A novel class of meso-tetrakis-porphyrin derivatives exhibit potent activities against hepatitis C virus genotype 1b replicons in vitro

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ABSTRACT

Recent years have seen the rapid advancement of new therapeutic agents against hepatitis C virus (HCV) in response to the need for treatment that is unmet by interferon-based therapies. Most antiviral drugs discovered to date are small molecules that modulate viral enzyme activities. In the search for highly selective protein-binding molecules capable of disrupting viral life cycle, we have identified a class of anionic tetraphenylporphyrins as potent and specific inhibitors of the HCV replicons. Based on the structure-activity relationship studies reported herein, meso-tetrakis-(3,5-dicarboxy-4,4′-biphenyl) porphyrin was found to be the most potent inhibitor of HCV genotype 1b (Con1) replicon systems but was less effective against genotype 2a (JFH-1) replicon. This compound induced a reduction of viral RNA and protein levels when acting in the low nanomolar range. Moreover the compound could suppress replicon rebound in drug-treated cells and exhibited additive to synergistic effects when combined with protease inhibitor BILN 2061 or with IFNα-2a. Our results demonstrate the potential use of tetracarboxyphenylporphyrins as potent anti-HCV agents.
INTRODUCTION

Hepatitis C virus (HCV) exerts an increasingly heavy burden on global healthcare and approximately 200 million people worldwide are infected (39). Chronically infected patients are often at risk for developing hepatic fibrosis, cirrhosis, and hepatocellular carcinoma (15). HCV is an enveloped virus that belongs to the Flaviviridae family, and seven recognized HCV genotypes and numerous subtypes have been identified. Genotype 1a is the most prevalent strain worldwide and genotype 1b is predominant in Europe and North America, whereas genotypes 2 is more prevalent in Asia (4, 29). The current standard of care pegylated IFNα combined with ribavirin is plagued with adverse effects and has sustained viral response in less than half of the patients with genotype 1 infections (11, 17, 25). Therefore more effective and better tolerated therapies are urgently needed, in particular for the treatment of non-responders to IFN-based therapies.

The HCV genome, which is a single-stranded positive-sense RNA about 9.6-kb in length, encodes a polyprotein that is cleaved by viral and host proteases into structural (core, E1, E2, and possibly p7) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (4). The nonstructural proteins NS3 through NS5B assemble on the cytoplasmic membranes into a well-organized macromolecular machinery called the HCV replicase that is essential for the viral RNA replication (8, 14, 32). Until recently, the development of anti-HCV drugs had been hindered by the lack of a robust cell culture model. The establishment and optimization of the replicon systems have extensively widened our knowledge of the HCV replication, and also proved a powerful tool for the discovery of novel agents that target the assembly and function of HCV replicase. HCV
replicons are subgenomic constructs capable of autonomous replication in heptoma cell lines, and the major viral components of the replicons consist of NS3 through NS5B (23). Amongst these nonstructural proteins, viral protease NS3/4A and RNA-dependent RNA polymerase NS5B are the most extensively explored targets for anti-HCV drug development (for reviews, see Ref (6, 24, 28)). However, due to the error-prone nature of NS5B, mutational escapes could rapidly emerge under selective pressures from viral-specific inhibitors (35, 40). Other modalities under investigation include immunal modulators and therapies targeting viral RNA.

Protein-protein interactions often involve substantial interfacial areas larger than 1000 Å² (34). Yet selective targeting of a surface region in order to alter a protein’s function or interaction with other biomolecules has not been extensively explored. In the current study, we have designed and synthesized a class of theoretical protein-binding molecules built on a porphyrin core, which is compatible with the biological milieu. The tetraphenylporphyrin scaffold provides a sizable platform allowing hydrophobic interactions with the target surface, while charged peptidic appendages projected from the periphery support electrostatic interactions with complementary groups on the target(s). This contact with large area may decrease the likelihood of high resistance developing of targeted virus. We explored the antiviral potential of this class of compounds against the HCV replicon systems. *Meso*-tetakis-(3,5-dicarboxy-4,4′-biphenyl) porphyrin (compound 6) was found to be the most potent and selective inhibitor of HCV genotype 1b Con1 replicons (EC₅₀ 0.024 ± 0.005 µM) with low cytotoxicity. While undertaking mechanistic studies to characterize the molecular target(s), we describe here the structure-activity relationships of tetraphenylporphyrin derivatives and the anti-HCV properties of
compound 6, which is a proof-of-concept model for the development of proteomimetics in HCV drug discovery.

MATERIALS AND METHODS

**Materials.** Meso-Tetra(4-carboxyphenyl)porphine (compound 1) was purchased from Frontier Scientific, Inc. The synthesis of compounds 4, 6 and 8 were described in previous publication (1) and the chemical synthesis procedures of the other analogues can be found in the supplementary materials. 5,10,15,20-Tetrakis (4-(trimethylammonio)-phenyl)-21H, 23H-porphine (TTMAPP) and 4,4',4'',4'''- (Porphine-5,10,15,20-tetrayl) tetrakis (benzenesulfonic acid) tetrasodium salt hydrate (TPPS$_4$) were purchased from Sigma-Aldrich. To protect photosensitive porphyrin compounds from degradation, compounds and compound-treated cell cultures were carefully shielded from light. NS3/4A protease inhibitor BILN 2061, developed by Boehringer Ingelheim (19), was a kind gift from Tsu-an Hsu from the National Health Research Institutes, Taiwan.

