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Published in:
Chemistry and Biology

DOI:
10.1016/j.chembiol.2014.10.023

2015

Research Article

Bioengineering and semisynthesis of an optimized cyclophilin inhibitor for treatment of chronic viral infection

Magnus Joakim Hansson[1, 2], Steven James Moss[3,5], Michael Bobardt[4], Udayan Chatterji[4], Nigel Coates[3,5], Jose A Garcia-Rivera[4], Eskil Elmér[1, 2], Steve Kendrew[5], Pieter Leyssen[6], Johan Neyts[6], Mohammad Nur-E-Alam[5], Tony Warneck[5], Barrie Wilkinson[3,5,7], Philippe Gallay[4]*, Matthew Alan Gregory[3,5]*.

Author affiliation
1 NeuroVive Pharmaceutical AB, Medicon Village, SE-22381 Lund, Sweden
2 Mitochondrial Medicine, Lund University, SE-22184 Lund, Sweden
3 Isomerase Therapeutics Ltd., Suite 9, Science Village, Chesterford Research Park, Cambridge, CB10 1XL, UK
4 Department of Immunology & Microbial Science, The Scripps Research Institute, La Jolla, California 92037, USA
5 Biotica Technology Ltd., Cambridge, CB10 1XL, UK
6 Department of Microbiology and Immunology, Rega Institute for Medical Research, K.U.Leuven, 3000 Leuven, Belgium
7 Department of Molecular Microbiology, John Innes Centre, Norwich, NR4 7UH, UK

*Corresponding authors
Matthew Alan Gregory, Isomerase Therapeutics Ltd., Suite 9, Science Village, Chesterford Research Park, Cambridge, CB10 1XL UK, matt.gregory@isomerase.co.uk
Philippe Gallay, Department of Immunology & Microbial Science, The Scripps Research Institute, La Jolla, California 92037, USA, gallay@scripps.edu

Summary

Inhibition of host-encoded targets, such as the cyclophilins, provides an opportunity to generate potent, high barrier to resistance antivirals for the treatment of a broad range of viral diseases. However, many host-targeted agents are natural products which can be difficult to optimize using synthetic chemistry alone. We describe the orthogonal combination of bioengineering and semisynthetic chemistry to optimize the drug-like properties of sanglifehrin A, a known cyclophilin inhibitor of mixed non-ribosomal peptide/polyketide origin in order to generate the drug candidate NVP018 (formerly BC556). NVP018 is a potent inhibitor of HBV, HCV and HIV-1 replication, shows minimal inhibition of major drug transporters and has a high barrier to generation of both HCV and HIV-1 resistance.

Significance

Hansson *et al*., describes the generation and preclinical analysis of a bacterial natural product with activity as a host targeted antiviral drug. This was generated by a combination of biosynthetic engineering and semisynthetic chemistry – bringing together the advantages of each method to more efficiently optimise a bacterially produced, genetically encoded natural product. The preclinical analysis reveals potent activity against HCV, HIV and HBV and a significantly improved profile as compared to both the parent natural product and the cyclosporine A (CsA) class of cyclophilin inhibitors, of which alisporivir is in phase III for treatment of chronic HCV infection.

Introduction

Treatment of chronic viral diseases by direct inhibition of viral targets frequently leads to rapid development of virally-encoded resistance. Therapies targeted to host proteins involved in the viral life-cycle offer an opportunity to both raise the barrier to generation of resistance and generate antivirals able to treat a broad range of viral diseases. However, this has been hindered in the past by the relative rarity of targets essential to the virus, but non-essential to the host, and the inherent complexity of discovering and developing drugs that effectively target these proteins.

From the 1940s to 2007, 73% of the 155 small molecules approved as anticancer drugs were of natural origin, either directly or derived[1]. As opposed to this, few natural products had been approved for use in antiviral therapy, although they were the inspiration for antiviral nucleoside analogues[2].

Many bacterial natural products (NPs), including the well-described polyketide class, have been shown to bind to and inhibit mammalian cellular proteins, including viral host-encoded targets such as cyclophilins, v-ATPases[3], CRM1[4] and Hsp90[5]. They can be produced at low cost of goods by fermentation and frequently the lead compound has both excellent potency against the target and good cellular penetration. However, natural products often display polypharmacology and have less than optimal mammalian pharmacokinetics and physicochemical properties.

The tools available for optimizing NPs are ever increasing. Specifically, bioengineering has been proposed as a way to reinvigorate natural products drug discovery[6,7]. In the past, semisynthetic approaches have been the most frequently used route to improve the drug-like properties of a natural product hit. However, the available semi synthetic options are pre-determined by the array of functional groups on the NP. In contrast, bioengineering options are pre-determined by the biosynthetic pathway. The potential changes are thus orthogonal to those available to semisynthesis.

Whole genome sequencing is now straightforward and easily affordable, and for NP classes such as the modular polyketide synthases (PKSs), knowledge of the DNA sequence encoding the biosynthetic gene cluster enables rapid understanding of gene product function. When combined with improved techniques for DNA transfer and the rapid targeted alteration of biosynthetic genes this provide a powerful platform for focused drug discovery efforts with the aim of improving drug-like properties, pharmacokinetics and reducing off-target effects. These bioengineering techniques are

readily combined with semisynthesis to identify molecules with further improved properties. In particular, inactivation of precursor pathways can allow mutasynthesis, the process of feeding a synthetic analogue of the precursor which is then incorporated, biosynthetically, into the final molecule[8,9]. This enables a combinatorial element to bioengineering.

Cyclophilins are a class of peptidyl-prolyl isomerases, proteins which catalyse the cis-trans isomerization of the peptide bond preceding prolyl residues. Knockout studies in several species, including mice and human cells, confirm that they have limited or no effect on cellular growth and survival[10-13]. However, cyclophilins recruited from host cells have been shown to have essential roles in many viral life-cycles. Initially, cyclophilin A was shown to be incorporated into HIV-1 virions[14,15], involved in viral replication, and its expression level in patients related to the speed of progression to AIDS[16]. Cyclophilin A isomerase activity, and potentially other cyclophilins such as B and cyclophilin 40, have been shown to be required for HCV replication[17-19]. Other viruses where cyclophilin involvement has been implicated in their life-cycle, or where cyclophilin inhibitors have shown inhibitory activity, include Vaccinia virus[20], West Nile virus, Dengue virus, Yellow fever virus[21], Hepatitis B virus[22], Human papilloma virus[23], Cytomegalovirus[24], SARS coronavirus[25], Japanese encephalitis virus[26] and Influenza A[27].

We now describe the use of combined bioengineering and semisynthetic approaches to optimize the drug-like properties of sanglifehrin A, a NP cyclophilin inhibitor, to generate NVP018 (formerly BC556). Preclinical analysis reveals NVP018 to be a molecule displaying *in vitro* inhibition of HBV and HCV and potent *in vitro* and oral *in vivo* inhibition of HIV-1.

Results

Bioengineering and mutasynthesis of *Streptomyces* sp. A92-308110

Previous semisynthetic derivatization to replace the spirolactam fragment of sanglifehrin A led to generation of the sangamides, molecules with improved solubility, potency and selectivity[28]. This approach moved the immunosuppressive NPs from tool compounds appropriate for chemical genetics to being compounds that could reasonably be pursued as therapeutic agents. We then used bioengineering to make further alterations to the macrocyclic portion of the molecule, changes which could not have otherwise been done without complex total synthesis. The major aim of this work was to further improve the drug-like properties of the final molecule by specific manipulation of the parts of the molecule involved in cyclophilin binding.

