efforts on peptidic inhibitors, with or without a serine trap [30, 31], peptides are in general unstable *in vivo* and permeate poorly across cell membranes hence focus was turned to small molecule ZIKV (pro) inhibitors. Nevertheless, the reported ZIKV(pro) inhibitors suffer from either instability towards hydrolysis [25, 29] or poor cell activity [32-34]. To date, compound NSC135618 that emerged from a HTS based on a conformational switch assay of the ZIKV protease designed to identify allosteric inhibitors of NS2B/NS3 [35] appears to be a reasonable base-line candidate with good *in vitro* biochemical and cell-based activity profile [NSC135618 IC₅₀ 0.38 μ M, EC₅₀ 1.00 μ M and SI ~ 48] may be viewed as a reference compound for ongoing efforts towards developing novel Zika protease. In this work we report examples of cell-active ZIKV protease inhibitors generated by rational design exhibiting IC₅₀ 0.5-0.8 μ M and EC₅₀ 1-2 μ M (CC₅₀ 37-48 μ M) essentially being equipotent with respect to reference compound NSC135618. Furthermore, we demonstrate our lead molecules to be amenable to further optimization.

Results

Validation of *in vitro* protease assay for inhibitor discovery

Before embarking on assessing novel molecules ZIKVpro assays were optimised by exploring a number of potential recombinant constructs. The cytosolic loop of the adjacent NS2B integral membrane protein is a cofactor required for the NS3 serine protease to adopt the enzymatically active conformation with a hydrophilic loop contributed by NS2B residues 42-97 [20, 36]. Previous studies have mostly used a covalently (G₄SG₄) linked NS2B/NS3pro [18], however the recent demonstration that the dual promoter expression bivalent NS2B/NS3pro can readily form the protease in the “closed” active conformation led us to include this construct in our study [21]. The bivalent (bZiPro) and G₄SG₄-linked (g-linked; gZiPro), as shown in Fig 2a, were utilized throughout this study. The Michaelis-Menten kinetics of each construct used in the study were determined using benzoyl-Nle-Lys-Arg-Arg-aminomethylcoumarin (Bz-nKRR-AMC) as a substrate, Fig S1, and found to be similar to previous reports [21]. Further validation of the assay was demonstrated by determining IC₅₀ values for aprotinin which were consistent with published results and utilized as controls throughout the study, Fig 2b.

Figure 2. Polyprotein of the dengue and zika viruses and NS2B-NS3pro constructs. (a) From top to bottom, dengue polyprotein, a glycine rich G₄SG₄ linker (gZiPro), and an unfused construct of NS2B and NS3pro expressed separately under two different T7 promoters (bZiPro). Legend: (g-) for glycine rich linker and (b-) for bivalent (b) Inhibition of the ZIKV protease constructs with aprotinin which is comparable with published IC₅₀ values of 76 and 12 nM for gZiPro and bZiPro [21] respectively.

Scaffold hopping to novel hits for flavivirus' protease inhibition

In our endeavor towards identifying a potent ZIKV NS2B/NS3(pro) inhibitor we decided to use the bis-amidine urea **14** as a starting point since this was selected by a rigorous chemoinformatic analysis for the related West Nile virus NS2B/NS3(pro) [27]. Furthermore, a similar bis-amidine compound was discovered from a DENV and WNV homology model *in silico/vitro* screening analysis [28]. The amidine groups in particular, were considered worthy to retain as mimics of the guanidine moieties in the arginine residues that form the P1 and P2 in the proteolytic cleavage recognition sites of the viral polyprotein. In an attempt to assess the contribution of the two amidine groups and their relative distance, scaffold hopping from **14** to the triphenyl analogue **15** was performed (Fig 3a). This

modification removed the hydrogen donor/acceptor capacity of the urea group but maintained the same distance between the amidine groups although in a more rigid fashion and reduced potency. Increasing the distance between the amidine groups through naphthalene analogue **16** effectively restored potency. Rewardingly, scaffold hopping to carbazole **17** resulted in significant potency increase with IC_{50} values of 2.83 and 0.52 μM in the g- and bZiPro assays respectively (Fig 3a). Despite the insertion of the 5-membered ring into the core of the naphthalene analogue **16**, the distance between the amidine groups in the carbazole analogue **17** is actually slightly reduced because this modification effectively bends the aryl-amidine arms towards one another. The increase in potency may be linked to positioning the two amidine groups at an appropriate distance from one another in order to allow for a more potent conformation to be adopted that maximizes interactions with hydrogen-bond-acceptor residues in the active site of the protease.

