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ORIGINAL RESEARCH

Metalloprotoporphyrin Inhibition of HCV NS3-4A Protease: Structure–Activity Relationships

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Background: Antiviral actions of tetrapyrroles have been described in a number of systems. Our goal was to evaluate antagonism of the HCV NS3-4A protease by a variety of common porphyrins and characterize structure–activity relationships that may be useful for future drug design of HCV and related *Flaviviruses*.

Methods: Using fluorometric assays, common metalloprotoporphyrins (MPP) all inhibited NS3-4A protease with IC_{50} values in low micromolar ranges [CoPP (1.4 μM) < ZnPP = MnPP = SnPP < CuPP < FePP (6.5 μM) = protoporphyrin].

Results: Lineweaver–Burk plots confirmed that MPP: NS3 inhibition was basically competitive. All tested MPPs inhibited HCV genotype 1A, 1B, 2A and 3A recombinant proteases with the same fidelity suggesting wide antagonistic capabilities. However, when the MPPs were tested in cellular incubations with HCV replicons only Zn, Fe and free-base protoporphyrin showed comparable EC_{50} and IC_{50} values suggesting that there may be critical differences in MPP uptake and intracellular availability. Meso, deutero, and isohematoporphyrin derivatives, with or without metal substitution, all showed less anti-protease and antiviral activities as compared to protoporphyrins, suggesting that the planar, vinyl side chains are important for protease active site binding. MPPs were also active against three common protease mutants (T54A, A156T, and V36M) with equivalent or better IC_{50} values as compared to wild type enzyme.

Conclusion: These findings document the versatility of MPPs as antiviral agents with an expanded sensitivity for HCV genotypes and resistance to some common viral mutations. The results also suggest that further study of MPP structure and function will be useful for the development of new antiviral agents.

Keywords: porphyrins, antiviral agents, hepatitis C virus, hepatitis C genotype

Introduction

Chronic hepatitis C virus, (HCV, *Hepacivirus*) infection is a serious health issue worldwide. In spite of potent antiviral agents, chronic infection can still lead to decompensated cirrhosis and hepatocellular carcinoma (HCC).[1] Recent development of multiple antiviral drugs that offer a cure for most HCV infected patients has been a dramatic medical achievement of this decade.[2] While the design of new antivirals for HCV is not a pressing concern per se, further study of HCV drug targets such as the NS3-4A protease is still useful for understanding related *Flaviviruses* such as Dengue, Yellow fever, West Nile and Zika.[3] These pathogens still present formidable treatment challenges.

The HCV NS3-4A protease-helicase is an attractive viral target for antiviral drugs. Enzymatically, the complex performs crucial protease and helicase steps in HCV RNA

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translation and polyprotein processing.[4] Oral, first-generation HCV protease inhibitors were the first drugs used in combination with conventional pegylated interferon and ribavirin therapies and significantly improved treatment outcomes.[5–7] Second generation protease inhibitors were subsequently shown to be effective adjunctive antiviral agents when used in combination with other drugs that target the HCV RNA polymerase or the NS5A protein.[8–10] Recently, new approved third-generation protease inhibitors have high potency with wide genotypic activity, yet they still have issues with viral resistance and treatment versatility.[11–17]

Iron protoporphyrin (heme) and structurally related metalloprotoporphyrins (MPP) have been shown to have antiviral activity for a wide range of viruses including HCV, HBV, and HIV[18] as well as related *Flaviviruses* such as Dengue.[19] Heme is a vital oxidative cofactor for mammalian cells and is tightly regulated through the induction of heme oxygenase-1 (HO-1). Enzymatically, HO performs the rate-limiting reaction for heme catalysis by oxidizing heme to biliverdin (BV) with the release of free iron and carbon monoxide. BV is then rapidly reduced to bilirubin (BR) through the coupled reactions of biliverdin reductase and NADPH oxidase.[20] Commercial preparations of heme (*Hemin and Panhematin*) are approved by the FDA for use in patients during attacks of acute porphyria.

The MPPs share many of the chemical properties of heme and interactions with cellular components. Recently, MPPs such as ZnPP have shown promise for use as antineoplastic or antiviral agents.[18,21] Although the antiviral behaviors of some common MPPs have been described in infected cells, relatively little is known about specificity, potency, and structure–function relationships. The goal of the present work was to evaluate anti-NS3-4A protease activities of a variety of common MPPs and protoporphyrin derivatives to further characterize structure–activity relationships. Our results identify the structural requirements for protoporphyrins that enhance anti-NS3 activity. These studies identify a class of antiviral agents with a broad range of HCV genotype activities and overall effectiveness against resistant protease mutations and suggest a new structural platform for further development.

Materials and Methods

Materials

Taq DNA polymerase (*Perkin-Elmer Cetus*, Norwalk, CT), and Moloney murine leukemia virus reverse transcriptase (*Gibco/BRL Life Technologies*, Gaithersburg, MD) were used in these studies. All porphyrins and metalloporphyrins were obtained from Frontier Scientific, Inc (Logan, UT) and were the purest forms available (>97% purity). All tetrapyrroles were dissolved in minimal volumes of Dimethyl sulfoxide (DMSO) and diluted into culture media or assay buffers to achieve the final concentration. Controls received an identical volume of diluted solvent only. HCV recombinant NS3-4A protease with *SensoLyte Assay*s utilizing Fluorescence Energy Transfer (FRET) probes were purchased from *AnaSpec*, Fremont, CA.

Antibodies

All secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-NS3-4A and NS5A antibodies were purchased from Meridian Life Science (ME) or Santa Cruz Biotechnology (CA).

Cell Lines and Cell Culture

The human hepatoma cell line (Huh 5.15NS replicon) with replicating sub-genomic HCV RNA (genotype 1b) was used as described.[22,23] The infectious HCV cell culture (HCV cc) strain (J6JFH) as described[24] was inoculated into Huh 7.5 cells and harvested using published protocols.[25] Both replicon cell lines and Huh 7.5 cells met Institutional Review board requirements at their institutions of origin.