The gene cluster required for biosynthesis of sanglifehrin A has been published[30]. Generation of the sanglifehrins requires thirteen typical PKS extension modules, and three NRPS (non-ribosomal peptide synthetase) modules incorporating L-valine, *meta*-L-tyrosine and L-piperazic acid. In addition, a crotonyl-CoA reductase/carboxylase (CCR) is present for generation of the PKS starter unit (2*E*)-2-ethylmalonyl-CoA, an iterative type I PKS is required for the supply of the unique (2*S*)-2-(2-oxo-butyl)malonyl-CoA extender unit, and a phenylalanine hydroxylase produces the NRPS extender unit *meta*-L-tyrosine from L-phenylalanine.

Analysis of the published crystal structures of sanglifehrin A[31] and an analogue with the spirolactam fragment removed[32], suggested that manipulation of the electronics on the *meta*-L-tyrosine moiety might lead to improved binding to cyclophilins. To achieve this, we inactivated *sfaA*, the gene encoding the phenylalanine hydroxylase responsible for *meta*-L-tyrosine biosynthesis. Deletion of *sfaA* was carried out by homologous recombination, following conjugation of a plasmid containing the appropriate deletion construct into *S.* sp. A92-308110 using standard techniques[33] and led to the strain BIOT-4585. Due to the lack of *meta*-L-tyrosine production BIOT-4585 did not produce sanglifehrin A unless fed with *meta*-L-tyrosine or an analogue.

Synthesis and selection of NVP018

A screen was then carried out by feeding a selection of appropriate *meta*-L-tyrosine analogues to BIOT-4585 in order to determine its ability to generate sanglifehrin analogues (see figure 1). Fermentations with those analogues successfully accepted as substrates were scaled up to allow isolation of new sanglifehrins. The antiviral activity

and ADME properties of each analogue were analysed, and the best of the analogues were subjected to semisynthesis to yield the corresponding sangamides as described previously[28]. The resulting sangamides were again analysed for antiviral activity and ADME properties, and from this NVP018 was chosen as a potential drug candidate (data not shown). NVP018 is derived from a sanglifehrin resulting from incorporation of 5-fluoro-*meta*-L-tyrosine. Semisynthesis replaced the spirolactam containing portion of the sanglifehrin with a tertiary hydroxamic amide moiety (table S1). Of the sangamides tested, NVP018 had the best combination of properties (table 1), including more potent inhibition of CypA PPIase activity and inhibition of HCV replicons.

NVP018 has reduced off target inhibition of drug transporters

One of the issues with cyclosporin-based cyclophilin inhibitors is off-target inhibition of transporter proteins. Alisporivir, for example, inhibits multiple transporter proteins, including OATP1B1, OATP1B3 and MRP2, which are involved in bilirubin transport. It is this inhibition which is now thought to be the mechanism for the dose limiting hyperbilirubinaemia[34]. These compounds also have drug-drug interaction concerns through inhibition of xenobiotic transporters such as Pgp and BSEP[35,36]. Comparison of the inhibition of these transporters using vesicular transport or uptake transporter inhibition assays, and *in vitro* inhibition of the xenobiotic transporters, Pgp and BSEP revealed that whilst alisporivir and CsA potently inhibited many of them, BC544 (figure 1, previously stated as compound **3o** in Moss et al., 2011) and NVP018 showed minimal or no inhibition (see table 2). To test the effect of NVP018 on bilirubin levels, we then dosed three mice with up to 250 mg/kg/day NVP018 for 7 days. In all cases, almost no increase was seen in either bilirubin or liver enzymes when measured at the end of the study (see supplementary information, table S2).

NVP018 displays *in vivo* pharmacokinetics suitable for once daily dosing

To confirm bioavailability following oral dosing, a single oral dose of 5 mg/kg was given to groups of mice, rats and dogs. In all cases, blood concentrations were maintained above the HCV EC_{90} for 24-48 h post dose (figure 2). Liver levels were found to be 3-6 fold higher than blood levels, and up to 230 fold higher than plasma levels, revealing further concentration within the target organ for treatment of hepatic disease (see supplementary information, table S3). Analysis of other organs in rats, including brain, heart and lungs revealed much lower exposure following oral dosing as compared to the blood and liver, probably due to first pass extraction by the liver.

NVP018 is an inhibitor of HCV replication *in vitro*

Until recently the standard of care treatment for chronic HCV infection has been a combination of interferon and ribavirin for 6-12 months. Use of recently approved high barrier to resistance drugs, such as the nucleotide polymerase inhibitor (NPI) sofosbuvir leads to high levels of viral clearance[37,38]. Cyclophilin inhibition is one of the few mechanisms of action of HCV inhibition which consistently shows a high barrier to resistance, and in Phase 2 trials, some patients were clear of the virus after being treated for 24 weeks with 1000 mg/day alisporivir monotherapy[39]. Alisporivir, a non-immunosuppressive semisynthetic derivative of cyclosporin A, is a potent cyclophilin inhibitor in Phase III trials for the treatment of HCV infection. NVP018 could provide a suitable alternative backbone for use in an interferon-free combination. Comparison of the inhibitory potential of NVP018 with the semisynthetic sangamide, BC544 and alisporivir against HCV replicons revealed that NVP018 was much more potent in all assays, and was similarly active against all genotypes tested (see table 3). Analysis of the effect of increasing levels of either human serum or FCS also confirmed that there was no effect on antiviral potency with addition of up to 40% serum (see supplementary information table S4).

To compare the barrier to resistance of NVP018 in HCV, Huh7-Con1 cells were serially passaged for 7 weeks in the presence of 300 μg/mL G418 and increasing concentrations of NVP018 or alisporivir. Colonies were amplified, cellular RNA extracted and analyzed by RT-qPCR. For all alisporivir-resistant replicons, only the previously described and clinically relevant D320E mutation was consistently found in all clones analyzed. In the case of NVP018 resistant replicons, all clones analyzed contained the same five mutations: L27I, R318W, D320E, S370P and C446S. When NVP018 was tested against separate replicons containing individual mutations from this list, only the D320E mutation was shown to confer more than 2-fold resistance alone. The combination of all five mutations only led to ~4-fold resistance compared to the wild-type.

NVP018 is an inhibitor of HBV replication *in vitro*

There have been previous reports that cyclophilin inhibitors such as cyclosporin A have an inhibitory effect on HBV replication[40]. We therefore tested NVP018 in two *in vitro* models to look for inhibitory activity. The first involved transfecting Huh7 cells with HBV DNA (8 μg) in the presence of 2 μM NVP018, alisporivir or DMSO. After days 0, 1, 2 and 3, amounts of *de novo* HBV proteins HBsAg and HBeAg present in cell extracts or supernatants were quantified by ELISA, in triplicate. Data is shown in Table 3 and revealed that NVP018 and alisporivir potently inhibited HBV replication at this concentration. An additional assay was run using HepG2 cells stably expressing

replicating HBV. These were treated with DMSO, NVP018 or alisporivir at three concentrations (0.1, 1 and 10 μM). The effect on HBV replication was monitored by treating HepG2 cells with test article at the relevant concentration for 3 days, removing the supernatant, retreating and analyzing after an additional 48 and 72 h by HBsAg ELISA, HBeAg ELISA and for HBV DNA, in triplicate, in both cell extracts and supernatants. This assay confirmed a dose dependent effect of NVP018 against HBV replication at the final time point.