**Cells.** HCV genotype 1b (Con1 isolate) subgenomic replicon cell line with luciferase reporter (Huh-luc/neo-ET) was kindly provided by Ralf Bartenschlager from the University of Heidelberg (37). Huh-luc/neo-ET cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM nonessential amino acids, and 250 µg/mL of G418 (Invitrogen). Genotype 2a (JFH-1 isolate) subgenomic replicon cells YSGR-JFH were cultured in DMEM containing 10% fetal calf serum, 1 mM nonessential amino acids and 400 µg/mL G418.
An additional genotype 1b (Con1) replicon cell line (429/BBix) was cultured in similar media but with 3 µg/mL blasticidin (22).

**Determination of antiviral activities.** Ff-luciferase reporter activity was used to monitor the replication of HCV replicons in Huh-luc/neo-ET cells free from G418. Replicon cells were seeded at the density of $5 \times 10^3$ cells per well in 96-well plates. The following day, replicon cells were incubated in duplicates with DMSO or serially diluted tetraphenylporphyrin (TPP) analogues at 37°C. 72 hrs after co-incubation, cells were lysed with ice-cold passive lysis reagent after PBS wash, and the luciferase activity was measured with the luciferase assay kit (Promega) and Tecan FARCyte luminometer (GE Healthcare) following the manufacturers’ descriptions. The relative light units (RLU) were adjusted as percentage readings of the compound-free controls and the 50% effective concentration ($EC_{50}$) was determined from dose-effect curve by nonlinear regression analysis using Origin 6.1 (OriginLab Software). TPP analogues were also screened in vitro against HIV-1 IIIB and HBV as previously described (26, 41). Briefly $1 \times 10^3$ MT-2 cells per well were exposed in triplets to $0.1TCID_{50}/cell$ (50% of the tissue culture infectious dose) of HIV-1 IIIB and cultured in the presence of compounds. The $EC_{50}$ values were estimated by MTT-based colorimetric quantitation of viral CPE after 5 days. Anti-HBV activities were evaluated in 2.2.15 cells by means of Southern DNA hybridization.

**Determination of cytotoxicity and mitochondrial DNA toxicity.** Exponentially growing Huh-7 cells were seeded at a density of $1 \times 10^4$ cells per well in a 24-well plate and incubated with TPP analogues for 3 days. Cells were fixed and stained with 0.5% methylene blue in 50% ethanol followed by extensive washing. After the plates were air
dried, cells were solubilized in 1% sarkosyl and cell growth was determined from the extent of absorption by spectrophotometric measurements at 595 nm (Biotek Instruments). For compounds whose dark colour interfered with 595-nm reading, cytotoxicity was measured by the CellTiter-Glo luminescent cell viability assay following manufacturer’s description (Promega). Cytotoxicity in MT-2 cells was determined from colorimetric quantitation of uninfected MT-2 cells after 5 days of co-incubation.

Quantitation of mitochondrial DNA content was performed as previously published (18). Briefly CEM cell lysates were spotted onto Hybond paper using the Miliford II slot blot apparatus (Schleicher & Schuell). MtDNA was detected with mtDNA-specific probe and then reprobed with Alu probe for internal control. The autoradiographic bands were quantified on scanning densitometer (Molecular Dynamics).

The impact of serum concentration in culture media on the antiviral activities of compound 6 and its analogues. Similar to the HCV replicon assay, Huh-luc/neo-ET cells were incubated with serially diluted compounds in the presence of 5%, 10%, 20% or 40% (v/v) FBS. Cells were harvested after 72 hrs for luciferase activity assays.

Quantification of HCV RNA and NS5A protein. Huh-luc/neo-ET cells were seeded at the density of $1 \times 10^5$ cells per well in 6-well plate and treated with either DMSO control or up to 250 nM of compound 6. Cells were harvested after 24, 48, or 72 hrs of incubation and subjected to luciferase activity assay, RNA quantification and immunoblotting. Results were averaged from three independent repeats. Luciferase
activity assay was done in triplets per experiment as aforementioned and RLU reading was normalized against the total protein level per sample determined from Bradford assay.

Total RNA was isolated using RNeasy Mini Kit (QIAGEN) and the RNA concentrations were measured by spectrophotometry (GE Healthcare) followed by dilution into 50 ng/µL. Replicon RNA was quantitated in triplicates by amplifying the HCV 5'UTR using one-step real-time reverse transcriptase polymerase chain reactions (qRT-PCR). Each 20 µL replicate contained 100 ng of total RNA, 100 nM probe (6FAM-5'-TATGAGTGTCGTACAGCCTCCAGG-3'-MGBNFQ, Applied Biosystems) and 200 nM forward and reverse primers (5'-CTTCACGCAGAAAGCGTCTA-3', and 5'-CAAGCACCTATCAGGCCAGT-3' respectively, Yale University W.M. Keck Facility) (21), together with iScript reverse transcriptase and reaction mix for one-step RT-PCR (Bio-Rad Laboratories). Reactions were run in the iCycler iQ RealTime thermocycler detection system (Bio-Rad Laboratories) as follows: 10 min at 50°C, 5 min at 95°C, followed by 42 cycles at 95°C for 15 sec and 60°C for 30 min. Results were normalized against the β-actin mRNA levels in each sample (20).