Figure 3. Scaffold hopping to 17 and biochemical and kinetic assessment of its inhibitory activity. (a) Scaffold hopping from urea-bisamidine **14** to carbazole **17** and assessment of inhibitory activity against g- and b-ZiPro constructs in protease assays. (b) The half maximal response was determined against EDEN2 and HPF2015 in Huh7 cells and plotted as plaque forming units (pfu) per mL vs log compound **17** concentration to determine EC_{50} 's. (c) Compound **17** inhibition against bZiPro was carried out in triplicate and plotted as % normalized response vs log compound **17** concentration to determine IC_{50} . Standard deviations for each data point are shown as error bars. (d) Lineweaver-Burk kinetics of compound **17** demonstrating a non-competitive form of inhibition. Determined by varying concentration of Bz-nKRR-AMC from 0 to 50 μM in triplicate for each concentration of compound **17**; tested and plotted as mean with error bars showing standard deviation.

It is worth noting that for all four compounds tested, the IC_{50} 's were consistently reduced for the bivalent NS3pro construct when compared with the g-linked construct possibly as a result of increased accessibility to the binding site in the absence of a steric linker. The *in cellulo* activity of these scaffold-hopping derived compounds was further investigated by screening for inhibition of ZIKV infection in Huh-7 cells (Fig 3b and c). To gain insights into the mechanism of inhibition of the new carbazole hit, the Michaelis-Menten kinetics for **17** were determined for bZiPro (Fig 3d). Enzyme kinetics analysis of bZiPro indicates a non-competitive form of inhibition in which **17** is able to bind both the free enzyme and the enzyme-substrate complex (Fig 3d) [37].

Synthesis

Based on the improved inhibitory activity of derivative **17**, the carbazole scaffold was selected for SAR studies aimed primarily at interrogating the nature of the N-substituent, the number of amidine groups as well as their relative positions within the carbazole core. Initially the synthesis of carbazole derivative **17** was attempted *via* a double Suzuki reaction of 3-cyano-boronic acid either directly on 3,6-dibromocarbazole **18** or the N-methylated derivative. These coupling reactions proved much more challenging than originally anticipated as the combined yield for the mono- and diarylated products never exceeded 25%. It is possible that the resonance electron donation by the N-Me or NH (including its deprotonated form under the reaction conditions) groups into the *para*-brominated positions impedes the oxidative addition of the palladium catalyst into the C-Br bond and perhaps an electron withdrawing group on the carbazole nitrogen atom could, at least in part, reverse this effect. Gratifyingly, when the Suzuki reaction was repeated with the N-Boc-3,7-dibromocarbazole **19** the diarylated product **20** was obtained in a few hours without contamination by the monoarylated derivative (Scheme 1).

Scheme 1. Synthesis of carbazole bis-amidine Series A. Reagents and conditions: i) B_2O_3 , DIMAP, MeCN, 2h, rt, ii) 3-CN-PhB(OH) $_2$, $\text{Pd}_2(\text{dba})_3$, K_3PO_4 , $\text{P}(t\text{-Bu})_3$, THF:H $_2\text{O}$ (3:2), 75°C, 1.5h, iii) TFA, DCM, 2h, rt, iv) for a: Me_2SO_4 , LHMDs, THF, r.t.; for b: 3-MeOPh-CH $_2$ Cl, NaOH, Bu_4NHSO_4 , acetone, 18h, r.t.; for c: 3-MeOPh-SO $_2$ Cl, DBU, DIMAP, MeCN, 18h, r.t., v) for a: $\text{NH}_2\text{OH}\cdot\text{HCl}$, TEA, NMP:MeOH (1:10), 2h, 80°C; for b and c: $\text{NH}_2\text{OH}\cdot\text{HCl}$, TEA, THF:MeOH (1:10), 18h, 60°C, vi) CDI, THF, 60°C, 18h, vii) Fe, AcOH, dioxane, 80-100°C, 2h.

Optimization of this step resulted in isolation of **20** after 1.5 hours in 90% yield and excellent quality without the need for chromatographic purification. Boc removal to **21** followed by installation of either the methyl or benzyl or benzenesulfonyl substituent, afforded the di-cyano intermediates **22a-c** in >70% yield over the two steps. Next, the direct conversion of the nitriles into amidines was attempted using standard protocols but without success. After considerable experimentation we decided to adopt a three-step process which involves initial conversion of the nitriles to the amidoximes **23a-c** using hydroxylamine, followed by reaction with CDI to form the corresponding oxadiazolones **24a-c** and finally reduction to the amidines with Fe/AcOH or Pd/HCO $_2\text{H}$. Intensive optimization efforts in each step led to the preparation of amidine **17** as the bis-formate salt without the need for column chromatography in any part of the synthesis.