NVP018 is an inhibitor of HIV-1 replication *in vitro*

Although no cyclophilin inhibitors are currently in clinical development for treatment of HIV-1 infection, alisporivir was originally developed as an inhibitor of HIV-1 replication, and showed a mean ~1 log drop in viral load following 14 days dosing in HIV/HCV co-infected patients[41]. We therefore tested NVP018 in comparison to BC544 and alisporivir in a CD4+ HeLa HIV-1 inhibitory FACS assay to see if improvements in inhibition of *in vitro* HCV replicons carried through to HIV-1 inhibitory assays. NVP018 was found to be both more potent as regards HIV-1 EC_{50} (see table 3) and in terms of maximal viral inhibition, which was seen to reach >95% inhibition with sanglifehrin analogues including BC544 and NVP018 but only 60% with cyclosporin A and alisporivir. This data was similar when repeated in Jurkat cells, activated CD4+ peripheral blood T-lymphocytes and macrophages. One of the concerns with development of alisporivir for treatment of HIV-1 was the presence of natural isolates with the H86Q alisporivir resistant genotype[22]. NVP018 was tested against both lab generated and natural H86Q isolates, and was found to be active in both cases.

NVP018 is a potent inhibitor of HIV-1 replication *in vivo*

As NVP018 had shown both potent *in vitro* HIV-1 inhibition and oral bioavailability, its effect on HIV-1 viral replication in the SCID-hu Thy/Liv mouse model was assessed. This model has been well validated against other standard HIV therapies and is thought to be a highly reproducible model with relevance to clinical effect[29].

To enable comparison with data from previous studies with approved agents in the model, NVP018 or alisporivir were dosed orally bi-daily for 14 days, starting one day before inoculation with the NL4.3 virus, at 50 mg/kg/day or 100 mg/kg/day. Administration of NVP018 gave >6 $\log_{10}$ reductions in HIV-1 RNA, and was more potent at all concentrations tested than alisporivir at protecting thymocytes, and in reducing HIV-1 RNA and p24 levels (see figure 3). No overt signs of toxicity were seen in any of the mice at any dose level . The potency of NVP018 compares well with data previously published by Stoddart et al. as the >6 $\log_{10}$ HIV-1 RNA log drop seen following NVP018 dosing was more pronounced than for approved therapies such as efavirenz, atazanavir,

emtricitabine and nevirapine, which showed 1-4 $\log_{10}$ viral RNA reductions after 21 days bi-daily dosing at 100 mg/kg.

Discussion

The cyclophilin inhibitor NVP018 was generated using a combination of semisynthetic and bioengineering methods. These are frequently used independently, but in the past have been less commonly used together[42,43]. This enabled a combinatorial approach to natural product drug discovery and led to the selection of NVP018, a compound with more potent cyclophilin inhibition, antiviral potency versus HBV, HCV and HIV-1, and reduced off-target transporter inhibition as compared to the parent compound, sanglifehrin A. A fluorine was introduced into the compound by mutasynthesis, a method which has been successful in introducing this substitution pattern in a less invasive way than semisynthetic chemistry[44,45,46,47,48].

Second generation cyclophilin inhibitors, such as NVP018, have the potential for therapeutic application in a number of areas, in particular chronic viral infection. This is due to their potency, high barrier to resistance, activity on multiple steps of the viral life-cycle and oral bioavailability. It is also worth noting the recent publications which suggest that these compounds might also have additional impact on Interferon Regulatory Factors, which have long been implicated in the escape of certain viruses, such as HCV and HIV, from the immune system[49,50]. Whilst cyclophilin inhibitors do not exhibit inhibition of all viruses, they have been shown to have activity against a wide variety of both RNA viruses (eg Coronaviruses, HIV and HCV) and DNA viruses (HCMV, HBV, VZV). It is therefore tempting to speculate that cyclophilin inhibitors may be a step in the direction of agents with broad antiviral activity[51].

We also anticipate that in the era of rapid genome sequencing and gene synthesis, bioengineering applications will continue to become more amenable and offer a more flexible route to improve natural products with potent inhibition of cellular targets, but with less than optimum drug-like properties. We hope that in the future, bioengineering and semisynthesis will be used more frequently in combination to optimize natural products with an aim to select candidates for clinical development.

Experimental Procedures

Compounds

BC544 was synthesized according to the method published in Moss et al., 2012 (compound 3o therein). Alisporivir (DEBIO-025/DEB025) was synthesized according to the method published in US6,927,208 from cyclosporin A (>99%) purchased from LC Labs, Woburn, MA, USA.

Genetic engineering of Streptomyces sp. A92-308110

In frame deletion of the *sfaA* gene, coding for the tyrosine hydroxylase, was carried out using double recombination. The recombinant strain generated, BIOT-4585, could then be fed (*S*)-methyl 2-amino-3-(3-fluoro-5-hydroxyphenyl) propanoate hydrochloride salt and grown under appropriate fermentation conditions to generate BC457. Further details are available in the supplementary information.

Semisynthesis of NVP018 from BC457

NVP018 was synthesized in three steps from BC457 using the same protocol as for compound BC544. Briefly BC457 was treated via modified Sharpless dihydroxylation conditions to form a diol which was subsequently cleaved to generate an allylic aldehyde by the action of sodium periodate. Finally the resultant allylic aldehyde was coupled with a suitable amide under Horner-Wadsworth-Emmons coupling conditions.

ADME Compound analysis

Solubility analysis, plasma protein binding, intravenous and oral pharmacokinetics and toxicology were carried out using standard methods at Shanghai Chempartner, Shanghai and efflux assays were carried out at Solvo Biotechnology, Hungary or Cyprotex, UK. Further details are available in the supplementary information.

ELISA Analysis of CypA-NS5A Interactions.

Test articles were tested for their capacities to block the interaction between CypA and HCV NS5A by ELISA. Specifically, we produced and purified recombinant GST, GST-CypA and Con1 NS5A-His proteins and conducted ELISA. Nunc MaxiSorb 8-well strip plates

were coated with GST or GST-CypA for 16 h at 4 °C and blocked. Recombinant NS5A-His (1 ng/mL) was added to wells in 50 µL of binding buffer (20 mM Tris pH 7.9, 0.5 M NaCl, 10 % glycerol, 10 mM DTT and 1 % NP-40) for 16 h at 4 °C. Captured NS5A-His was subsequently detected using mouse anti-His antibodies (1 µg/mL) (anti-6xHis, Clontech) and rabbit anti-mouse-horseradish peroxidase phosphatase (HRP) antibodies (1:1000 dilution). To test the effect of test articles on the CypA-NS5A complex formation, increasing concentrations of each test article were added to GST-CypA together with recombinant NS5A. All experiments were conducted twice. Data (triplicate) are presented (IC_{50}).