For immunoblot analyses, cells were lysed in 100 µL of lysis/loading buffer (30 mM Tris 6.8, 12.5% glycerol, 1% SDS, 5% β-mercaptoethanol, and 0.01% bromophenol blue). Samples were electrophoresed by 8% SDS-PAGE and transferred onto a nitrocellulose membrane for 30 min at 15V using Trans-Blot semi-dry transfer apparatus (Bio-Rad Laboratories). The membrane was blocked with 5% non-fat dry milk in PBS for 1 hr and probed by mouse monoclonal antibody (7D4) specific for Hepatitis C Virus NS5A (Santa Cruz Biotechnology, Santa Cruz, CA) or monoclonal
antibody specific for human α-tubulin (Sigma-Aldrich) at 4°C overnight followed by washing in PBS with 0.2% Tween 20. After incubation with goat anti-mouse Ab (Sigma-Aldrich) for 1 hr at room temperature, the membrane was washed extensively and detected by chemiluminescent procedures according to manufacturer’s instructions (Perkin Elmer).

Reversibility of the action of compound 6 against genotype 1b HCV replicon. Huh-luc/neo-ET cells were seeded at a density of $2 \times 10^5$ cells per well in a 6-well plate, and were incubated for 12 days with DMSO control or up to 1 µM of compound 6 in the absence of G418. Cells were split every three days when media and compounds were replenished, and samples were collected for RNA quantification. Compound 6 was removed on Day 12 when cells were split and cultured in the presence of 250 µg/mL of G418. Replicon cells were continuously monitored for another 12 days, during which cells were split and sampled whenever reaching confluence. Cell viability was measured by CellTiter-Glo luminescent cell viability assay following manufacturer’s procedures (Promega), and the HCV RNA was quantitated by qRT-PCR and normalized as described above.

Activity of compound 6 against genotype 2a (JFH-1) replicons. In parallel to Huh-luc/neo-ET cells, $1 \times 10^5$ YSGR-JFH cells per well were incubated with DMSO control, compound 6, or recombinant human IFNα-2a (Pestka Biomedical Laboratories) in 6-well plates. Cells were harvested after 72 hrs of co-incubation and HCV RNAs were quantitated by qRT-PCR and normalized as described above.

Combination studies. Huh-luc/neo-ET cells were seeded at the density of $5 \times 10^3$ cells per well in 96-well plates. In the following day a mixture of two components
(compound 6 with IFNα-2a, or compound 6 with BILN 2061) were applied in serial dilution and hence kept at constant ratio. A total of eight different mixtures were assayed in duplicates such that the potency ratio of the two compounds ranged from emphasizing Drug 1 / de-emphasizing Drug 2 to de-emphasizing Drug 1 / emphasizing Drug 2. 72 hrs after co-incubation, cells were harvested for luciferase activity assay and the median-effect equation was used for dose-effect analysis. The doses of Drug 1 and Drug 2 required to inhibit HCV replication by x% when used alone were denoted as \( (D_x)_1 \) and \( (D_x)_2 \), whereas the apparent isoeffective doses needed to achieve x% inhibition when used in combination were denoted as \( (D)_1 \) and \( (D)_2 \). The ratios \( (D)_1/(D_x)_1 \) were plotted against \( (D)_2/(D_x)_2 \) in antiviral isobolograms, in which the hypotenuse represents the line of additivity. If the experimental isobole bows below the hypotenuse, the combination is considered to be synergistic; if the isobole bows above the hypotenuse, antagonism is suggested. Synergy index (SI) values were calculated as the fractional distance from the origin to the intersection of isobole and hypotenuse, with the total distance (half the length of hypotenuse) designated as value 1.00. The smaller the SI value the stronger the degree of synergism. Another parameter for quantitative determination of combination is the combination index (CI, calculated as \( (D)_1/(D_x)_1 + (D)_2/(D_x)_2 \)). CI values within the range 1 ± 0.1 indicates additivity. CI values above or below the boundary suggest antagonism or synergism respectively (5, 13, 33). P value was determined by two-way ANOVA test using GraphPad Prism 4.0.
RESULTS

Structure-activity relationships of the tetraphenylporphyrin analogues.

From a small library of porphyrin analogues that we initially explored for antiviral application, compound 2 emerged as a micromolar inhibitor of HCV replicons in vitro and provided the first insight into the development of meso-tetrakis-phenylporphyrin (TPP) derivatives as anti-HCV agents (Table 1). Over 7-fold improvement in the anti-HCV EC_{50}, together with a decrease of cytotoxicity and less effect on mitochondrial DNA synthesis, was observed in its synthetic precursor compound 1 in which the aspartic acid side chains were replaced with more rigid and planar carboxylic acids. Substitution of the carboxylic acids in compound 1 with sulfonic acids as in TPPS_{4} led to complete loss of anti-HCV activity in vitro (EC_{50} 51.24 ± 8.577 µM). Reversing the charge of functional groups in the example of TTMAPP also compromised the activity against HCV (EC_{50} 3.58 ± 0.208 µM). Structures of the TPP analogues along with synthesis intermediates can be found in Fig S1.