Effects of MPP on Cellular Viability and Proliferation

The most active MPPs were tested for effects on replicon cell proliferation and viability using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye conversion assay (*Cell Titer 96, Promega*) essentially as described by the manufacturer with some modifications. Huh 5.15 replicon cells were plated into 96-well plates and allowed to attach overnight. At 24-hrs intervals, MTT reagent was then added to cultures and absorbance measured at 570 nm. Because of variable background absorbances to MPPs noted by others,[26] controls also included subtraction of blanks containing just vehicle and MPP concentration. Only viable cells convert MTT into formazan which is measured by absorbance (abs) at 570 nm.[27] The formula used for determination of viable cells relative to controls was

$$\%\,\text{viable cells} = \left(\text{abs}_{\text{sample}} - \text{abs}_{\text{blank}}\right) / \left(\text{abs}_{\text{control}} - \text{abs}_{\text{blank}}\right) \times 100$$

As we have reported previously, MTT assay closely corresponded with cell counting and trypan blue staining.[28]

RNA Isolation and Real-Time RT-PCR

Total RNA was extracted from cells using Trizol reagent (*Invitrogen)*, treated with Turbo RNase-free DNase (*Ambion*, TX), and processed as described.[23] The cDNA was synthesized with a superscript first-strand synthesis system (*Invitrogen)*. Real-time RT-PCR was performed using Taq DNA polymerase with the SYBR green Universal PCR Master Mix Protocol (Perkin Elmer Applied Biosystems, Foster City, CA). Quantitation was performed using the Comparative Cycle Threshold (ΔC_T) method using GAPDH housekeeping gene as standard.[29]

HCV NS3 Recombinant Protease Assays

Recombinant NS3-4A protease activities (1A, 1B, 2A, and 3A genotypes) were determined fluorometrically with *SensoLyte* HCV Protease Assay Systems (*AnaSpec*, Fremont, CA) using FRET assays.[25,28] Wide (591 nm/622 nm) short (490nm/520nm), or narrow (340nm/490nm) wavelengths for excitation/emission were used as listed in Table 1. Wavelengths used for each compound were determined empirically from autofluorescence experiments that defined the optimal signal-to-noise ratios. All assays were performed in parallel with control incubations containing porphyrin only to correct for autofluorescence. IC_{50} was defined as the concentration of antagonist necessary to achieve 50% inhibition of NS3-4A protease activity as determined in the enzymatic assays and was calculated by regression from activity vs antagonist concentration sigmoidal plots. To ensure assay performance, a known inhibitor, *Anaspec 25346*, was routinely tested in each batch of assays.[25] The inhibitor was employed at 10–20 μM (about 2-5x its IC_{50}) and led to 60–80% inhibition of the NS3-4A recombinant enzyme.

Competitive inhibition constants (K_i) of MPPs were determined on Lineweaver–Burk plots. Slopes of 1/V vs 1/S for each competitor concentration were determined by linear regression and Ki determined graphically.

The effects of various tetrapyrroles on HCV replication in replicon cells were determined in a semiquantitative fashion, measuring HCV RNA in cells treated with inhibitor as compared to vehicle only controls. Replicon cells were seeded into culture dishes and allowed to attach overnight then incubated with various quantities of porphyrin or control vehicle only. Forty-eight hr later, total RNA was extracted using *Trizol* reagent and the amount of HCV RNA determined by real-time RT-PCR as described above. EC_{50} was defined as the concentration of antagonist necessary to achieve 50% reduction of HCV RNA synthesis in cells in vitro as compared to controls. EC_{50} values were derived by regression analyses using activity vs inhibitor concentration sigmoidal plots. For all replicon assays danoprevir,[30] a known NS3-4A antagonist was used as a positive control to ensure replicon and assay performance. Concentrations used were 5-10x Ki (about 10 nM) and showed 80–90% inhibition.

Western Blot Analyses

Western blots (WB) were performed as previously described using enhanced chemiluminescence for signal detection (*ECLTM*, Amersham).[29]

Statistical Determinations

All mean FRET values were determined using 3–4 replicates per point. A completely randomized design with multiple treatment groups was used for the analysis of variance for each experiment and variances then pooled among experiments using appropriate degrees of freedom for among and within-group comparisons. IC_{50} and ED_{50} values were determined by regression assuming sigmoid curve inhibition and tested either pairwise using Fisher's Least Significant Difference test or among treatment groups using F statistic. All experiments were repeated at least twice. *Graphpad Prism* or *Excel* software was used for least-squares regression and calculation of all variances; however, final multiple group statistical comparisons were set up conventionally and performed by hand using standard tables of t and F values. *P* values that were greater than 0.05 were not considered significant.

Results

Porphyrin Inhibition of HCV Protease and HCV Replication

Structurally, all porphyrin compounds have a macrocyclic ring composed of 4 pyrroles joined by conjugated methine bridges (Figure 1). Substitutions at the R positions form deuteroporphyrin (H), mesoporphyrin (C-C), protoporphyrin (C=C) and isohematoporphyrin (C-C-OH). While heme (FePP) is by far the most common MPP, a variety of synthetic MPPs with different coordination metals was available for evaluation. We tested each compound for both enzymatic inhibition of the NS34A protease and attenuation of viral replication in cell-free and whole-cell assays, respectively. Background fluorescence of the porphyrins was reduced by the choice of optimal excitation

Table 1 IC_{50} and EC_{50} Values Were Determined for Inhibition of NS3-4A Protease Activity and HCV RNA Replication, Respectively

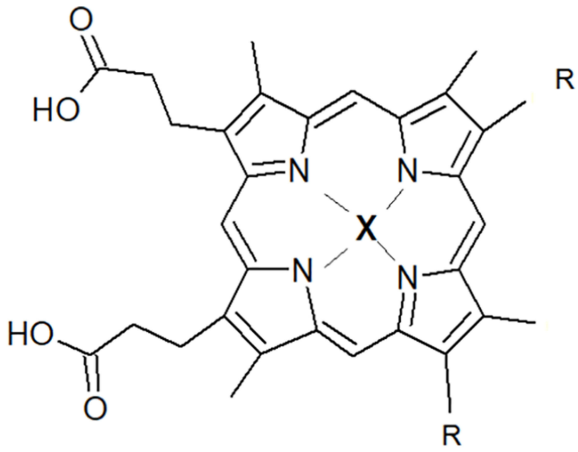
Porphyrin	Wavelength (nm)	NS3/4a Protease	HCV Replicon
		IC_{50} (μM)	**EC_{50} (μM)**
A. Metalloprotoporphyrins			
CoPP	620	*1.4	10.0
ZnPP	520	2.5	**3.0
MnPP	520,490	2.5	>20
SnPP	620	2.6	>100
CuPP	620,520	3.8	>10
FePP	620	6.5	4.0
		*CoPP < ZnPP = MnPP= SnPP (P < 0.05) < CuPP < FePP (P < 0.01)	**ZnPP < FePP (p < 0.05) < CoPP < MnPP, SnPP, CuPP (P < 0.001)
B. Metalloporphyrins			
Fe Deuteroporphyrin	620	#>20	##> 20
Cu Deuteroporphyrin	620	> 50	>20
Zn Mesoporphyrin	520	>50	>100
Sn Mesoporphyrin	620	>20	>20
Cu Isohematoporphyrin	620	> 50	>20
Fe Isohematoporphyrin	620	>20	>20
		# Metalloprotoporphyrin < all metalloporphyrins, p< 0.001	##Zn protoporphyrin < all metalloporphyrins p < 0.001
C. Free-base porphyrins			
φProtoporphyrin	520	φ7.5	φφ6.1
Deuteroporphyrin	520	>50	>20
Mesoporphyrin	520	11	10.0
Isohematoporphyrin	520	>50	>20
		φProtoporphyrin < mesoporphyrin (P< 0.01)	φφProtoporphyrin < mesoporphyrin (P< 0.01)
D. Positive Controls			
Anaspec 25,346		4.9–5.5 uM	nd
Danoprevir		nd	0.5 −1.0 nM