PPIase Inhibition Analysis

The inhibition of the PPIase activity of CypA was used to compare the inhibitory potential of sanglifehrin derivatives as an indication of their binding affinity to CypA. The PPIase activity of recombinant CypA, produced by thrombin cleavage of GST-CypA, is determined by following the rate of hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide by chymotrypsin. Chymotrypsin only hydrolyzes the *trans* form of the peptide, and hydrolysis of the *cis* form, the concentration of which is maximized by using a stock dissolved in trifluoroethanol containing 470 mM LiCl, is limited by the rate of *cis*-*trans* isomerization. CypA was equilibrated for 1 hour at 5 °C with selected sanglifehrin derivatives using a drug concentration range from 0.1 to 20 nM. The reaction was started by addition of the peptide, and the change in absorbance was monitored spectrophotometrically at 10 data points per sec. The blank rates of hydrolysis (in the absence of CypA) were subtracted from the rates in the presence of CypA. The initial rates of the enzymatic reaction were analyzed by first-order regression analysis of the time course of the change in absorbance. All sanglifehrin derivatives exhibited anti-PPIase activity that correlated well with their capacities to prevent CypA-NS5A interactions analyzed by ELISA or pulldown.

HCV Antiviral Assay Huh 5-2

Anti-HCV assay in Huh 5-2 cells was performed by seeding 6.5 x 10^3 cells per well in a tissue culture treated white 96-well view plate (Packard, Canberra, Canada) in

Dulbecco's modified essential media (DMEM) supplemented with 250 µg/ml G418. Following incubation for 24 h at 37 °C (5 % CO_2) medium was removed and 3-fold serial dilutions in complete DMEM (without G418) of the test compounds were added in a total volume of 100 µl. After 4 days of incubation at 37°C, cell culture medium was removed and luciferase activity was determined using the luciferase assay system (Promega, Leiden, The Netherlands); the luciferase signal was measured using a Safire2 (Tecan, Switzerland). Relative luminescence units were converted to percentage of untreated controls. The 50 % effective concentration (EC_{50}) was defined as the concentration of compound that reduced the luciferase signal by 50%.

Cytostatic Assay

For the assessment of the potential cytostatic effect of the evaluated inhibitor, cells were seeded at a density of 6.5 x 10^3 cells per well of a 96-well plate in complete DMEM. Serial dilutions of the test compounds in complete DMEM were added 24 h after seeding. Cells were allowed to proliferate for 3 days at 37 °C, after which the cell number was determined by means of the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium)/phenazinemethosulfate (MTS/PMS) method (Promega). The CC_{50} (value derived from the dose-response curve) represents the concentration at which the metabolic activity of the cells would be reduced to 50 % of the metabolic activity of untreated cells. The selectivity index (SI), indicative of the therapeutic window of the compound, was calculated as the CC_{50}/EC_{50}.

HCV Replicon antiviral assays

The replicon cells (subgenomic replicons of genotype 1a (H77), 1b (Huh7) and 2a (JFH-1)) were grown in DMEM, 10 % fetal bovine serum (FBS), 1 % penicillin-streptomycin (pen-strep), 1 % glutamine, 1 % non-essential amino acids, 250 µg/ml G418 in a 5% CO_2 incubator at 37 ºC. All cell culture reagents were purchased from Mediatech (Herndon, VA). The replicon cells were trypsinized and seeded at 5 x 10^3 cells per well in 96-well plates with the above media without G418. On the following day, the culture medium was replaced with DMEM containing compounds serially diluted in the presence of 5 % FBS. The cells containing the HCV replicon were seeded into 96-well plates and test articles were serially diluted with DMEM plus 5 % FBS. The diluted compound was then

applied to appropriate wells in the plate. After 72 hours incubation at 37 °C, the cells were processed. The intracellular RNA from each well was extracted with an RNeasy 96 kit (Qiagen). The level of HCV RNA was determined by a reverse transcriptase-real time PCR assay using TaqMan® One-Step RT-PCR Master Mix Reagents (Applied Biosystems, Foster City, CA) and an ABI Prism 7900 sequence detection system (Applied Biosystems). The cytotoxic effects were measured with TaqMan® Ribosomal RNA Control Reagents (Applied Biosystems) as an indication of cell numbers. The amount of the HCV RNA and ribosomal RNA were then used to derive applicable EC_{50} values (concentration that would inhibit the replicon replication by 50%).

In vitro assays for assessment of HBV antiviral activity

HepG2 cells stably replicating robustly HBV were treated with DMSO, tenofovir, BC544, NVP018 and ALV at 3 concentrations (0.1, 1 and 10 µM). Effect on HBV replication was monitored by treating HepG2 cells with test article at the relevant concentration for 3 days, removing the supernatant, retreating and analyzing after an additional 72 hours by HBsAg ELISA, HBeAg ELISA and HBV DNA in triplicates in cell extracts. These data were used to generate test/control values which were plotted against the test article concentration and used to calculate IC_{50} values.

Huh7 cells were transfected with HBV DNA (8 µg) in the presence of DMSO, tenofovir, BC544, NVP018 and ALV (alisporivir) at 2 µM. After day 3, amounts of *de novo* HBsAg protein present in cell extracts or supernatants was quantified by ELISA in triplicate. This was used to calculate a test/control value.

In vitro assay for assessment of HIV antiviral activity

Antiviral efficacy against HIV may be tested as follows: Blood derived CD4+ T-lymphocytes and macrophages are isolated as described previously[52]. Briefly, human PBMCs were purified from fresh blood by banding on Ficoll–Hypaque (30 minutes, 800 g, 25 °C). Primary human CD4+ T cells were purified from PBMCs by positive selection with anti-CD4 Dynabeads and subsequent release using Detachabead. Cells were cultured in RPMI medium 1640 (Invitrogen) supplemented with 10 % FCS, MEM amino acids, L-glutamine, MEM vitamins, sodium pyruvate, and penicillin plus streptomycin and were subsequently activated with bacterial superantigen staphylococcal

enterotoxin B (SEB; 100 ng/ml) and mitomycin C-killed PBMC from another donor (10:1 PBMC:CD4 cell ratio). Three days after stimulation, cells were split 1:2 in medium containing IL-2 (200 units/ml final concentration). Cultures were then split 1:2 every 2 days in IL-2 medium and infected with HIV at 7 days after stimulation. For generating primary human macrophages, monocytes were purified from human PBMCs by negative selection and activated and cultured at a cell concentration of 10^6/ml in DMEM, supplemented with 10 % FCS, MEM amino acids, L-glutamine, MEM vitamins, sodium pyruvate, and penicillin (100 units/ml), streptomycin (100 mg/ml), and 50 ng/ml recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) and maintained at 37 °C in a humidified atmosphere supplemented with 5% CO_2. To obtain monocyte-derived macrophages, cells were allowed to adhere to plastic and cultured for 6 days to allow differentiation.

CD4+ HeLa cells, Jurkat cells, activated CD4+ peripheral blood T-lymphocytes and macrophages (500,000 cells/100 µL) were incubated with pNL4.3-GFP (X4 virus) or pNL4.3-BaL-GFP (R5 virus) (100 ng of p24) in the presence of increasing concentrations of test article, 48 hours later, infection was scored by analyzing the percentage of GFP-positive cells by FACS and EC_{50} calculated.

SCID-hu Thy/Liv mouse model

Stocks of NL4-3 (X4 virus) were prepared by transfection of 293T cells and collection of supernatants on day 2. Supernatants were filtered, aliquoted and frozen at -80 ˚C until use. Amounts of virus were quantified by p24 ELISA and infectivity of viral stocks verified using CD4+ HeLa-betagalactosidase reporter cells.