The synthesis described above was successfully scaled up and provided multigram quantities of intermediate **21** which allowed for diversification in N-substituted derivatives. Employing the same transformations on the cyano groups of analogues **22**, amidines **25b** and **25c** were synthesized as either the bis-pentanosulfonate and hydrochloride salts respectively (Scheme 1). The pentanosulfonate salt version was obtained by organic solvent extraction from an aqueous solution of the amidine to which excess of sodium pentanosulfonate is added. This anion exchange process was subsequently utilized in all cases where the initially formed formate, acetate or hydrochloride amidine salts were too hydrophilic to be extracted effectively into organic solvents.

In order to examine the necessity for the second amidine group, two series of derivatives were prepared possessing only one amidine functionality. Series B (**27a-c**, Scheme 2) maintaining the second aryl substituent on the carbazole, was accessed from a selective mono amidoxime formation of di-cyano intermediates **22a-c**. Series C (**30a-c**, Scheme 2) lacking the second aryl substituent on the carbazole, was accessed by a selective Suzuki monoarylation reaction of intermediate **19** with 3-cyano phenylboronic acid.

The monoarylation Suzuki reaction of **19** required considerable optimisation which involved screening of more than 70 combinations of palladium pre-catalysts, ligands, bases, solvents reaction temperatures and times. This study allowed us to examine a wide spectrum of catalytic activities and determine optimum conditions for both the diarylation and monoarylation processes. In this way we established particularly active conditions for the full diarylation of **19** which we also tested with derivatives **19a-c**. With this set of conditions (3-CN-PhB(OH) $_2$, $\text{Pd}_2(\text{dba})_3$, K_3PO_4 , $(t\text{-Bu})_3\text{P}$, THF:H $_2\text{O}$

(3:2), 75°C), **19c** was also diarylated fully in contrast to **19a** and **b** which performed poorly, thus confirming the requirement for an electron withdrawing group on the nitrogen for successful Suzuki reactions with these systems. This allowed for a more direct synthesis of **22c** and related derivatives without having to install and remove the Boc group thus shortening the synthesis by two steps. The mono-amidoximation of **22c** also proved challenging in terms of maximising conversion and limiting formation of the bis-amidoxime.

Scheme 2. Synthesis of carbazole mono-amidine Series B and C. Reagents and conditions: i) 3-CN-PhB(OH)₂, Pd₂(dba)₃, K₃PO₄, (*t*-Bu)₃P, THF:H₂O (3:2), 75°C, 1.5h, ii) 3-CN-PhB(OH)₂, DPEPhos, Pd(OAc)₂, K₃PO₄, THF:H₂O (85:15), r.t., 18h, iii) TFA, DCM, r.t., 2h, iv) for a: Me₂SO₄, LHMDs, THF, r.t., 18h; for b: 3-MeOPhCH₂Br, LHMDs, THF, r.t., 18h; for c: 3-MeOPhSO₂Cl, DBU, MeCN, r.t., 16h, v) NH₂OH·HCl, TEA, NMP:MeOH (1:1), 85°C, 2h vi) CDI, THF, 60°C, 18h vii) Fe, AcOH, dioxane, 80-100°C, 2h.

An additional complexity we encountered with all amidoxime formations is the partial conversion of the nitrile to the primary amide and this is consistent with previous reports [39, 40]. Optimization of this step led to the isolation of monoamidoximes **26a-c** which were converted to the monoamidine derivatives **27a-c** by treatment with CDI followed by reduction with Fe/AcOH or Pd/HCO₂H.

For the synthesis of derivatives **28a-c**, en route to series **30a-c**, the wide spectrum of catalytic activities/protocols studied for the Suzuki diarylation of **19**, allowed us to determine balanced conditions enabling maximum consumption of the dibromocarbazole while limiting the formation of the diarylated product. We found that selectivity ratios of up to 9:1 favoring monoarylation of **19** could be achieved using Pd(OAc)₂, DPEPhos ligand, K₃PO₃, THF/water (85:15) at ambient temperature and 0.7eq of the boronic acid. The sub-stoichiometric amounts of the boronic acid ensured minimum over-arylation of the desired product even after 18 hours albeit at the expense of

overall conversion. Progression of the monoarylated-**19** to derivatives **28a-c** followed by conversion to amidoximes **29a-c** and reduction to amidines **30a-c** was achieved in the same way as described above.