With the goal of improving hydrophobic surface recognition, we extended each peptidic appendage by one phenyl ring giving rise to the family of meso-tetrakis-biphenylporphyrins (TBPs) that included compounds 4 and 5. The distance from opposite para positions of the phenyl groups was thus extended from approximately 15.5 Å in TPPs to 24.0 Å in TBPs, thereby increasing the total recognition area by 100 Å^{2} (34). Expansion of surface area from compound 2 to compound 5 was accompanied by a 12-fold improvement in anti-HCV EC_{50}. However, this was not the case in the comparison of compound 1 to its larger homolog 4 which showed reduced the antiviral activity.
We then increased the total negative charges of the peripheral groups from four to eight in order to enhance electrostatic interactions with potentially complementary regions on the target(s). While compound 3 was relatively ineffective against HCV replicons, compound 6 proved to be the most potent nanomolar inhibitor in our study, with an EC$_{50}$ of 0.024 ± 0.0051 µM that represented a 75-fold improvement over the lead compound 2. Given the extremely low cytotoxicity in naïve Huh-7 cells, compound 6 offers a favourable selective index in culture (CC$_{50}$ / EC$_{50}$) of over 2000. Additionally compound 6 did not alter the amount of mitochondrial DNAs. No changes in cellular morphology were observed in compound 6-treated replicon cells, confirming the lack of subtle cytotoxicity. Removal of one carboxylic acid from the meta position on each phenyl ring gave rise to compound 9 with decreased antiviral activity, suggesting that the projection of all eight negative charges are indispensable for potent inhibition of HCV replicons. As the functional groups became bulkier and more flexible in the cases of compounds 7 and 8, the antiviral activity was substantially decreased. Overall there was no evidence of increased cytotoxicity over time. To study if metallation of the porphyrin rings can influence the antiviral function, we synthesized zinc-, copper- and iron-conjugates of compound 6 and compared their EC$_{50}$s. Results suggested that metallation of porphyrin core did not significantly alter the anti-HCV activity (Table S1).

In addition to anti-HCV SAR, we also examined the nine TPP analogues against HBV and HIV-1 IIIB to establish antiviral specificity (Table 1). None of the compounds were able to inhibit HBV; whereas analogues bearing tetrabiphenylporphyrin motifs (compounds 5-8) exhibited micromolar activity against HIV-1 IIIB.
Compound 6 suppressed viral macromolecules in genotype 1b replicon cells in a time-dependent and dose-dependent manner.

The anti-HCV activities of compound 6 in Huh-lucneo-ET cells were characterized by studying different viral parameters: the luciferase reporter activity, HCV RNA level, and the protein level of NS5A (Fig 1). The replicon luciferase activity was markedly inhibited by compound 6 in a dose-dependent manner and the EC₅₀s decreased with incubation time indicating improved efficacy. The 24-hr and 48-hr EC₅₀s of compound 6 were 57.8 nM and 19.2 nM respectively; the 72-hr EC₅₀ was 17.6 nM, an over 3-fold improvement compared with that of 24-hrs incubation (Fig 1A).

Since luciferase activity indirectly reflects the overall level of viral replication, we expected the HCV RNA to be suppressed in a similar fashion post exposure to the inhibitor. Quantitative amplification of the HCV 5’-UTR demonstrated that compound 6 indeed led to a reduction of the HCV RNA level in replicon cells in a time- and dose-dependent manner (Fig 1B). The relative HCV RNA level was expressed as percentage of the mock-treated control. The 24-hr and 48-hr EC₅₀s of compound 6 were estimated to be 67.8 nM and 36.2 nM respectively; the 72-hr EC₅₀ was 29.2 nM, an over 2-fold improvement compared with that of 24-hr incubation. When viral protein amount was quantitated by western blot analyses, a similar reduction of the NS5A protein level in drug-treated replicon cells was observed (Fig 1C and D).

The antiviral activities were confirmed in 429/BBiX (EC₅₀ 0.014 μM), a Huh-7.5 cell line carrying genotype 1b replicon that confers blasticidin resistance and does not carry luciferase reporter gene (Fig S2). In addition we confirmed that compound 6 did not affect the luciferase reporter activities in Ff-luciferase expression systems that carried
NFκB, TGF-β and AP-1 response element respectively (data not shown). Together this ruled out the possibility that compound 6 exerted its action by interacting with non-viral components of the replicon, i.e., Ff-luc, neo' genes and their gene products.

The effect of serum concentration in culture media on the anti-HCV activity of compound 6 & its analogues.

Sequestration of compounds by serum proteins could decrease the availability of free agent but might also improve the uptake of hydrophobic derivatives. Hence we studied the effects of serum protein binding on the antiviral activity of compounds 1, 4 & 6 using different amounts of FBS in the media. Huh-luc/neo-ET cells were incubated with compound 1, 4 or 6 for 72 hrs and the anti-HCV activity was evaluated by measuring the reduction of reporter luciferase activities. Up to 40% (v/v) FBS in media did not alter the luciferase activity of Huh-luc/neo-ET cells. In all cases the EC$_{50}$ increased with percentage FBS, which could reflect a decrease of available compounds due to sequestration by serum proteins. Extrapolation of the plot of EC$_{50}$ vs serum amount provided an estimate of the theoretical EC$_{50}$ values at 0% FBS (Table 2). Comparison of the relative fold changes in EC$_{50}$ with increasing percentage of FBS showed that compound 1 was more affected by serum binding than compound 6. Compound 4 appeared to be least affected by serum concentration and the fold increase was non-linear unlike the other two analogues (Fig 2).
The activity of compound 6 against genotype 1b (Con1) replicon was reversible but longer treatment with higher dosages could prevent viral rebound.

The goal of anti-HCV treatment is to completely eliminate the virus from infected cells. In order to assess the reversibility of the antiviral action and the possibility of replicon clearance, we incubated Huh-luc/neo-ET cells with increasing concentrations of compound 6 for 12 days free from G418 (Fig 3). On Day 12 compound 6 was removed and 250 µg/mL G418 was reintroduced while the replicon RNA level and cell growth were monitored for another 12 days. Due to the inhibition of viral replication by compound 6, cells that have lower levels of replicons should become more sensitive to G418. Therefore the percentage of cells killed reflected the percentage of cells “cured” by compound 6.