Human fetal thymus and liver were obtained from Advanced Bioscience Resources in accordance with federal, state, and local regulations. Co-implantation of thymus and liver fragments under the kidney capsule to create SCID-hu Thy/Liv mice and inoculation of the Thy/Liv implants with HIV-1 (1000 TCID50 HIV-1 per Thy/Liv implant by direct injection) were carried out as described[53,54]. Male C.B-17 SCID (model #CB17SC-M, homozygous, C.B-Igh-1b/IcrTac-Prkdcscid) mice were obtained at 6–8 weeks of age from Taconic, and cohorts of 50–60 SCID-hu Thy/Liv mice were implanted with tissues from a single donor. Implants were inoculated 18 weeks after implantation with 50 µL of stock virus or complete DMEM (control infection) by direct injection. Animal protocols were approved by the TSRI Institutional Animal Care and Use Committee. Groups of 6 mice

each were treated with NVP018 or alisporivir (ALV) at 50 or 100 mg/kg/day by twice-daily oral gavage until implant collection 2 weeks after inoculation.

Implants were dispersed through nylon mesh into single-cell suspensions and assessed for p24 by ELISA (p24 (ELISA): intracellular Gag pg/10^6 cells), for viral RNA (bDNA assay: copies/10^6 cells), and for depletion of thymocyte subsets by flow cytometry (% of CD3, CD4, CD8) as described previously[29,53,55]. Specifically, implant cells were stained with phycoerythrin cyanine dye CY7-conjugated anti-CD4 (BD Biosciences), phycoerythrin cyanine CY5.5-conjugated anti-CD8 (Caltag), allophycocyanin cyanine CY7-conjugated anti-CD3 (eBiosciences), and phycoerythrinconjugated anti-W6/32 (DakoCytomation). Cells were fixed and permeabilized with 1.2 % paraformaldehyde and 0.5 % Tween 20, stained with fluorescein isothiocyanate-conjugated anti-p24 (Beckman Coulter), and analyzed on an LSR II (BD Biosciences). After collecting 100,000 total cell events, percentages of marker-positive (CD4+, CD8+, and CD4+CD8+) thymocytes in the implant samples were determined by first gating on a live lymphoid cell population identified by forward- and side-scatter characteristics and then by CD3 expression. Total RNA was extracted from frozen thymocyte pellets using Trizol LS (Invitrogen) and resuspended in nuclease-free water. The capsid region of the HIV-1 gag was amplified by RT-PCR using 10 µL of purified RNA and AmpliTaq Gold (Applied Biosystems) according to the manufacturer's instructions.

Acknowledgements

We thank Drs. Wakita and Chisari for Huh-7.5.1 replicon cells, Shanghai Chempartner for chemical synthesis and ADMET studies and Cyprotex, UK and Solvo Biotechnology for ADME studies. Animal protocols were approved by Shanghai Chempartner or the TSRI Institutional Animal Care and Use Committee.

This work was supported by the U.S. Public Health Service grant no. AI087746 from the National Institute of Allergy and Infectious Diseases (NIAID).

NVP018 was formerly described as BC556 and is now referred to as NV556/OCB-030. It is being developed as an intravenous treatment by Neurovive Pharmaceutical AB as NVP019 and as an oral treatment for HBV by OnCore Biopharma, Inc. as OCB-030.

Figure/Table legends

Figure 1: Overview of (bio)synthetic medicinal chemistry route to generate BC544 and NVP018.

Table 1: Comparison of cyclophilin inhibition, HCV NS5A-cyclophilin A complex disruption and genotype 1b replicon inhibition for cyclosporin and sanglifehrin analogues.

Table 2: Comparison of solubility, plasma protein binding and transporter inhibition.

Table 3: EC_{50} of cross genotype HCV replicon inhibition and IC_{50} of HIV-1 inhibition. IC_{50} of HepG2 cells stably replicating HBV following treatment with DMSO, tenofovir, BC544, NVP018 and Alisporivir and test/control value after 3 days of Huh7 cells following transfection with HBV DNA in the presence of DMSO, tenofovir, BC544, NVP018 and Alisporivir at 2 µM.

Figure 2: Mean blood concentration-time profiles of NVP018 following a single po dose of 5 $mg.kg^{-1}$ to CD-1 mice, SD rats or beagle dogs. Values represent the average concentrations calculated in groups of 3 mice with standard deviation shown with error bars.

Figure 3: Comparison of implant P24 levels, T-lymphocytes and HIV-1 RNA in the SCID-hu Thy/Liv mouse model, following bi-daily doses of either 50 or 100 $mg.kg^{-1}$ alisporivir (ALV) or NVP018 for two weeks. *=$p \leq 0.05$, compared with untreated mice. Values represent the average concentrations/numbers calculated in a group of 6 mice with standard deviation shown with error bars..