It is worth noting that the purification of monoamidoximes **26a-c** and monoarylated-**19** are the only points requiring chromatography in the entire synthetic work presented here.

In the last subset of compounds synthesized, Series D (Scheme 3), one amidine functionality was positioned in the southern hemisphere at the N-substituent rather than on the aryl arms that extend out of the carbazole rings. In this series, the aromatic rings in the northern hemisphere bear either bromine atoms (**32a,b**) or 3-cyano-aryl groups (**34a,b**) or an additional amidine functionality (**36**).

Scheme 3. Synthesis of carbazole amidine Series D. Reagents and conditions: i) for **a**: 3-CN-PhCH₂Br, LHMDs, THF, r.t., 18h; for **b**: 3-CNPhSO₂Cl, DBU, MeCN, r.t., 18h, ii) NH₂OH·HCl, Et₃N, MeOH:THF (1:10), 60°C, 18h, iii) CDI, THF, 60°C, 18h, iv) Fe, CH₃COOH, dioxane, 80-100°C, 2h, v) 3-CN-PhB(OH)₂, Pd₂(dba)₃, K₃PO₄, (*t*-Bu)₃P, THF:H₂O (3:2), 75°C, vi) TFA, DCM, r.t., 2h.

Attachment of either the 3-cyanobenzyl or 3-cyanobenzenesulfonyl group to the nitrogen atom of 3,6-dibromocarbazole **18**, followed by reaction with hydroxylamine produced the amidoxime intermediates **31a** and **31b** which were subsequently converted to the amidine derivatives **32a** and

32b by reaction with CDI followed by reduction with Fe/AcOH, a process that preserves the C-Br bonds. The synthesis of derivatives **34a** and **34b** formally requires the selective conversion of the cyano group attached on the benzenesulfonyl substituent to the corresponding amidine, leaving the other cyano groups intact. We overcame this challenge by discriminating the said cyano group by transforming it to amidoxime (**31a,b**) prior to the installation of the 3-cyanophenyl substituents *via* the double Suzuki reaction (**33a,b**).

Rewardingly, using our standard Suzuki conditions, amidoximes **31a** and **31b** were converted into the bis-3-cyanophenyl analogues **33a** and **33b** respectively and subsequently into amidines **34a** and **34b** in the usual way. The last member of our library was prepared from monoarylated-**19** (**28** Scheme 2) which after Boc removal, installation of the 3-cyanosulfonyl group on the carbazole nitrogen atom, followed by reaction with excess hydroxylamine gave the bis-amidoxime **35**. This was converted to the bis-amidine **36** where the amidine groups extend out from opposite directions of the carbazole core.

Biochemical and cellular evaluation of carbazole amidines

All amidines described above were tested for toxicity and their inhibitory activity was assessed against the bZiPro construct and in an appropriate cell-based assay (Table 1). Rewardingly, several compounds exhibited significant inhibition against bZiPro within the biochemical assay achieving sub-micromolar potencies in several cases. The 3,6-diaryl carbazole scaffold of series A and B, with at least one of the aryl substituents bearing an amidine group, displayed the greatest inhibitory activity (IC_{50} 0.5-1.75 μ M). Whilst in series C, where one of the aryl groups is replaced by bromine, potencies are 5-10 fold lower suggesting that an important hydrophobic / π -stacking interaction may be provided by the second aryl ring. The inclusion of the N-benzyl group proved to be more important for the monoamidines in series B and C reducing the IC_{50} by up to 3-fold when compared with their N-methyl counterparts. Equally, in series D, "walking" the amidine group(s) around the carbazole core, also results in a 3-25 fold drop in potency suggesting that the positioning of the aryl-amidine moiety(ies), relative to one another and to the carbazole core, is pivotal for enhanced potency.

The amidines showed a strong trend throughout scaffolds A-D where the monoamidines appear to be more toxic than their double amidine counterparts. Within series A and B the N-sulfonyl analogues **25c**, **27c** appear to be less toxic than the N-methyl and N-benzyl analogues with **25c** being the safest across the whole batch ($CC_{50}>100\mu$ M). This trend does not extend to all monoamidine derivatives within series D where at least one of the amidine functionalities is positioned in the N-substituent, yet a sulfonyl analogue, **36**, is the least toxic in this series too. In series C all three types of N-substitutions produce similarly low values of CC_{50} .