Notes: NS3-4A protease activity was determined with recombinant genotype 1b enzyme and IC_{50} values calculated with regression analyses on plots of activity vs [inhibitor] as shown in Figure 2. All compounds were tested along with a known antagonist *Anaspec 25346* as a positive control to ensure enzyme performance as stated by the manufacturer. For EC_{50} determinations, log-phase replicon cells were incubated with porphyrin or control vehicle 48 hr and the relative amount of HCV RNA then determined by the comparative cycle threshold level (ΔCT) as described in methods. EC_{50} was then extrapolated by regression on plots of HCV RNA vs [Inhibitor] curves as shown in Figure 2. All compounds were tested along with a known positive control, danoprevir[30] as described in methods to ensure replicon and assay performance. Each IC_{50} or EC_{50} value is the mean of at least two separate experiments with 3–5 determinations per point and variances were pooled using analysis of variance. Statistical values were determined using Fisher's Least Significant Difference (LSD) test for multiple comparisons.

and absorption wavelengths (Table 1) which were determined empirically for each compound tested.

In the first experiments, plots of NS3-4A enzymatic activity (Figure 2A) and HCV RNA (Figure 2B) in the presence of various MPs were determined using recombinant genotype 1B NS3-4A protease or HCV replicon cells, respectively. All MPPs significantly inhibited recombinant NS3-4A and showed greater activity than biliverdin, the heme oxidation product.[25] As summarized in Table 1A, the MPPs exhibited IC_{50} in low micromolar range: CoPP (1.4 μM) < ZnPP = MnPP = SnPP < CuPP < FePP (6.5 μM). While the coordination metal did significantly impact IC_{50} of the protoporphyrins, all values were within a 5-fold micromolar range suggesting that the metal has only mild effects on enzyme inhibition (Table 1A). Moreover, FePP (IC_{50} = 6.5 μM) was only slightly better than that of the free-base protoporphyrin (IC_{50} = 7.5 μM) in support of this notion. It should be noted that IC_{50} values of Table 1 and Figure 2 plots may differ slightly because the tabular values are the cumulation of multiple experiments.

In contrast to the IC_{50} values, there was considerable disparity in the EC_{50} of the MPPs, and only ZnPP and



Deuteroporphyrin R = H
Mesoporphyrin R = CH_2CH_3
Protoporphyrin R = CH=CH2
Isohematoporphyrin R = CH_2CH_2OH
X = null, Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+} Sn^{2+}, Cu^{2+}, Co^{2+}

Figure 1 Porphyrin structure nomenclature.

FePP showed comparable IC_{50} and EC_{50} values (Table 1A, Figure 2B). At this time, we suspect that these findings may reflect differences in MPP cellular uptake, intracellular distribution, and cellular replication. Nevertheless, Western blot analyses showed that ZnPP, CoPP and CuPP decreased expression of HCV proteins NS3-4A and core in both infected hepatoma cells and replicons roughly comparable to EC_{50} values which further confirms their antiviral activities (Figure 2C).

Assay of various metal-substituted porphyrin derivatives showed that removal of the vinyl R group of the protoporphyrins (Figure 1) and substitution with deutero-, isohemato, or meso groups resulted in the loss of activity (Table 1B). We next tested the antiviral behavior of metal-free, ie, "free base" porphyrins (Table 1C) containing the same R substitutions. None of the porphyrin variants was as effective as protoporphyrin for anti-protease activity or inhibition of viral replication although mesoporphyrin did show appreciable anti-NS3-4A and intracellular activity. The isohemato- and mesoporphyrin R substitutions both contain saturated alkyl groups with free rotation that are not resonance stabilized with the planar porphyrin ring, in contrast to the vinyl group of protoporphyrin.

The performance of HCV replicons is known to be influenced by multiple factors such as cellular proliferation, viability, and culture confluency.[31,32] Consequently, we tested whether the major, active MPPs, as well as a known NS3 protease inhibitor, danoprevir,[30] had effects on cellular proliferation and/or viability in Huh 5.15NS replicon cells (Table 2). Huh 5.15NS replicon cells were seeded onto plates, allowed to attach overnight, then MPP (10 or 20 μM) or control vehicle added. At 24 and 48 hrs intervals, cultures were assayed with MTT assay as detailed in the methods. Of the compounds tested, ZnPP was most active and reduced the amount of viable cells 40–50% at both intervals while CoPP had no detectable effects at either time point. Neither FePP or SnPP had significant effects at 24 hr but both caused a small reduction of viable cells (12–19%) by 48 hrs. Because HCV inhibitors can potentially influence replicon cellular proliferation through inhibition of the replicon pathway,[31,32] we also tested the effects of Danoprevir, a known NS3 inhibitor with the MTT assay. Danoprevir had little if any effects on viable cells at 24 or 48 hrs at concentrations equivalent or in excess of its EC_{50}[33] (Table 2).

Kinetics of NS3-4A Protease Inhibition by Porphyrins and Derivatives

Reciprocal plots were performed to determine the inhibitory parameters of three active MPPs (Zn, Co, and Sn) (Figure 3A–C, respectively) for genotype 1B. These plots demonstrated that enzymatic inhibition was basically competitive and all three MPPs showed similar Ki (0.48, 0.43 and 1.7 μM, ZnPP, CoPP, and SnPP respectively).

Genotype Selectivity of MPP:NS3-4A Protease Inhibition

In general, first and second-generation HCV anti-proteases tend to show selectivity for genotypes 1 and 2.[10,34] Consequently, it was important to determine whether MPPs behaved in a similar fashion. We tested whether CoPP and ZnPP inhibited NS3-4A constructs from genotype 1A, 1B, 2A, and 3A viruses (Figure 4A and B, respectively). Both MPPs showed protease inhibition plots and IC_{50} values that were statistically indistinguishable across the four tested genotypes. These findings suggest that the antiviral activities of MPPs are versatile across HCV genotypes and that the MPPs may also be useful for related viruses.