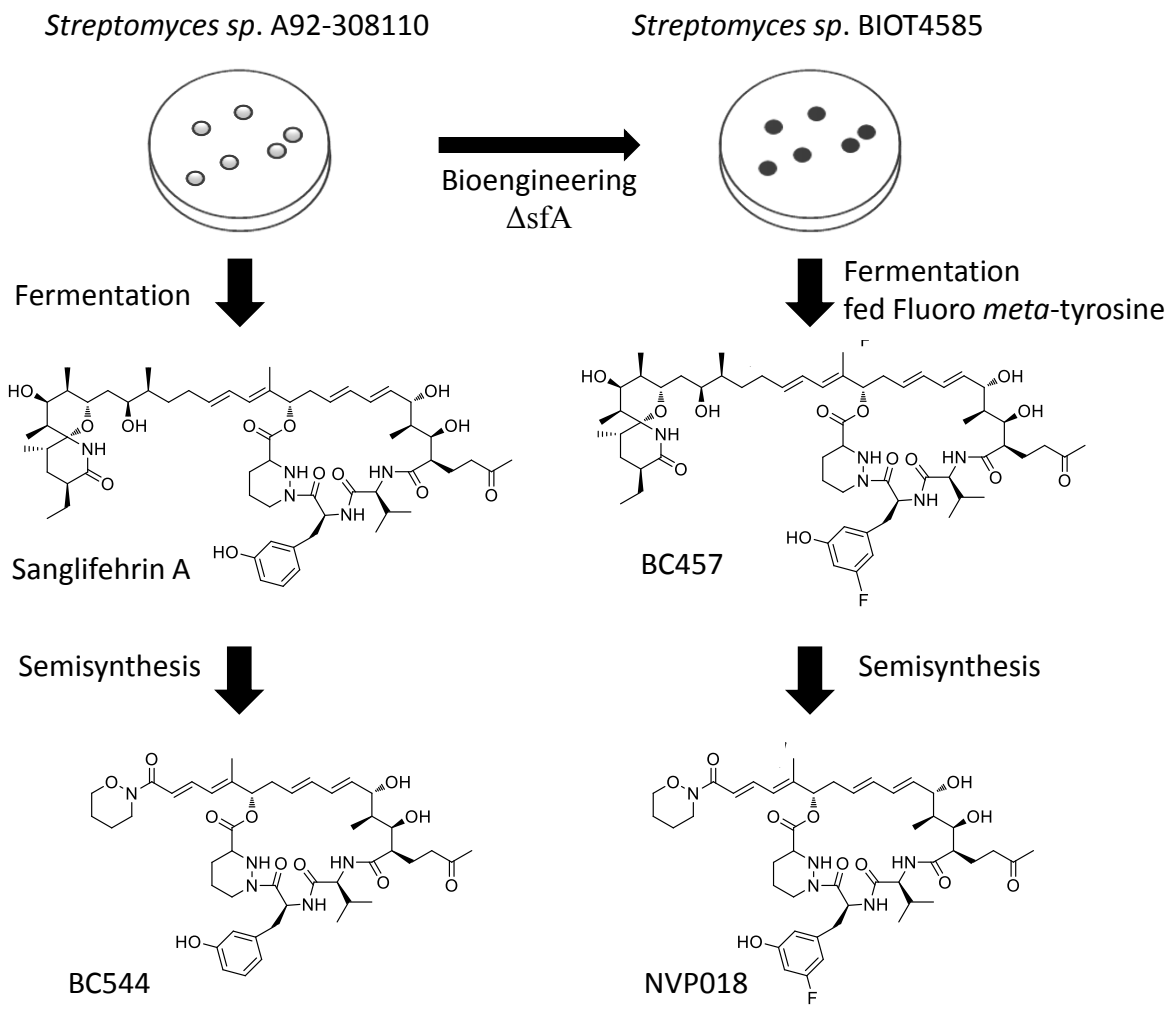

Streptomyces sp. A92-308110
Streptomyces sp. BIOT4585
Bioengineering
ΔsfA
Fermentation
Fermentation
fed Fluoro *meta*-tyrosine
Sanglifehrin A
BC457
Semisynthesis
Semisynthesis
BC544
NVP018

Table 1: Comparison of cyclophilin inhibition, HCV NS5A-cyclophilin A complex disruption and genotype 1b replicon inhibition for cyclosporin and sanglifehrin analogues

	CypA PPIase IC_{50} (nM)	NS5A-CypA disruption IC_{50} (μM)	HCV Huh5.2 Gt1b replicon EC_{50} (nM)	HCV Gt1b replicon CC_{50} (μM)
CsA	9.7 ± 1.30	0.98 ± 0.08	306 ± 141	4.4
Alisporivir	0.8 ± 0.05	0.24 ± 0.02	96 ± 23	11.2
BC544	2.7 ± 0.2	0.36 ± 0.03	125 ± 22	>100
NVP018	0.3 ± 0.03	0.10 ± 0.01	33 ± 10	>100

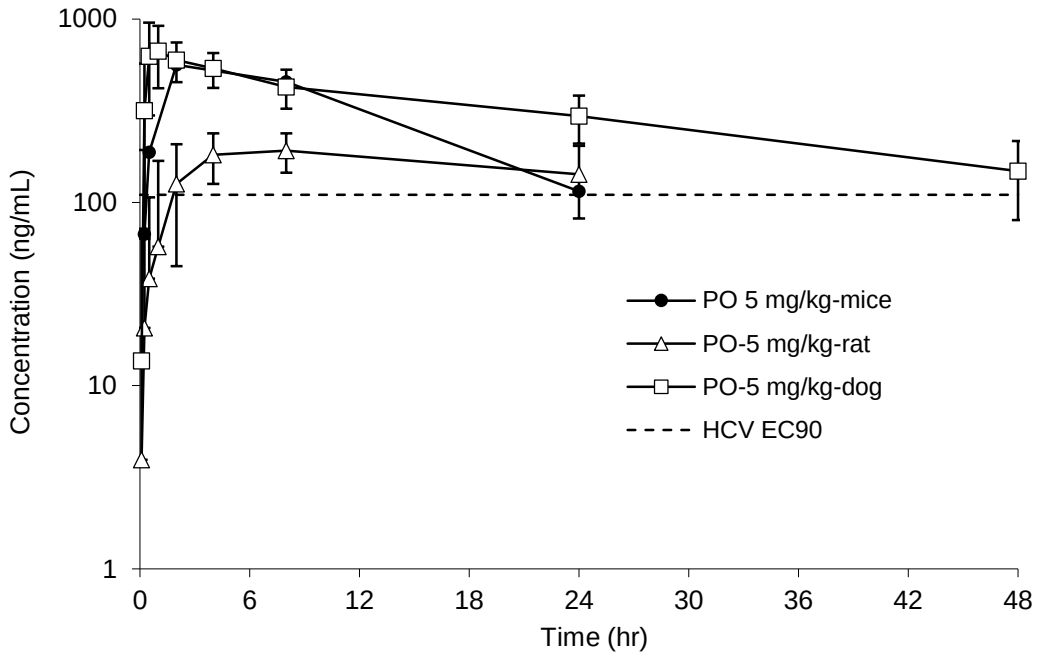
Table 2: Comparison of solubility, plasma protein binding and transporter inhibition

	Solubility in PBS pH7.4 (uM)	Human PPB (fraction unbound)	OATP1B1 IC_{50} (μM)	OATP1B3 IC_{50} (μM)	MRP2 IC_{50} (μM)	Pgp IC_{50} (μM)	BSEP IC_{50} (μM)
CsA	36	<1%	0.85	0.13	4.1	0.73	0.46
Alisporivir	15	<4%	0.45	0.19	~16 (>49%)	0.72	0.18
BC544	330	28%	10.97	5.40	>50	>50	29.3
NVP018	140	22%	4.31	1.75	>50	>50	12.3

Table 3: EC50 of cross genotype HCV replicon inhibition and IC50 of HIV-1 inhibition. IC50 of HepG2 cells stably replicating HBV following treatment with DMSO, tenofovir, BC544, NVP018 and Alisporivir and test/control value after 3 days of Huh7 cells following transfection with HBV DNA in the presence of DMSO, tenofovir, BC544, NVP018 and Alisporivir at 2 µM

	HCV			HIV-1	HBV			
	HCV Huh7 Gt1a replicon EC_{50} (nM)	HCV Huh7 Gt1b replicon EC_{50} (nM)	HCV Huh7 Gt2a replicon EC_{50} (nM)	HIV-1 HeLa cells IC_{50} (nM)	HBV Huh7 stable HBV DNA IC_{50} (nM)	HBV Huh7 stable HBsAg IC_{50} (nM)	HBV Huh7 stable HBeAg IC_{50} (nM)	HBV HepG2 transfected HBsAg T/C d3 at 2uM (%)
CsA	198 ± 13	137 ± 4	455 ± 37	5300	N/A	N/A	N/A	N/A
Alisporivir	98 ± 11	76 ± 5	102 ± 8	1500 ± 240	>10000	>10000	>10000	18
BC544	63 ± 4	58 ± 5	89 ± 6	310 ± 20	610	600	280	9.6
NVP018	18 ± 2	13 ± 1	21 ± 3	130 ± 15	950	520	350	8.8

Mean blood concentration-time profiles of BC556 after a PO dose of 5 mg/kg in CD-1 mice, SD rats or beagle dogs (N=3)