In terms of cellular activity, some of the compounds displaying poor protease inhibition in the biochemical assay and/or low CC_{50} values appeared disproportionately potent in the cell assay,

possibly due to non-selective/off-target effects and high toxicity respectively. In order to discern meaningful trends we decided to disregard these as being false positives and considered only those derivatives with $CC_{50} > 20$ namely amidines **17**, **25b**, **25c**, **27c**, and **36**. Of these compounds it is interesting to note that the N-sulfonyl analogues are strongly represented in **25c**, **27c**, and **36** however the lowest EC_{50} is **17** at 1.25 μ M. The cellular activity data from this subset of compounds suggest that these derivatives possess appropriate solubility and permeability to produce significant inhibitory effects at the cellular level (EC_{50} 1-25 μ M) and more importantly that they are most probably genuine protease inhibitors since their EC_{50} values are consistent (typically 1-10fold higher) with their IC_{50} values.

Table 1. Evaluation of amidine inhibitors in DENV2 and ZIKV biochemical and cell assays

Series	Entry	R	bZIKV IC_{50}	CC_{50}	ZIKV EC_{50}	SI ZIKV
A	17	Me	0.52	47.47	1.25	37.98
	25b	3-MeOPh-CH ₂	1.75	22.7	6.66	3.4
	25c	3-MeOPh-SO ₂	0.99	>100	23.40	>4
B	27a	Me	1.70	6.25	NI	ND
	27b	3-MeOPh-CH ₂	1.40	3.30	1.97	2.8
	27c	3-MeOPh-SO ₂	0.81	37.88	1.56	24.27
C	30a	R = Me	13.86	6.57	1.21	5.42
	30b	3-MeOPh-CH ₂	4.04	5.3	2.29	2.3
	30c	3-MeOPh-SO ₂	6.45	5.22	NI	NI
D	32a	R ₁ , R ₂ = Br, X = CH ₂	25.6	12.79	7.34	1.74
	32b	R ₁ , R ₂ = Br, X = SO ₂	14.15	6.58	1.59	4.14
	34a	R ₁ , R ₂ = 3-CNPh, X = CH ₂	14.9	7.48	NI	ND
	34b	R ₁ , R ₂ = 3-CNPh, X = SO ₂	3.50	3.35	NI	ND
	36	R ₁ = Br, R ₂ = 3-amidinoPh, X = SO ₂	9.19	35.73	8.50	4.20

Discussion

Post translational processing of the flaviviral polyprotein by the viral protease is essential for viral infection and provides an attractive target for the development of antiviral drugs, especially given the success of HCV protease inhibitors [41]. Despite the increasing interest in the development of ZIKV protease inhibitors there has been only limited progress when cellular activity is assessed; peptides proved too unstable and/or unable to permeate cell membranes adequately, serine traps generally have toxicity issues while small molecule inhibitors have met mainly with lack of permeability and off-target effects. In terms of protease affinity and inhibition the modest results reported to date may be attributed to the flat, hydrophilic active site with several basic residues similar to those of various human serine proteases [38]. In order to overcome these issues we decided to exploit a key feature present in early hits of flaviviral protease inhibitors namely the two positively charged amidinium groups that serve as mimics for the two arginine residues present in the recognition triplet KRR of the viral polyprotein (the natural substrate of the protease). A scaffold hopping exercise was undertaken to determine the optimum distance between the two amidinium residues followed by a targeted SAR to examine their relative position around the carbazole core and the nature of the N-substituent. In this context, four series of carbazole-based mono- and bis-amidines were generated while the N-substituent was varied as either methyl or benzyl or benzenesulfonyl group.

Following biochemical and cellular evaluations of the carbazole amidines compound **17**, a bis-amidine derivative of N-Me carbazole, was found to be the most potent compound across both the ZIKV NS3pro biochemical and ZIKV cellular infectivity assays (IC_{50} 0.52 μ M, EC_{50} 1.25 μ M). Increasing the size and hydrophobicity of the N-substituent first to *meta*-methoxybenzyl then to *meta*-methoxybenzenesulfonyl maintained the reasonable protease inhibition but led to attenuation of cellular potency. This is demonstrated by compound **25c**, with comparable bZiPro inhibitory activity (IC_{50} 0.99 μ M) and superior toxicity profile (CC_{50} >100 μ M), which was much less potent in the ZIKV cell-based assay (EC_{50} 23.4 μ M) suggesting that N-substitution with the larger and more hydrophobic benzenesulfonyl group may not aid cell permeability. In contrast, in **27c**, removal of the second charged amidinium residue and inclusion of the benzenesulfonyl group appears to restore cell permeability with retention of significant inhibitory activity in both the biochemical and ZIKV cell-based assays (IC_{50} 0.82 μ M, EC_{50} 1.56 μ M).