Up to 1 µM compound 6 did not cause significant toxicity in cells during the 12-day treatment. A steady decrease in viability was initially observed when replicon cells were co-cultured with G418, followed by gradual rebound (Fig 3B). Cells that were treated with higher concentrations of compound 6 experienced significant delay in rebound. 8.5% of the cells treated with 50 nM of compound 6 survived 6 days after the removal of inhibitor and replicon-positive cells slowly rebounded to 13.1% after a lapse of another 6 days. Only 0.007% of the cells treated with 100 nM of compound 6 survived under the selective pressure of G418 by the end of the experiment; no rebound was observed and the replicon RNA level once fell beneath detection limit. Cells that were exposed to 300 nM and 1 µM of compound 6 were no longer viable 9 days after co-incubation with G418, which indicated complete “cure”. Concentrations of compound 6 of 300 nM and above induced approximately 4.5-log₁₀ reduction in the HCV RNA levels.
after 12 days of exposure. HCV RNA level in cells treated with lower concentrations of compound 6 rebounded faster than in cells treated with higher dosages (Fig 3A).

In a separate experiment we treated the replicon cells with compound 6 for 9 days followed by a 15-day rebound period in the presence of G418. Only 0.4% of the cells treated with 300 nM of compound 6 survived with a lack of rebound and 1 µM of compound 6 achieved complete “cure” (data not shown).

**Genotype 2a (JFH-1) replicon cells were more resistant to both compound 6 and IFNα-2a.**

In contrast to Huh-luc/neo-ET replicon cells, YSGR-JFH – a genotype 2a JFH-1 isolate replicon cell line – was more resistant to treatment with compound 6 as well as IFNα-2a. Replicon cells of the two genotypes were incubated with various concentrations of compound 6 or IFNα-2a for 72 hrs and the HCV RNA level was quantitated by qRT-PCR. The antiviral EC50 of compound 6 against YSGR-JFH replicon cells was 1.38 ± 0.148 µM, which was over 57-fold higher than the EC50 against Huh-luc/neo-ET replicons (Fig 4A). The antiviral EC50s of IFNα-2a were 2.39 ± 1.765 IU/mL and 25.99 ± 4.119 IU/mL in Huh-luc/neo-ET and YSGR-JFH replicon cells respectively, representing an approximately 11-fold difference (Fig 4B). YSGR-JFH replicon cells were also less responsive to compound 1 as expected (EC50 1.9 µM).
Compound 6 exhibited additive to synergistic effect when combined in vitro with BILN 2061 or IFNα-2a.

The development of more effective and nontoxic combinations of therapeutic agents has become an important goal in the management of HCV infection. We assessed the combination of compound 6 and established anti-HCV agents with respect to their antiviral activities when used alone. In a classical isobologram, the synergy index (SI) represents the fractional distance from the origin to where the isobole and hypotenuse intersect. Hence SI > 1 indicates antagonism and SI < 1 synergism. The smaller the SI value the higher the degree of synergism. The intersection also represents the most optimum potency ratio (theoretically \( \frac{D_1}{D_2} = \frac{D_3}{D_4} \)) to achieve the highest degree of synergy at a given effect level when the isobole is of symmetrical distribution.

As shown in Fig 5A and 5C, the combination of compound 6 with BILN 2061 or IFNα-2a was near additive at the 50% effect level (EC\(_{50}\)), with SI values around 1.00. Synergistic effect became more apparent at the 90% inhibition level when SI value was around 0.70 and the two isoboles differed significantly as indicated by P value < 0.0001 (Fig 5B, D). The combination of compound 6 with BILN 2061 (optimum potency ratio \( \approx 0.34 : 0.36 \)) was slightly more synergistic than combination with IFNα-2a (optimum potency ratio \( \approx 0.38 : 0.37 \)). Calculation of the combination index (\( CI = \frac{D_1}{D_2} + \frac{D_3}{D_4} \)) provided a similar conclusion on drug combination: CI\(_{90}\) values of compound 6 in combination with BILN 2061 (Fig 5E) or IFNα-2a (Fig 5F) confirmed additive to synergistic interactions between the compounds.
DISCUSSION

In recent decades, efforts in medicinal chemistry have been concentrated in the development of small molecule inhibitors that are selective and high-affinity binders of active sites in the protein cavities with the goal of disrupting protein-protein or protein-ligand interactions. In contrast, the protein exterior surfaces frequently employed in specific recognition during intermolecular interactions have been less explored. Specific targeting of such large interfacial areas with their complex topological distribution of hydrophobic, polar and charged residues can potentially be achieved by molecules that mimic protein surface structures. Porphyrins, peptidocalixarenes and α-helical mimetics are examples of macromolecules that have been designed to bind to protein surface and modulate protein-protein interactions (for a review, see ref (9)). Porphyrins are attractive macrocyclic scaffolds due to their intrinsic compatibility with the biological milieu and their physicochemical properties along with synthesis procedures are also well documented. The photoinactivation of viruses by tetrapyrroles has been widely studied. Porphyrins and metalloporphyrins have also demonstrated light-independent activity in the micromolar range against HIV and vaccinia virus (7, 38). In particular anionic tetrapyrroles including sulfonated porphyrins such as metallo-TPPS₄ were shown to inhibit HIV-1 infection by blocking cell fusion induced by the envelope protein and also possibly by disruption of gp120-CD4 binding (38). Interestingly, an uncharged molecule TPP[2,6-(OH)₂] was equally active against vaccinia virus suggesting that the interaction between charged groups may not be the sole basis for its antiviral activity (3). Exploration of the four-fold symmetry of porphyrin derivatives is best illustrated in the rational design of tetraphenylporphyrins to reversibly block the conductance of voltage-
gated potassium channels, which are homotetrameric molecules essential for numerous cellular functions. As synthetic mimics of peptide toxins, these cationic porphyrins appear to bind the channel pore and also mediate polyvalent interactions with the conserved acidic residues on the channel subunits (12).