For two metalloporphyrins, Zn and Sn, it was possible to directly compare the activities of their respective meso variants with the parental MPP across different HCV NS3-4A genotypes (Figure 4B and C). These studies

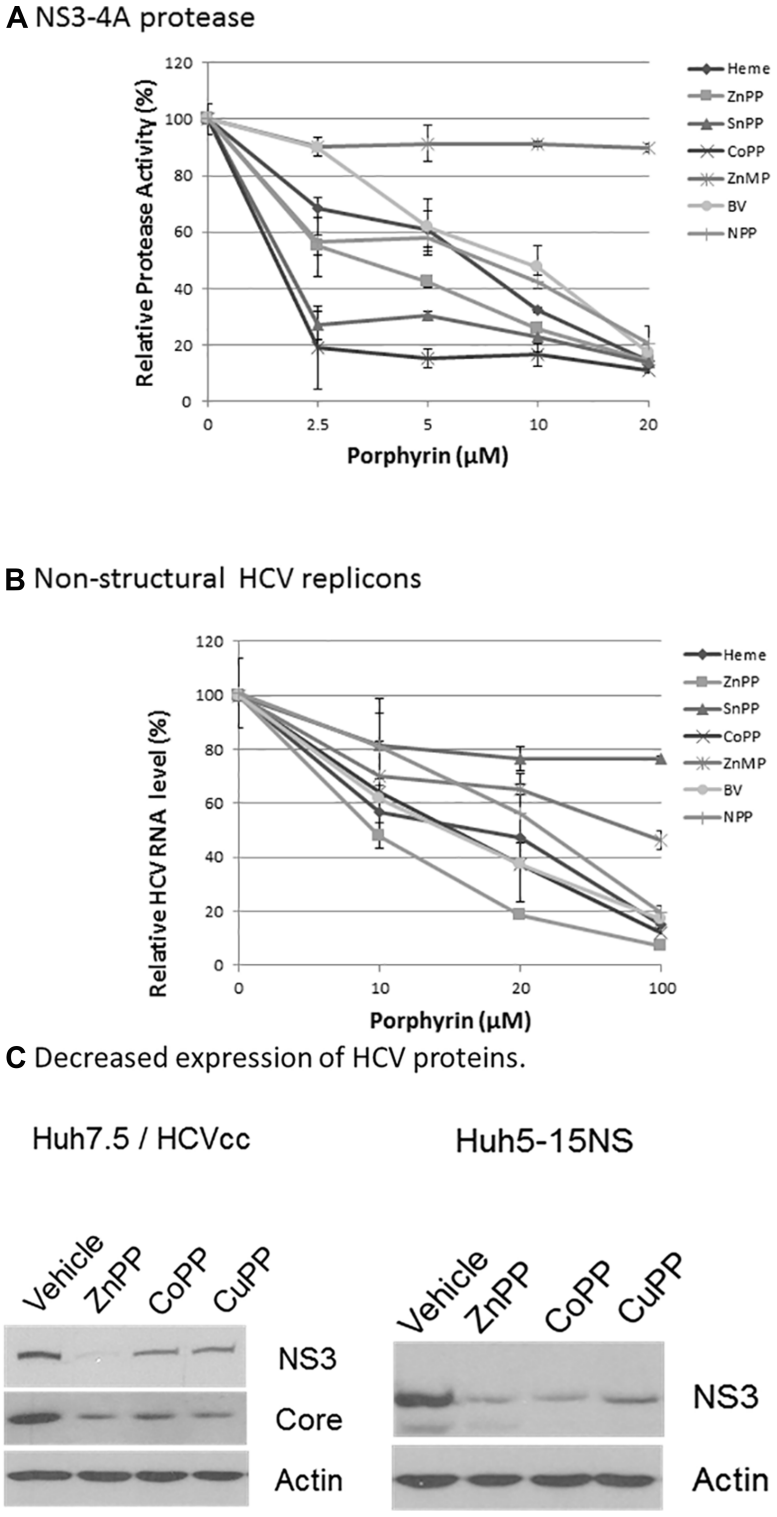


Figure 2 Porphyrin inhibition of HCV protease and HCV replication. (**A**). NS3-4A protease activity was determined fluorometrically using genotype 1B recombinant NS3/4A enzyme throughout. Appropriate wavelengths for measurement of fluorescence emission were determined empirically for each porphyrin to avoid quenching and/or background interference fluorescence (see Table 1). Each point value is the mean ±SEM with 3–5 determinations per point. Statistically significant differences between IC_{50} were ranked using Fischer's Least Significant Difference (LSD) test for multiple comparisons (Table 1). For $\alpha = 0.01$, LSD = 1.57. (**B**). Log-phase Huh 5.15 NS replicons were treated for 48 hrs with various concentrations of porphyrin and HCV RNA then quantified using *Real-Time* comparative threshold (ΔC_T) assay. Each point value is the mean ±SEM with 3–5 determinations per point. Statistical significant differences between EC_{50} values were determined using Fischer's Least Significant Difference (LSD) test for multiple comparisons (Table 1). For $\alpha = 0.01$; LSD = 1.83. (**C**). Western Blots for HCV proteins after HCV infection of Huh 7.5 or Huh 5.15NS replicon cells. Log-phase, J6/JFH infected, Huh 7.5 cells (left panel) were treated with MPP (10 μM) for 48 hrs. Cells were then lysed and viral protein expression evaluated on WB as described in methods. Log-phase Huh 5.15NS replicons (right panel) were treated with indicated MPP (10 μM) for 24 hrs and WB analyses performed similarly.

Table 2 Semi-Confluent HCV Replicon Cells in 96 or 24-Well Plates Were Incubated in Triplicate or Quadruplicate with the Indicated Compounds or Control Vehicle for 24 or 48 hrs

Compound	Conc (μM)	24 hrs			48 hrs		
		%	CV	P[a]	%	CV	P[a]
FePP	0	100	0.74		100	6.00	
	10	91.3	1.71	ns	88.0	4.47	ns
	20	96.3	2.05	ns	81.0	9.50	< 0.05
ZnPP	0	100	1.02		100	7.12	
	10	50.0	3.74	< 0.01	53.0	5.10	< 0.01
	20	54.6	4.00	< 0.01	59.0	7.24	< 0.01
CoPP	0	100	1.71		100	2.86	
	10	103	3.89	ns	102	3.06	ns
	20	104	3.48	ns	100	0.95	ns
SnPP	0	100	5.87		100	3.30	
	10	94.4	4.36	ns	86.0	7.10	< 0.05
	20	88.8	6.99	ns	86.0	1.20	< 0.05
	conc (nM)						
Danoprevir	0	100	4.05		100	5.85	
	0.5	99.1	1.60	ns	91.2	3.96	ns
	1.0	88.5	11.0	ns	92.9	6.66	ns

Notes: The relative amount of viable cells (control vs MPP or danoprevir) was measured with MTT assay as described in methods. Taking controls as 100%, mean percentage values were calculated and variability normalized as the coefficient of variation (CV). Since t statistic cannot test percentages, T-tests were performed pair-wise on the mean absorbance values and standard error of the means before normalizing data to percentage values.