Compounds **17** and **27c** appear to be genuine inhibitors of the ZIKV protease as their EC_{50} values are consistent with their IC_{50} counterparts, varying only 2-3 fold and, more importantly, their overall profile IC_{50} 0.5-0.8 μ M and EC_{50} 1-2 μ M (SI 24-38 μ M) is comparable to the reference ZIKV protease inhibitor NSC135618 (IC_{50} 0.38 μ M, EC_{50} 1.00 μ M and SI ~ 48) [35]. Furthermore, they constitute the first examples of rationally designed cell active ZIKV protease inhibitors with capacity for further optimization at the N-substituent of the carbazole core. With this in mind and intrigued by the fact

that similar potencies towards the ZIKV protease and cellular activities are obtained by bis-amidine **17** and mono-amidine **27c** we decided to explore further this potential in lead diversification.

In the original scaffold hopping exercise the two amidine groups were maintained as they mimic the guanidine groups present in the arginines of the viral polyprotein occupying positions P1 and P2 in flaviviral proteases. The natural substrate for the ZIKV protease however has K and R residues occupying the P1 and P2 sites [19] respectively rather than R and R in the DENV proteases [17, 18]. This may partly explain the tolerance of the ZIKV protease towards both mono- and bis-amidine derivatives such as **17** and **27c**. In order to further explore this assertion we undertook a molecular docking study with compounds **17**, **25c** and **27c** into the ZIKV NS2BNS3pro structure (PDB ID: 5GPI) (Fig 4a-c). These show a consistent double charge-charge interaction between one of the amidine groups of the inhibitor with the carboxylate of D129 and the carbonyl oxygen of Y130. The S1 pocket of the ZIKV NS2BNS3pro is mainly formed by NS3 residues A132 and Y161 [19] which sandwich the benzene ring bearing the amidine group, itself interacting with D129 and Y130. The second amidine group present in **17** and **25c** locates itself in pocket S1' which is formed by V36, H51, K54 and S135 in NS3, forming a hydrogen bond with the main chain carbonyl oxygen of H51 (Fig 4a,b). The 3-MeOPh-SO₂ group of **25c** and **27c** enters pocket S2, which is formed by S81, D83 of NS2B and H51, D75 of NS3. As such, pocket S2, is not considered hydrophobic and it is therefore intriguing as to how it accommodates the benzenesulfonyl substituent of the inhibitor. This may be attributed to potential water mediated interactions between the SO₂ group and the S81 and/or D83 in NS2B. Such an interaction is supported by the reduced activity of derivatives **25b** and **27b** which lack the SO₂ moiety and also by the increased potency of derivative **27c** which does possess it.

It appears that with mono-amidine **27c** (Fig 4c) the interaction of the SO₂ group combined with a strong interaction between the cyano group and ammonium group of the K54 side-chain, offset the interaction lost between the second amidine group and the amidine-H51 carbonyl oxygen. The stabilizing interaction of the cyano group is further corroborated by the reduced potency of derivatives **30a-c** which contain bromine atom instead of the cyano-aryl group.

Figure 4. Docking of 17, 25c and 27c to Zika and DENV2 proteases. Docking poses of compounds (a) **17** (in cyan), (b) **25c** (in light green) and (c) **27c** (in orange) to the Zika protease site. NS3 is shown in yellow cartoon style and NS2B in light grey. Electrostatic interactions are depicted in magenta dashed lines.

Conclusions

In our pursuit of potent ZIKV protease inhibitors we have discovered novel carbazole derivatives capable of inhibiting the enzyme with cellular potencies where both bis- and mono-amidine derivatives were found to be active. It was also established that carbazole N-substitution is tolerated both in terms of potency and toxicity, an aspect that is currently being exploited through additional SARs, enabled at large by a robust multi-step chemical synthesis we have developed that does not require chromatography. The potential of these novel compounds will be further explored with the aim of developing a broad spectrum flaviviral protease inhibitor with acceptable toxicity and pharmacokinetic profile.

Methods and Materials

Expression constructs

To simultaneously express NS3pro and its NS2B cofactor (bDEN2Pro) at the same time NS3pro (1-180) and NS2B (47-97) were cloned into the NcoI/HindIII and NdeI/XhoI sites of pETDUET-1, respectively and confirmed by nucleotide sequencing as described previously [21, 23].