For the development of HCV enzyme-specific therapies, viral protease NS3/4A and RdRP NS5B are the most intensely exploited targets. Successful examples of small molecule inhibitors include protease inhibitor telaprevir (VX-950) and boceprevir (SCH503034), nucleoside polymerase inhibitor R7128 and non-nucleoside NS5B inhibitor VCH-222. The macrocyclic inhibitor of NS3 – BILN 2061, despite being suspended in clinical development, is a proof-of-principle peptidomimetic compound that was designed to mimic the conformation of substrate-based hexapeptides bound to NS3 and is active both in vitro and in vivo (19, 36). In the present study we report the development of tetracarboxyphenylporphyrins for feasible interaction with biomolecules involved in HCV replication. This class of tetraphenylporphyrins (TPPs) offers a rigid scaffold capable of forming hydrophobic interactions with protein exteriors or solvent-exposed shallow clefts. The binding of the synthetic ligands could be further strengthened through electrostatic interactions with the cationic groups on the targets. The inherent four-fold symmetry of TPPs can potentially lead to simultaneous binding to several components/subunits of a heteromeric or homomeric complex. The structural features of TPPs could be of particular interest in antiviral drug discovery, because the virus would require multisite mutations (possibly spanning more than one target protein) to become highly resistant, an event with significantly lower probability than single-site mutation that is often sufficient for conferring resistance to small molecule inhibitors.
Based on a lead, compound 2, that exhibited micromolar activity against HCV replicons, we explored TPP analogues with different structural features in search of a selective inhibitor active in the low nanomolar range. The following key factors were taken into consideration during our structural optimization: (1) surface area, (2) charge, size and flexibility of the peptidic appendages, (3) the projection of functional groups relative to the hydrophobic core, and finally (4) solubility and serum sequestration. An interesting feature of tetraphenylporphyrin (TPP) and tetrabiphenylporphyrin (TBP) derivatives is that the first phenyl ring is perpendicular to the porphyrins core whereas the second phenyl ring lies perpendicular to the first ring and in the same plane as the porphyrin core. Consequently compounds 1 and 4, for example, represent a completely different projection of anionic appendages. As shown in Table 1, SAR analysis revealed that the most optimum structure against HCV in vitro is that of an octaanionie tetrabiphenylporphyrin – compound 6 (EC50 0.024 ± 0.0051 µM), which represented a 75-fold improvement in EC50 over the lead compound and is comparable to other anti-HCV agents developed to date. Moreover the carboxylic acids could not be replaced with sulfonate, trimethylammonium, the more flexible aspartic acid, or bulkier moieties, nor could the number of negative charges be decreased – all of which led to a reduction in activity. Metallated derivatives of compound 6 demonstrated anti-HCV activity similar to the parent compound, suggesting that contact with the porphyrin core does not contribute towards anti-HCV activity, or compound 6 itself becomes metallated upon entering the cells. Expansion of the hydrophobic surface area improved antiviral efficacy except in the case of compound 1 to 4, which may be due in part to their different projections of anionic groups and their differences in serum binding. Sequestration by serum has the
potential to decrease the availability of free drug, but may also improve its solubility and promote uptake into the hepatocytes. Compound 1 appeared to have the highest degree of binding to serum proteins and its anti-HCV EC$_{50}$ increased linearly with percentage serum in the media (Fig 2). In contrast, compound 4 has the lowest degree of serum association, which could hinder its uptake. Sharing the same hydrophobic core, compound 6 however benefits from a greater number of electrostatic interactions that could help towards uptake into cells. The activity of compound 6 against HCV replicons was confirmed by the suppression of viral RNA and protein levels of two independent genotype 1b (Con1) replicons established in Huh 7 and Huh 7.5 cells respectively (Fig 1, S1). If compound 6 disrupts the assembly of the HCV replication complex, active replicates that have already assembled prior to drug treatment could continue viral replication until turnover, which may explain the initial increase of antiviral potency over time as shown in Fig 1.

Compounds 1, 4, 5, 6 and 9 are selective inhibitors of HCV in vitro and are relatively inactive against DNA virus HBV and RNA virus HIV-1 IIIB. Although compounds 5, 6 and their bulkier derivatives showed micromolar inhibition of HIV-1 IIIB comparable to tetraporphines that are under development as microbicides, there was no correlation between the trend of anti-HCV and anti-HIV efficacy therefore it is unlikely that the two types of antiviral activities share the same mechanism of action. The micromolar anti-HIV activities of compound 5 to 8 suggested that tetrabiphenylporphyrin scaffold and flexibility but not bulkiness of the peptidic appendages may be favourable to the inhibition of HIV-1 IIIB. Hamilton et al. had previously shown that tetracarboxyphenylporphyrin derivatives bind cytochrome c. Compounds 1, 4, 6 and 8
were found to bind cyt c with $K_d$ values of $0.95 \pm 0.25, 17 \pm 0.84, 1.5 \pm 0.17$ and $1.7 \pm 0.097 \mu$M, respectively (1, 16), but this property did not correlate with the SAR in the present study. We treated Huh-luc/neo-ET cells with up to 1 $\mu$M of compound 6 for 9 days, during which the media were replenished every 3 days and the cells were passaged once. Live cells were stained with the ratiometric indicator JC-1 (Invitrogen) in order to measure the mitochondrial potential using confocal microscopy. Compared with mock-treated control, compound 6 did not affect the mitochondrial membrane potential unlike the classical uncoupler valinomycin (Calbiochem). In light of the extremely low toxicity on cells and particularly on the amount of mitochondrial DNA, it is unlikely that the potent anti-HCV activity of compound 6 is mediated through cyt c binding.