Abbreviations: ns, not significant; conc, concentration.

demonstrated, most dramatically for ZnPP, that the protoporphyrin was a significantly better anti-protease as compared to the mesoporphyrin.

The findings of Figure 4 were further tested with statistical cross-comparisons of MPP vs meso MP vs genotype from multiple experiments and summarized in Table 3. IC_{50} values for either CoPP or ZnPP across each genotype were not significantly different; however, CoPP was a better inhibitor than ZnPP for all genotypes ($p < 0.05$) (Table 3A). In contrast, both ZnPP and SnPP were better inhibitors than their meso variants (Table 3B). These comparisons emphasize the importance of the vinyl R group of the MPP for anti-protease activity.

MPP Inhibition of NS3-4A Mutant Enzymes

Mutant enzymes appear in response to nearly all HCV anti-proteases and potentially can limit the scope and efficacy of antiviral therapies. We tested the activities of three MPPs, Zn, Cu and Mn for three major NS3 mutations, V36M, A156T, and T54A (Figure 5A–C, respectively). V36M is a common mutation that emerges in nearly all genotypes. Protease positions 54, 155, and 156 are "hot spots" for linear antivirals, such as first-generation telaprevir and boceprevir. Additionally, mutations at position 156 also arise after treatment with next-generation anti-proteases such as simeprevir and grazoprevir.[35] Enzyme constructs containing these mutations were tested for MPP inhibition as compared to WT enzymes. All three MPPs inhibited the mutant proteases as well or slightly better than the WT enzyme.

Discussion

Rapid advances have been achieved in HCV antiviral research; however, structure–function studies of new agents against HCV targets remain useful. First, there is still a need for new HCV protease inhibitors with a wider range of genotype and performance capabilities. Second, other *Flavivirus* pathogens share structural and functional features with HCV and knowledge gained from HCV can be compared and occasionally extrapolated to other viruses. Third, studies with HCV enzymes such as the NS3-4A protease enhance enzymatic research and benefit drug modeling for other serine-activated proteases.

HCV has high viral sequence variability, multiple genotypes, and an error-prone RNA polymerase that facilitates resistance-associated substitutions (RAS).[36] These issues have presented challenges for the development of HCV anti-protease agents.[37] First-generation protease inhibitors were designed using a substrate-based approach that modeled peptide analogs, "peptidomimetics".[38] These efforts were followed by the development of first and then second-generation macrocyclic drugs which yielded considerable improvement in dosing and performance. Although active against HCV genotypes 1, 2, and 4, most early macrocyclics were ineffective against genotype 3. Three third-generation macrocyclics, glecaprevir, voxilaprevir and grazoprevir were developed not only to target genotype 3 but to expand drug coverage to genotypes 1–6.[12] All of these drugs showed pan-genotypic inhibition of NS3 protease in vitro,[39–41] however, grazoprevir was a significantly less effective inhibitor for genotype 3 as compared to genotype 1 (90-fold difference in Ki).[41] Glecaprevir[13,17] and grazoprevir[15,16] are now approved for use in combination with NS5A inhibitors (as *Mavyret* and *Zepatier, respectively*), while Voxilaprevir[11,14] is approved for use with NS5A and NS5B inhibitors (as *Vosevi*). Overall these drugs have greatly expanded patient coverage, especially for those treatment-experienced individuals and for some resistant infections with RAS mutations;