Protein expression and purification

Proteins were expressed as described previously [21, 23] and resuspended in His buffer A (20 mM phosphate buffer, pH 8.0, 500 mM NaCl, 2 mM β -mercaptoethanol, 10 mM imidazole and 5% glycerol), lysed by sonication and clarified by centrifugation at 15,000 rpm for 1 hour. Protein was purified by FPLC (Akta) using a Ni-NTA HisTrap FF column (GE Healthcare). After washing with His Buffer A protein was eluted using a linear gradient of imidazole from 10 to 500 mM. Proteins were dialysed for 18 h against S200 buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 5% glycerol) with concomitant cleavage of the His-tag by thrombin. Tags and uncleaved proteins were removed by washing back over the Ni-NTA HisTrap FF column and flow-through concentrated prior to size exclusion chromatography over a HiPrepSephacrylS-200 HR (GE Healthcare) column pre-equilibrated in S200 buffer with 2 mM DTT. Protein purity was confirmed by SDS-PAGE and concentrated to 20 mg/mL.

Protease assays

Enzyme activity was measured by quantifying cleavage of Bz-nKRR-AMC substrate which releases the fluorescent product AMC as described previously [18, 22]. The assay was performed in a final volume of 30 μ L in a costar 384-well black flat plate (Corning, NY, USA). For the determination of kinetic parameters activity of NS3pro at a concentration of 5 nM was measured by the addition of

varying concentrations of substrate Bz-nKRR-AMC from 0-200 μ M in buffer containing 50 mM Tris pH 8.5, 20% glycerol and 1 mM CHAPS. Fluorescence intensities were measured in a Tecan Spark 10M plate reader (Tecan, Durham, NC, USA) for 30 min at 30 sec intervals using an excitation wavelength of 380 nm and emission wavelength of 460 nm. Experiments were repeated twice at 37°C. Initial velocity was measured and expressed as μ M of AMC released during the reaction per second. Data were fitted to the Michaelis-Menten equation ($Y = V_{max}*[S]/K_m+[S]$) to calculate apparent K_m , K_{cat} and V_{max} by non-linear regression using GraphPad Prism (version 5.00, GraphPad Software, San Diego, CA, USA).

Inhibition assays

For inhibition assays purified protein at a concentration of 1.6 nM for ZIKV NS3pro constructs were pre-incubated for 15 mins at 30°C with small molecule inhibitors in a concentration range of 0.007-100 μ M. Fluorescence intensities were measured as per protease assays after the addition of Bz-nKRR-AMC substrate to a final concentration of 16.7 μ M. Initial velocities were normalized relative to wells containing substrate and no inhibitor and data were analysed using the Transform function (GraphPad Prism). Sigmoidal dose response curves were plotted using non-linear regression function $\log(\text{inhibitor})$ vs normalised response in GraphPad Prism to determine IC_{50} (IC_{50} : concentration at which the biochemical activity is reduced to 50%).

Cell lines, viruses and compounds

HuH-7 cells (human hepatocarcinoma cells, ATCC) was maintained in 4.5g/L glucose DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). BHK-21 cells (baby hamster kidney fibroblast, ATCC) was maintained in RPMI 1640 medium (Gibco) with 10% FBS and 1% P/S. All mammalian cell lines were cultured in a humidified incubator at 37°C with 5% CO_2 . C6/36 (an *Aedes albopictus* cell line) was cultured in RPMI 1640 medium (Gibco) with 25mM HEPES, 10% FBS and 1% P/S, and incubated at 28°C in the absence of CO_2 .

The ZIKV strain H/PF/2013 (GenBank accession: KJ776791.2) used in this study was grown in C6/36 cells. Virus supernatants were clarified, filtered through 0.2 μ m membrane, aliquoted and stored at -80°C. Virus titer was determined by standard BHK-21 plaque assay.

All compounds tested were synthesized as described in the supplementary material; typically in >95% purity. For *in vitro* and cellular assays, the solid compounds were reconstituted with 100% DMSO to stock concentrations of 20 or 100mM. For *in vivo* study, compound **25c** was reconstituted with 100% DMSO to a stock concentration of 100mg/ml. All reconstituted compounds were stored at -20°C until use.

Ethics statement

ZIKV H/PF/2013 was a gift from Cécile Baronti at Aix Marseille Université. Institutional approval has been granted by Duke-NUS Medical School to perform experiments with ZIKV.