Compared with subgenomic genotype 1b (Con1) replicons, genotype 2a (JFH-1) replicon appeared to be more resistant to IFNα-2a with 11-fold difference in the anti-HCV EC$_{50}$, in accordance with literature (27). Con1 and JFH-1 isolate differ significantly in their replicase coding region. Surprisingly genotype 2a (JFH-1) replicon was also more resistant to compound 6 and the anti-HCV EC$_{50}$ fell into micromolar range, being 57 times less effective than the activity against genotype 1b (Con1) replicons. The HCV RNA levels were similar between the two cell lines indicating that differences in replication capacity could not be the major contributing factor. Such different sensitivity towards compound 6 suggests the possible involvement of a cellular interactor of the viral replicase, which however could be dispensable for the replication of genotype 2a (JFH-1) replicons. Besides the significant impact of genetic variability on the drug sensitivity, the observed differential response to IFNα-2a and compound 6 between the two subgenomic replicons could be correlated. HCV is known to suppress host immune
responses and reduction of viral load restores the production of IFNα/β and related antiviral signalling pathways (10, 30). Therefore the antiviral activity exerted by compound 6 could be augmented through the action of revived host defences and the IFN amplification loops. Such amplification could be more significant in genotype 1b (Con1) replicon-containing cells due to their intrinsic IFN sensitivity and this potential “dual inhibition” could be masked in cells harbouring genotype 2a (JFH-1) replicons.

Unlike the treatment of HIV, HCV therapy can lead to complete eradication of virus in a significant proportion of patients. We demonstrated that the antiviral activity of compound 6 was irreversible if the treatment period is sufficiently long and the dosages adequate (Fig 3). Moreover the longer the treatment, the further was the delay in viral rebound. Whether the percentage of replicon-positive cells that survived the treatment under high concentrations of compound 6 could have involved the development of resistance remains to be addressed. The limitation of the replicon model, however, could be the relationship between the viral load per cell and the sensitivity of host cells to G418. If the HCV replicon falls below a threshold level enough to subject hepatocytes to geneticin toxicity, the remaining replicon is beneath detection limit due to decreased cell viability. On the other hand, geneticin selectively amplifies replicon-positive cells above the threshold. We have also carried out rebound studies in the absence of geneticin; however the HCV RNA level in mock- and drug-treated cells all reduced with time due to the lack of selective pressure.

As in HIV management, combination therapy is an important focal point in the development of anti-HCV agents. Optimum combination of drugs with different mechanisms of action should improve efficacy with a wider therapeutic window and
reduced viral resistance. It is important that the combination should produce at least additive effects with no antagonism. Our *in vitro* synergy studies showed that the combination of compound 6 with BILN 2061 or with IFNα-2a was additive to synergistic at the effect levels studied, more so at 90% inhibition (Fig 5). According to the antiviral isobolograms, approximately equipotent combination of compound 6 and BILN 2061 (~0.350EC_{90}) or compound 6 and IFNα-2a (~0.375EC_{90}) was sufficient to inhibit HCV replication by 90%. The difference between the degree of synergism at the 50% and 90% response level illustrated how the nature of drug-drug interactions may vary depending on the dose ratio in combination and on the endpoints of choice (5, 33).

The antiviral specificity and genotypic selectiveness of compound 6 suggest that the compound could be targeting the viral replicase. Whether the binding of octaanionic tetrabiphenylporphyrin to viral protein blocks the interaction with HCV genome, other proteins in the replicase or with host factors is under investigation. If the synthetic agent targets highly conserved sequences that are essential for viral replication, mutations at these hot spots should have decreased probability and as a result it could be difficult for the virus to develop high resistance. *In vitro* resistance characterization and transient replication assays will be employed in future research to elucidate the mechanism of action of compound 6 and to establish its cross-resistance profiles involving known inhibitors of the HCV NS3/4A as well as NS5B. While undertaking detailed mechanistic studies, we present here the proof-of-concept design and antiviral results for compound 6, which shows great potential as a potent and selective inhibitor of HCV.
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FIGURE LEGENDS

**Fig 1.** Time- and dose-dependent reduction of viral parameters in genotype 1b (Con1) replicon cells induced by compound 6. Huh-luc/neo-ET cells were incubated with serially diluted compound 6 for 24, 48, or 72 hrs. Results were expressed as percentage of mock-treated controls. (A) Reduction of reporter luciferase activity, which indirectly reflects the replication level of HCV replicons. (B) Reduction of HCV RNA level normalized against the mRNA level of human β-actin. (C) Reduction of the NS5A protein level as in one experiment. (D) NS5A protein level quantitated and normalized against the protein level of α-tubulin (mean ± S.D. from three independent experiments).