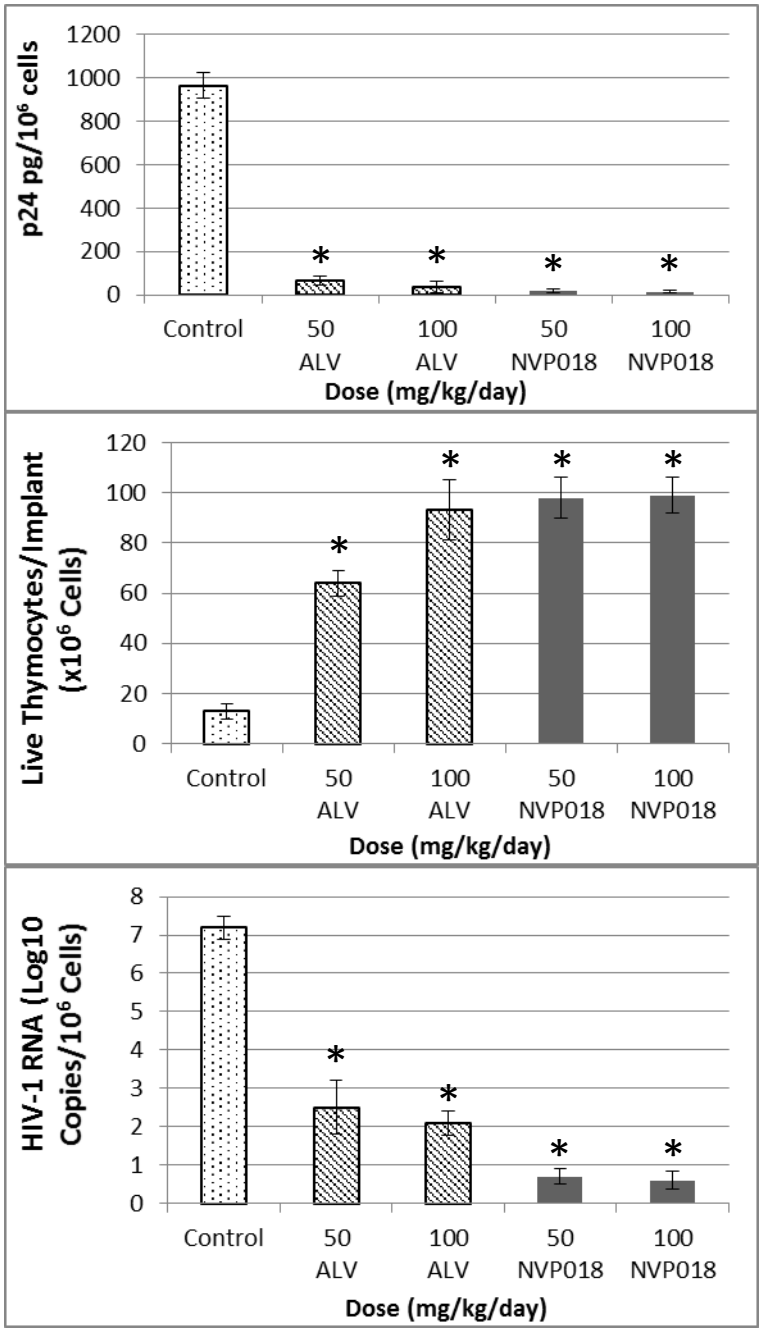

p24 pg/10⁶ cells
1200
1000
800
600
400
200
0
Control
50 ALV
100 ALV
50 NVP018
100 NVP018
Dose (mg/kg/day)
Live Thymocytes/Implant (x10⁶ Cells)
120
100
80
60
40
20
0
Control
50 ALV
100 ALV
50 NVP018
100 NVP018
Dose (mg/kg/day)
HIV-1 RNA (Log10 Copies/10⁶ Cells)
8
7
6
5
4
3
2
1
0
Control
50 ALV
100 ALV
50 NVP018
100 NVP018
Dose (mg/kg/day)