Dose-dependent inhibition assay

1×10^5 HuH-7 cells were infected with ZIKV-H/PF/2013 at MOI 0.5. Following infection, the cells were then treated with the compounds at the indicated concentrations ranging from 100 μ M to 0.01 μ M for 24 (ZIKV infection). Supernatants were collected at the specific timepoint, clarified and subjected to plaque quantification by BHK-21 plaque assay. A sigmoidal dose response curve of virus titer (pfu/ml) against log concentration of the compound was plotted and the effective concentration 50 (EC₅₀; concentration at which the virus titer is reduced to 50%) was determined using GraphPad Prism 5.

Cell cytotoxicity test

2×10^4 HuH-7 cells were seeded onto a 96-well flat bottom white plate and treated with the compounds at the indicated concentrations for 48 h. Cell viability of the treated cells was quantified using the CellTiterGlo Luminescent kit (Promega) according to manufacturer's instructions. The cytotoxic concentration 50 (CC₅₀; concentration at which 50% of the cells are dead) was determined using GraphPad Prism 5.

Molecular modeling

For docking on the Zika virus protease, the crystal structure of unlinked NS2B-NS3 was used (pdb code 5GPI). For the docking, selected compounds were docked into the binding sites of receptor using AutoDock Vina [39] and AutoDockTools for file preparation. The protease was held rigid during the docking process, while ligands were allowed to be flexible. Docking simulations in both cases were performed using a grid box with dimensions of 45 x 45 x 45 Å, a search space of 10 binding modes and exhaustiveness was set to 20. The best docking pose for each compound was selected on the basis of the lowest energy docked conformation.

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Author Contributions

G. R. designed all syntheses and SAR studies and wrote the article; V. Z., A. K., A. C., and D. K. did all syntheses optimisation and preparation of compounds in gram scale; C. M. D. S., K. W. K. C., S. A. C., C. P. G., S.W., prepared all protein constructs and developed and performed all biochemical and cell assays; in addition V. Z. and C. M. D. S. wrote the article; M-T. M performed the computational models and docking simulation work; J. L. and D. L. worked on the study of mechanism of inhibition; S. G. V. conceived the study, obtained the funding and supervised all of the biochemical and cell-based assays and wrote the article.

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Graphical Abstract

Rational Design of cell active ZIKV(pro) inhibitors

Highlights

- According to WHO Zika virus is a serious health concern and there is no approved treatment.
- The NS2B/NS3 protease is largely conserved across flaviviruses and constitutes a validated target for antiviral research
- Using rational design, novel carbazole derivatives, bearing at least one amidine, were identified as potent and cell-active NS2B/NS3(pro) inhibitors
- A targeted SAR study has revealed that certain N-substitutions are tolerated which will be exploited to further improve the pharmacokinetics of the newly found lead-molecules

ACCEPTED MANUSCRIPT

Cell-active carbazole derivatives as inhibitors of the Zika virus protease

Gerasimos Rassias,^{1,*} # Vasiliki Zogali,¹ # Crystall M.D. Swarbrick,^{2, 3} # Kitty Wing Ki Chan,² Shu Ann Chan,² Chin Piaw Gwee,² Sai Wang,² Entzy Kaplanai,¹ Aleksander Canko,¹ Dimitrios Kioussis,¹ Julien Lescar,⁴ Dahai Luo,⁵ Minos-Timotheos Matsoukas,⁶ Subhash G. Vasudevan.^{2,*}

¹ Department of Chemistry, University of Patras, Patra, 26504, Greece

² Program in Emerging Infectious Diseases, Duke-NUS Medical School, 8 College Road 169857, Singapore

³ Institute for Glycomics, Griffith University, Gold Coast Campus, Australia

⁴ School of Biological Sciences, Nanyang Technological University, Singapore

⁵ Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore

⁶ Department of Pharmacy, University of Patras, Patras 26504, Greece

* Correspondences: rassiasg@upatras.gr & subhash.vasudevan@duke-nus.edu.sg

Equal first author contribution

Keywords: Flavivirus; Zika; protease; inhibitors; carbazoles; amidines;

Highlights

- The flavivirus NS2B/NS3 protease is a conserved and validated antiviral target
- Novel carbazole derivatives designed with at least one amidine are potent inhibitors
- The profile of the lead compound identified is among the best reported in the literature
- A targeted SAR revealed N-substitutions that are tolerated with respect to safety and potency
- The mechanism of inhibition appears to be via partial and reversible occupancy of the active site