**Fig 2.** Effect of serum binding on the EC$_{50}$s of compounds 1, 4 & 6. The fold changes in EC$_{50}$ were plotted against percentage serum using the EC$_{50}$ at 5% FBS as one fold (mean ± S.D. from three independent experiments). The antiviral activities of compounds 1 and 6 decreased linearly with increasing serum, and also differed significantly from each other at the 95% confidence intervals. Change in the EC$_{50}$s of compound 4, which was the least affected by serum binding, was nonlinear. P value was determined by two-way ANOVA test using GraphPad Prism 4.0.

**Fig 3.** Compound 6 could prevent the rebound of genotype 1b (Con1) replicons. Replicon cells were incubated with increasing concentrations of compound 6 for twelve days free
from G418. At the end of incubation, compound 6 was removed and 250 µg/mL G418 was reintroduced. (A) The level of HCV RNA in cells was quantitated by qRT-PCR. RNA copy number per µg of total RNA was expressed as ratio relative to the mock-treated controls. During the rebound period, replicon cells incubated with 300 nM and 1 µM of compound 6 were not confluent enough for sampling. (B) Cell viability was shown on log_{10} scale as percentage of mock-treated controls. Replicon cells that were treated with 300 nM and 1 µM of compound 6 were no longer viable by Day 21.

**Fig 4.** Compared with genotype 1b (Con1) replicons, genotype 2a (JFH-1) replicon was more resistant to both compound 6 and IFNα-2a. Genotype 1b (Con1) replicon cells Huh-luc/neo-ET and genotype 2a (JFH-1) replicon cells YSGR-JFH were incubated with increasing concentrations of (A) compound 6 or (B) IFNα-2a. Cells were harvested 72 hrs after incubation. The HCV RNA level was quantitated by qRT-PCR and expressed as percentage of mock-treated controls (mean ± S.D. from three independent experiments).

**Fig 5.** Antiviral isobologram and CI\textsubscript{90} plot of compound 6 in combination with BILN 2061 or IFNα-2a \textit{in vitro}. Huh-luc/neo-ET cells were co-incubated for 72 hrs with various concentrations of Drug 1 or 2 alone or the two in combination at different potency ratios. (A, C) The ratios of the apparent EC\textsubscript{50} of each drug in combination over its EC\textsubscript{50} when applied alone were plotted against each other in isobolograms. The hypotenuse represents linear additive response to the action of two therapeutic agents. Isoboles that bow below the hypotenuse indicate synergism, and isoboles that bow above the hypotenuse indicate antagonism. Experimental data points on the isobole represents a combination that inhibits the HCV replication by 50% and hence isoeffective with the line of additivity. The EC\textsubscript{90} isobolograms of compound 6 in combination with BILN 2061 or IFNα-2a were shown in (B) and (D) respectively (mean ± S.D. from at least four independent experiments). Different degrees of synergism/antagonism are expected at different effect levels. The combination index (CI) at 90% effect level for the combination of compound 6 with BILN 2061 or IFNα-2a were plotted in (E) and (F) respectively. Mean CI\textsubscript{90} value for each dose ratio was indicated above the bars. CI = 1 ± 0.1 suggests additivity as indicated by the dashed line. CI value below the boundary indicates synergism and above, antagonism.

**Fig S1.** Dose-dependent reduction of viral parameters in genotype 1b (Con1) cell line 429/BBix 72 hrs after incubation with compound 6. (A) Reduction of HCV RNA level. Results were normalized against the mRNA level of human β-actin and expressed as percentage of mock-treated control (mean ± S.D. from three independent experiments). (B) Reduction of the NS5A protein level.

**Fig S2.** Chemical structures of the tetraphenylporphyrin analogues compounds 1 ~ 3, and the tetrabiphenylporphyrin analogues compounds 4 ~ 9 were shown with their synthesis intermediates.
REFERENCES


27. Miyamoto, M., T. Kato, T. Date, M. Mizokami, and T. Wakita. 2006. Comparison between subgenomic replicons of hepatitis C virus genotypes 2a (JFH-1) and 1b (Con1 NK5.1). Intervirology 49:37-43.
Fig 1. Time- and dose-dependent reduction of viral parameters in genotype 1b (Con1) replicon cells induced by compound 6.
Fig 2. Effect of serum binding on the 72-hr EC₅₀'s of compounds 1, 4 & 6. Effect of serum concentration on the EC₅₀'s of compounds 1, 4 & 6.
Fig 3. Compound 6 could prevent the rebound of genotype 1b (Con1) replicons.
Fig 4. Compared with genotype 1b (Con1) replicons, genotype 2a (JFH-1) replicon was more resistant to both compound 6 and IFNα-2a.
A. $SI = 0.96 \pm 0.051$

B. $SI = 0.70 \pm 0.050$

C. $SI = 1.13 \pm 0.010$

D. $SI = 0.74 \pm 0.180$
Fig 5. Antiviral isobologram and CI90 plot of compound 6 in combination with BILN 2061 or IFNα-2a in vitro.
<table>
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<th>Antiviral Activity $EC_{50}$ (μM)</th>
<th>Cytotoxicity $CC_{50}$ (μM)</th>
<th>Mitochondrial DNA Toxicity $IC_{50}$ (μM)</th>
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<td></td>
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Table 1. Structure-activity relationships of anionic tetraphenylporphyrin analogues (mean ± S.D. from three independent experiments).
* Not enough compounds to complete the toxicity studies

Table 2. The effect of serum concentration in culture media on the anti-HCV EC\textsubscript{50}s of compounds 1, 4 & 6. (mean ± S.D. from three independent experiments)

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<th>Compound</th>
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<th>10% FBS</th>
<th>20% FBS</th>
<th>40% FBS</th>
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