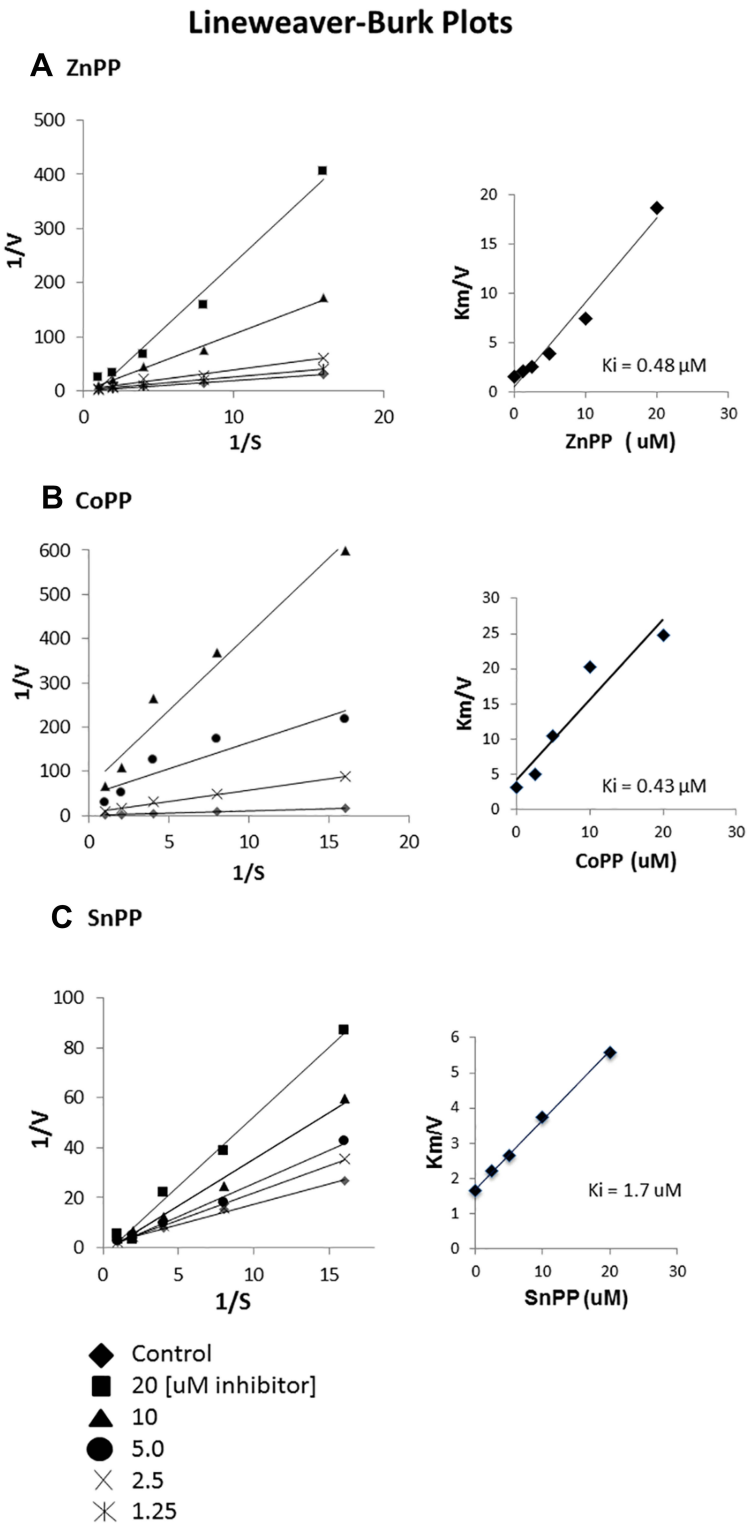


Figure 3 Lineweaver–Burk (LB) plots of NS3-4A protease Inhibition (**A**-**C**). Left Panels. LB plots of substrate concentration versus NS3-4A enzyme activity (genotype 1b) in the presence of various concentrations of MPP inhibitor. FRET assays of recombinant NS3-4A protease activity were conducted as described in methods. Right Panels. Secondary plots of Km/V (slope) versus MPP concentration were used to derive Ki for each MPP. Plots of [MPP] vs Km/V showed highly significant linearity for all three MPPs (r^2 = 0.97, 0.90 and 0.99 for ZnPP, CoPP and SnPP respectively, P< 0.005) strongly suggesting competitive inhibition of MPP for NS3/4A protease.

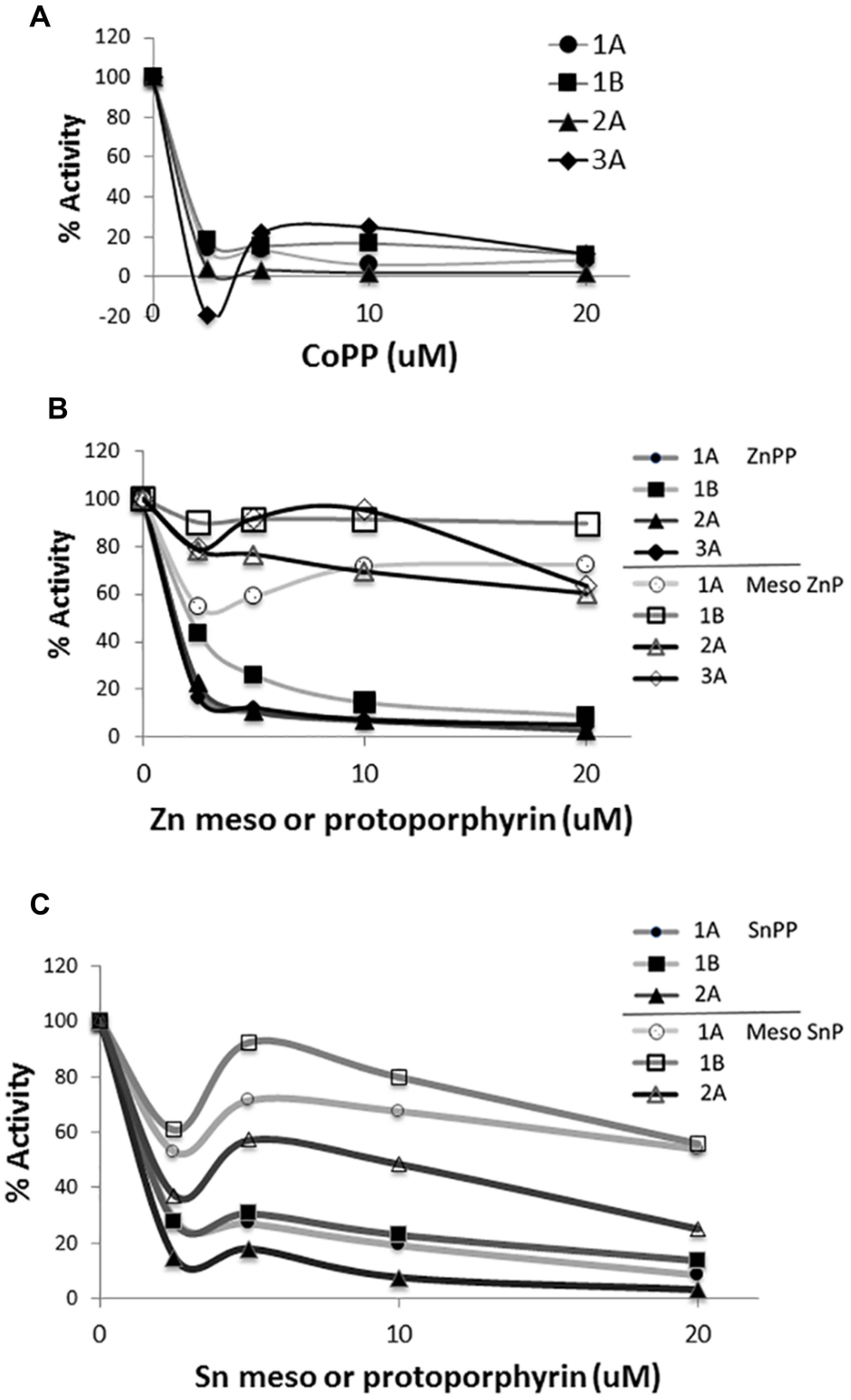


Figure 4 MPP and MP inhibition of HCV genotypes 1A, 1B, 2A and 3A NS3-4A proteases. NS3-4A protease activities were determined with FRET analyses using recombinant enzymes as described in methods. Each point is the mean ± SEM of three to five determinations per point, normalized to percentages. Error bars were omitted from this figure to improve the clarity of the individual curves. Data were analyzed as shown in Table 3A. (**A**) CoPP inhibition of various genotypes. (**B**) ZnPP and meso ZnP inhibition of HCV genotypes. (**C**) SnPP and meso SnP inhibition of HCV genotypes. Between-group comparisons were tested with a randomized block design for analysis of variance and *F*-test (Table 2).

Table 3 IC_{50} Values Were Determined for Inhibition of NS3-4A Proteases of the Indicated Genotypes Using Co or Zn Protoporphyrins (A) or Zn and Sn Protoporphyrins and Mesoporphyrins (B), respectively

A. Genotype vs Metalloprotoporphyrin		
	IC_{50} (uM)	
Genotype	***CoPP**	****ZnPP**
1A	1.4	1.5
1B	1.4	2.5
2A	1.2	1.6
3A	1.0	1.4

B. Metalloprotoporphyrin vs Metallomesoporphyrin IC_{50} (uM)						
Genotype	**ZnPP**	**ZnMP**	**P**	**SnPP**	**SnMP**	**P**
1A	1.5	>20	<.01	2.6	>20	<.01
1B	2.5	>20	<.01	2.6	>20	<.01
2A	1.6	>20	<.01	2.2	10	<.01
3A	1.4	>20	<.01	nd	nd	nd

Notes: To determine significant differences between IC_{50} values, a completely randomized design with equal (A) or unequal (B) group sizes was used for the analysis of variance and differences between genotypes vs MPs and MPPs determined by F statistic. Each IC_{50} is the mean of at least two separate experiments and variances were pooled among experiments for the statistical determinations. *CoPP vs genotype = ns; **ZnPP vs genotype = ns. All genotypes CoPP< ZnPP = $p<0.05$.
Abbreviations: ns, not significant; nd, not determined.

however, issues such as availability, tolerability, and suitable target populations remain.

Metalloporphyrins (MP) have been widely investigated for use as antiviral agents for a number of human and animal pathogens. They were proposed as general virucidal agents as well as highly specific compounds capable of targeting viral enzymes.[18] While a wide variety of synthetic MP and MPP have been described, mammalian cells chiefly synthesize heme or Fe protoporphyrin IX, which is used ubiquitously for many oxidative and metabolic reactions. Heme inhibits HIV and HBV reverse transcriptases[42,43] as well as HCV replication in vitro.[28] Biliverdin, the linear tetrapyrrole product of heme oxidation, was shown to inhibit HCV as well as HIV proteases.[25,44] Furthermore, the anti-HCV protease capabilities of heme and related MPPs appear to extend beyond the inhibition of viral replication. Heme and ZnPP were shown to rescue innate immune signaling in infected hepatocytes by inhibition of NS3-4A cleavage of mitochondrial antiviral signaling protein (MAVS), thus enhancing innate immune type I interferon response.[28]

Related pathogenic *Flaviviruses* such as Dengue (DENV), West Nile, and Yellow Fever share important structural and enzymatic properties with HCV. For DENV, there has been no approval of a specific anti-dengue drug although antiviral therapy in acute infection may reduce the risk for severe DENV.[45] However, Tseng et al[19] recently demonstrated that MPPs such as ZnPP and CoPP inhibited NS2-NS3 DENV protease with a mechanism remarkably similar to what our group reported for HCV NS3-4A.[23,25] Furthermore, biliverdin or CoPP treatment of infected DENV cells restored innate type I interferon signaling similar to our results with HCV.[28] Taken together, the findings of Tseng et al[19] and our report are useful for further development of MPPs or related tetrapyrroles as anti-DENV agents.

Herein, we investigated the basic structural requirements for MPs, as a class, to optimally target the NS3-4A protease. MPPs were the most effective inhibitors of all the porphyrins and MPs tested. MPPs contain two vinyl groups (Figure 1) which extend the resonance stabilized planarity of the porphyrin ring. Porphyrins with other substitutions such as meso (ethyl), isohemato (ethanolic), and deutero (hydrogen) were clearly less effective NS3-4A protease inhibitors. These results suggest that R-groups extend the planarity of the ring and act to enhance or stabilize active site binding. Planar compounds such as the macrocyclics[46] can optimally accommodate the long and shallow NS3-4A protease active site groove[47] and it appears that MPPs behave in a similar fashion. These observations also support our past work which showed a marked difference between the tetrapyrroles biliverdin (BV) and bilirubin (BR) for NS3-4A inhibition. BV, a flat compound with vinyl R groups, has an IC_{50} for NS3 protease 30 fold less than BR,[25] which has a folded "ridge-tile" hydrophobic structure.[48]

Classical double reciprocal, Lineweaver–Burk (LB) plots showed that MPPs bound NS3-4A with a basic competitive behavior similar to what we have shown previously for BV.[25] CoPP and ZnPP were the most active protease inhibitors tested and showed Ki values 10–20 fold stronger than biliverdin. Interestingly, the identity of the coordination metal had only minor effects on protease IC_{50} and calculated Ki. Moreover, free-base protoporphyrin, the immediate precursor to heme in the cellular synthetic pathway, was noted to have IC/EC_{50} values similar to FePP. These findings suggest that the coordination metal only minimally impacts protease inhibition per se; however, the metal may alter pharmacodynamics such as cellular uptake

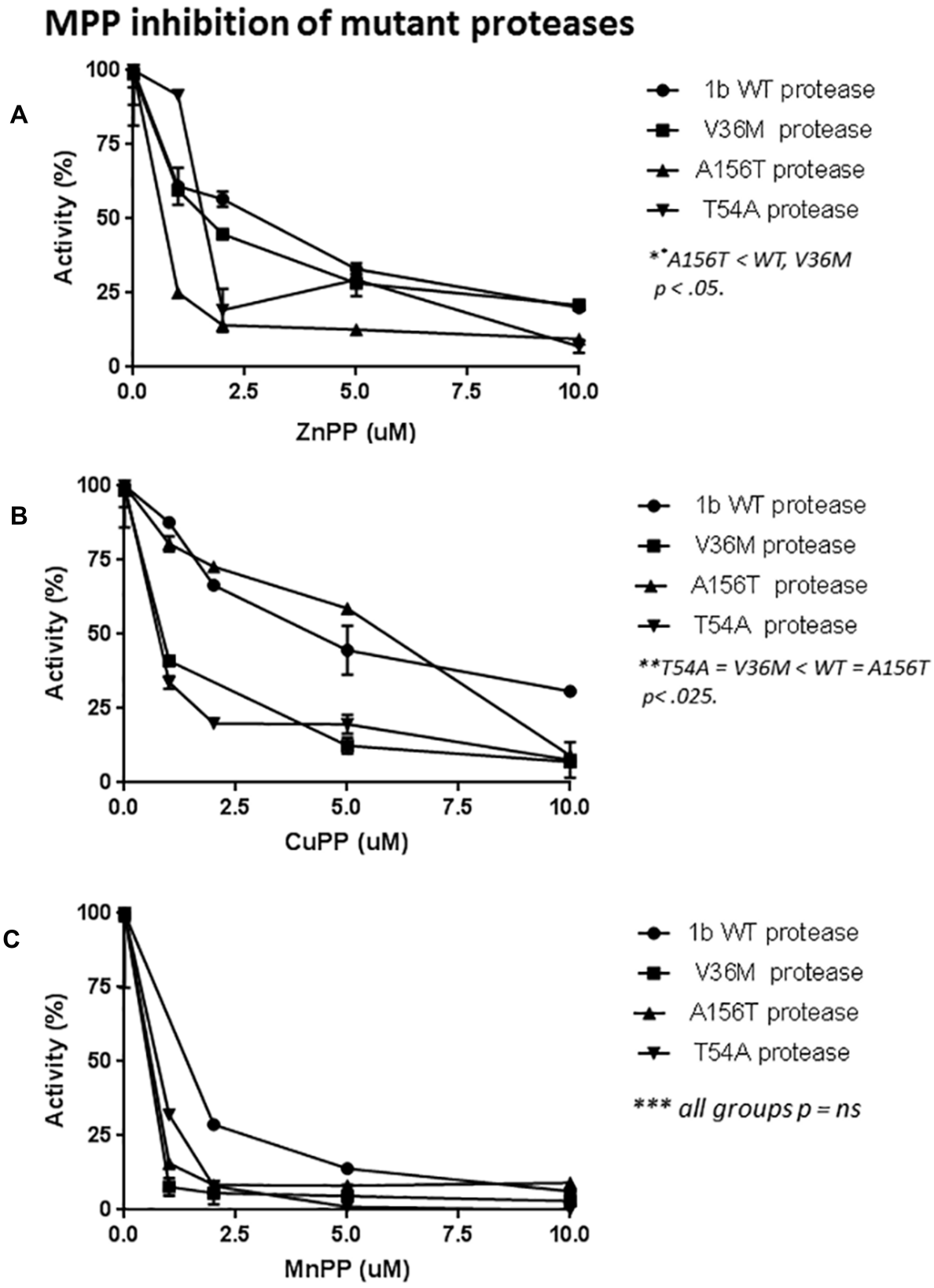


Figure 5 Inhibition of mutant NS3-4A proteases by MPP. Mutant [V36M, T54A, or A156T] or wild-type (WT) NS3-4A recombinant proteases were assayed fluorometrically in the presence of various concentrations of (**A**) ZnPP, (**B**) CuPP, and (**C**) MnPP as described in the methods. Each point is the mean ± SEM of three to five determinations per point and normalized to percentages. Between-group comparisons were tested with a randomized block design for analysis of variance and *F*-test.

and distribution much more dramatically. Generally, pharmacodynamics is not well understood for the MPPs as a class. Even the cellular uptake of heme is complex and can involve multiple mechanisms in addition to hemopexin binding and receptor-mediated uptake.[49–51] Related non-heme protoporphyrins such as ZnPP, CoPP and SnPP are known to interact differentially with heme uptake systems, and they also show marked differences intracellularly for interactions with cellular enzymes such as HO-1. Consequently, pharmacodynamic parameters appear important for the eventual choice of antiviral MPP candidates to consider for further development.

In addition to structural requirements for optimal NS3-4A inhibition, our data showed that three MPPs: CoPP, ZnPP and SnPP, were equally active against four of the major viral genotype enzyme constructs, ie 1A, 1B, 2A, and 3A, (Table 3). The weaker inhibitory activity of mesoporphyrins was also noted across these genotypes. The effectiveness of the MPPs against genotype 3A protease is especially attractive as it is the most resistant of all genotypes to first and most second-generation oral antiproteases[10] suggesting consideration for use as a pan-genotypic agent.

Three MPPs were also tested against three major NS3-4A mutations, V36M, T54A and A156T. While V36M and T54A chiefly arise with the use of the linear peptidomimetic compounds, A156T arises during treatment with nearly all linear and macrocyclic compounds developed to date.[52,53] ZnPP, CuPP and MnPP inhibited protease constructs with these mutations with the same fidelity as the wild-type enzyme, suggesting that MPPs could be designed for use on resistant proteases.

While our data indicate a direct interaction of MPP with the NS3-4A protease, it is important to note that protoporphyrins and MPPs might also impact HCV through other host enzymes and innate antiviral pathways. MPPs such as ZnPP and SnPP influence cellular levels of heme precursors in the protoporphyrin synthetic pathway through feedback and also induce HO-1 expression and heme oxidation possibly implicating multiple sites of antiviral activity.[18,54–56] Overexpression of HO-1 has been shown to directly inhibit HCV replication.[23] Consequently, in addition to NS3-4A inhibition, MPPs offer further possibilities for interactions with host cell enzymes that are likely to enhance the direct HCV antiviral actions.

HCV replicons were used to quantify the intracellular activities of the MPPs. However, viral synthesis in replicon cells is known to be affected by agents that also influence other cellular activities such as stability, replication, and growth.[31,32] Testing of four of the most potent MPPs, ZnPP, CoPP, SnPP and FePP showed differences in the ability of these compounds to influence viability or cellular proliferation in addition to their antiviral activities. Of the four compounds tested, ZnPP showed the most effect on viability as compared to CoPP which had no influence. Marginal effects were seen with SnPP and FePP. These results might be expected since ZnPP, but not other MPPs, has been reported to regulate pathways important for cellular replication such as Wnt/β-Catenin[57,58] and cyclin D1.[26] Our preliminary studies indicate that ZnPP directly interferes with DNA synthesis and also inhibits telomerase and chromosomal telomere repair mechanisms.[59] While these effects lead to a modest increase in apoptosis, ZnPP tends to be more cytostatic than cytotoxic however this difference cannot be determined from our data here. Consequently, it is not yet clear how or where the cellular activities of ZnPP may influence antiviral behavior but these topics require further study.

Of all the MPPs evaluated here, ZnPP and CoPP were found to be the most effective MPPs in enzymatic assays. Thus, it is important to note that the lower levels of ZnPP found to be effective in vitro can be achieved in cells during some disease states. Normally ZnPP is present at about 0.5 μM in reticulocytes; however, during pathologic states such as iron deficiency, it can increase tenfold to 5 μM.[60] ZnPP, or a derivative, is potentially a drug with multiple uses: it was proposed for use in jaundice of the newborn,[61] as well as chemotherapy,[21,62,63] suggesting versatile applications.

Finally, the possibility that liberated zinc or iron ion from the oxidation of the parental MPP significantly contributes to NS3-4A inhibition appears remote. While free iron and zinc have been shown to inhibit HCV replication, free ion concentrations 10–100-fold higher than either Fe or ZnPP values noted here were required.[25,64,65]

Conclusions

In summary, we have shown that porphyrins can be useful inhibitors of the HCV NS3-4A protease. The optimal activity appears to depend on the planarity of the R side chains while the identity of the coordination metal has only minimal impact. As a group, the metalloporphyrins show strong inhibitory activities across genotypes 1–3 and full inhibition of three common NS3 mutations. Our findings suggest that MPPs as a class should be investigated

further for use as antiprotease and antiviral agents in HCV and potentially other *Flavivirus* applications.

Abbreviations

abs, absorbance; BR, bilirubin; BV, biliverdin; $EC_{50,}$ concentration of agent to inhibit HCV replication by 50%; FRET, fluorescence energy transfer; HCV, Hepatitis C virus; HCVcc, Hepatitis C virus cell culture; HO, heme oxygenase; $IC_{50,}$ concentration agent to inhibit NS3-4A protease activity by 50%; MP, metalloporphyrin; MPP, metallo-protoporphyrin; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); NS, nonstructural; PCR, polymerase chain reaction; "X"MP, X-mesoporphyrin (where X = heavy metal); "X"PP, X-protoporphyrin (where X = heavy metal).

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Disclosure

JB is an employee and shareholder of Frontier Scientific Inc. CAT is an employee of Curza Global LLC. The authors report no other conflicts of interest in this work.

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