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Figure S1

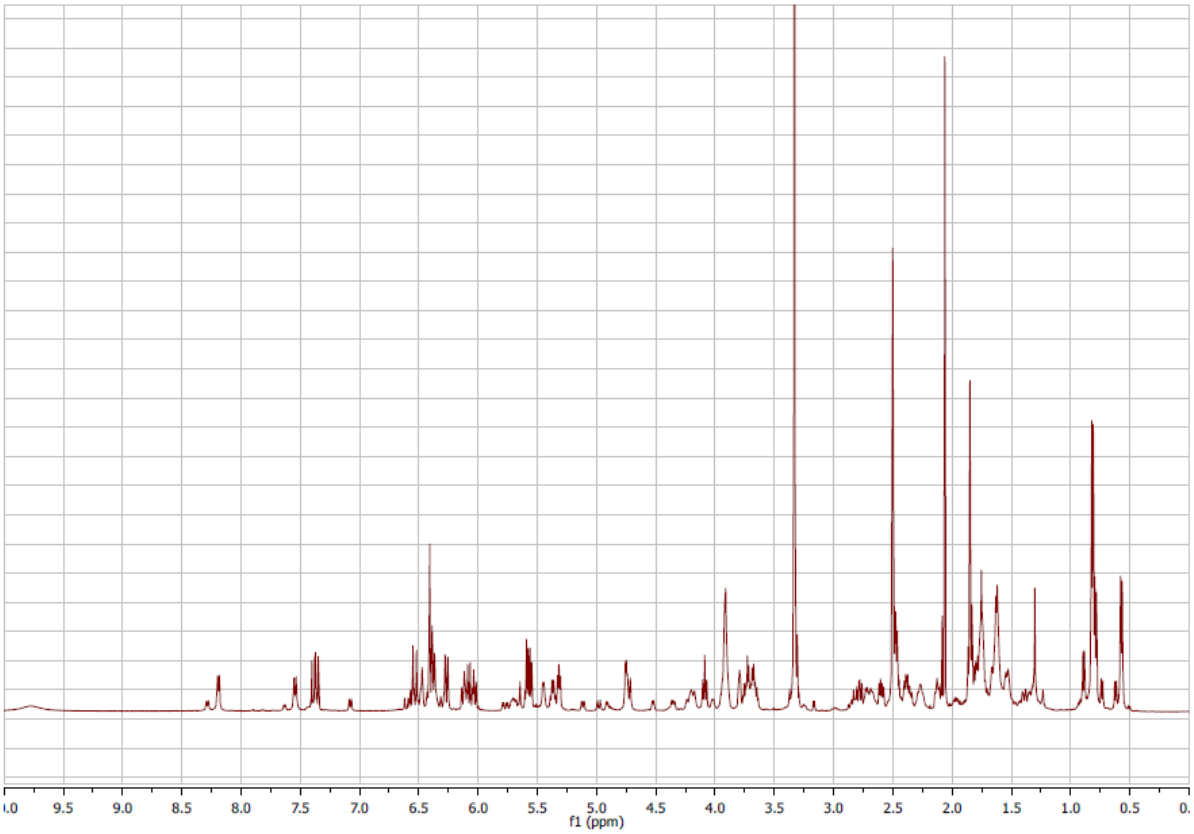


Figure S2

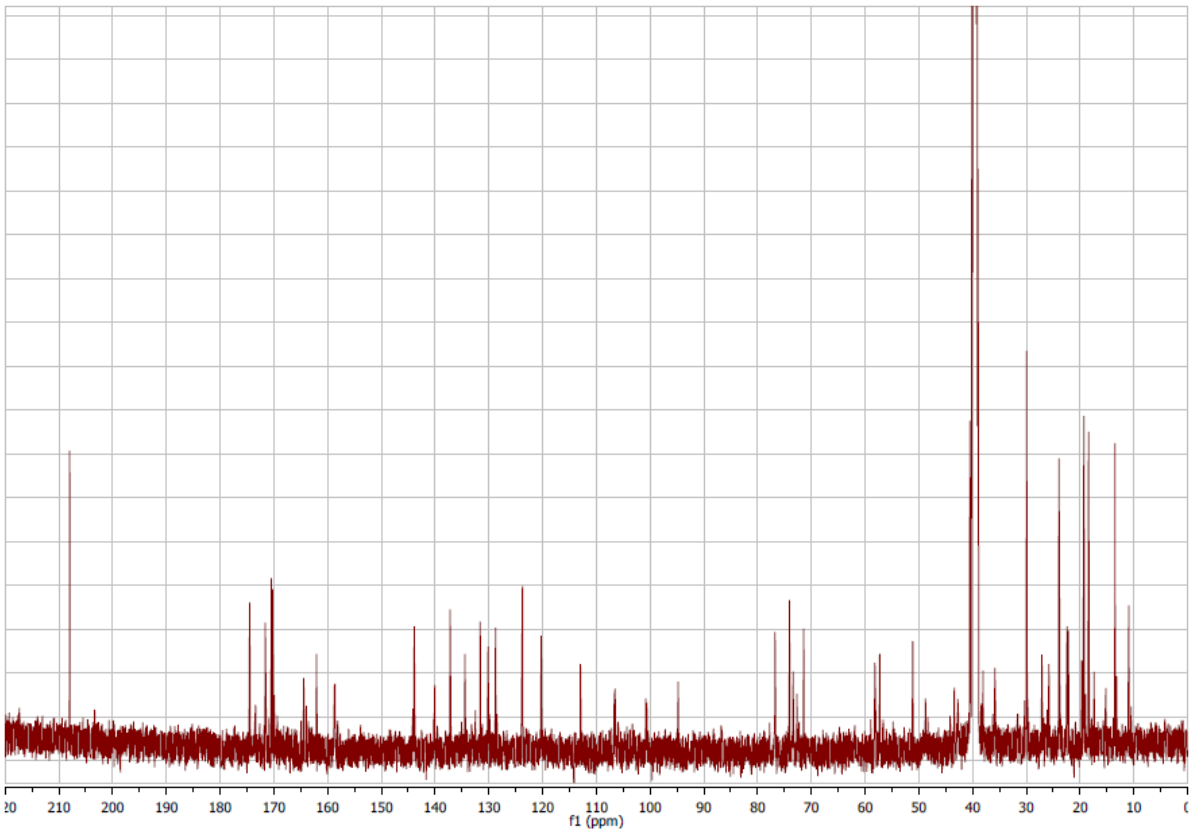


Figure legends

Figure S1: ^{1}H NMR spectra for NVP018, Related to Experimental Procedures

Figure S2: ^{13}C NMR spectra for NVP018, Related to Experimental Procedures

Table S1: ^{13}C NMR (126 MHz, DMSO) - ^{1}H NMR (500 MHz, DMSO), Related to Experimental Procedures

Table S2: Serum chemistry at day 7 following administration of NVP018 to CD1 mice, Related to Experimental Procedures

Table S3: AUC_{24} after 5 mg/kg po dose to SD rats, Related to Experimental Procedures

Table S4: Effect of serum on HCV inhibition, Related to Experimental Procedures

Table S1:

signal	*position*	multiplicity	^{13}C δ [ppm]		^{1}H δ [ppm]	$^{3}J_{H}$ [Hz]
1	41	s	207.95			
2	13	s	174.47			
3	7	s	171.57			
4	1	s	170.42			
5	10	s	170.20			
6	28	s	164.39			
7	34	s	162.02		-	
8	32	s	158.64			
9	24	s	143.85			
10	30	s	140.10			
11	26	d	137.17		**7.36**	15.0, 11.5
12	18	d	134.43		**5.54**	
13	20	d	131.57		**6.09**	
14	19	d	130.11		**6.02**	
15	21	d	128.72		**5.56**	
16	25	d	123.71		**6.26**	11.5

17	27	d	120.24		**6.52**	
18	31	d	112.93		**6.40**	
19	35	d	106.48		**6.36**	
20	33	d	100.75		**6.40**	
21	23	d	76.72		**5.31**	
22	17	d	74.04		**3.90**	
24	15	d	71.38		**3.79**	
25	2	d	58.14		**2.25**	
26	11	d	57.25		**4.08**	
27	14	d	51.10		**2.11**	
28	8	d	48.74		**5.39**	
29	16	d	43.38		**1.60**	
31	40	t	40.47		**2.45, 2.37**	
32	5	t	40.35		**4.18, 2.66**	
33	**29**	t	38.01		**2.77, 2.58**	
34	22	t	35.82		**2.46**	
35	42	q	30.03		**2.05**	
36	36	d	29.93		**1.75**	
37	3	t	27.08		**1.52, 1.37**	
38	39	t	25.76		**1.73, 1.81**	
40	4	t	22.39		**1.62, 1.24**	
42	**37-Me**	q	19.26		**0.81**	
43	**38-Me**	q	18.39		**0.80**	
44	**24-Me**	q	13.46		**1.84**	
45	**16-Me**	q	10.91		**0.56**	7.0
23	28e	t	73.37		**3.89**	
30	28b	t	42.73		**3.73, 3.64**	
39	28b	t	23.90		**1.61**	
41	28g	t	22.15		**1.73**	
	32-OH	-			**9.77**	

	15-OH	-			**5.44**	4.6
	17-OH	-			**4.74**	4.6
	9 -NH				8.18	7.3
	12-NH				7.53	8.8
	6a-NH				4.72	11.8

Table S2:

Dose level	Total bilirubin (µM)	Ratio to 0mg/kg
0 mg/kg	3.4	1.0
5 mg/kg	2.9	0.9
50 mg/kg	4.4	1.3
250 mg/kg	3.7	1.1

Table S3:

Organ	AUC_{24}	Fold vs plasma
Plasma	142	-
Blood	5310	37x
Liver	32800	230x
Lung	2740	19x
Heart	2040	14x
Brain	0	-

Table S4:

Serum added	Percentage addition	NVP018 Huh7-Con1-Luc EC_{50} (nM)
Human serum	5%	11 ± 2
	10%	15 ± 2
	20%	14 ± 1
	40%	13 ± 2
Foetal bovine serum	5%	15 ± 1
	10%	15 ± 2
	20%	12 ± 2
	40%	14 ± 2

Supplemental procedures

Experimental procedures

All purchased chemicals and solvents were of reagent or HPLC grade unless otherwise stated and were used directly as obtained from the manufacturer unless stated otherwise. Tetrahydrofuran was freshly distilled from sodium under an atmosphere of N2. LC-MS of intermediates was conducted on an Agilent HP1100 or HP1200 operating in positive mode. Chromatography was achieved on a Waters Sunfire C18 column (length 50 mm, diameter 4.6 mm, 3.5 μm). Solvent A was water (0.05% TFA) and solvent B was acetonitrile (0.05% TFA). LC method: T = 0 min; 1% B; T = 1.7 min, 99% B; T = 2.6 min, 99% B; T = 2.7 min, 1% B. NMR of intermediates was recorded on a Bruker Avance500 spectrometer at 500 MHz (^{1}H NMR). NMR of final products were acquired on a Bruker Avance500 spectrometer fitted with a 5 mm triple resonance inverse automatic tuning and matching (TCI ATM) cryoprobe with Z gradients running at 298 K and operating at 500 MHz and126 MHz for ^{1}H and ^{13}C, respectively. Chemical shifts are reported in parts per million and are referenced relative to the solvent resonance. Coupling constants are given in hertz. LC-MS of final products was recorded on an integrated Agilent HP1100 HPLC system in combination with a Bruker Daltonics Esquire 3000+ spectrometer fitted with an electrospray source. LC-MS analysis was performed on an Agilent HP1100 equipped with a Gemini NX C18 110 Å column (150 mm × 4.6 mm, 3 μm, Phenomenex) heated to 40 °C. The gradient elution was from 10 % B held for 2 min followed by a linear increase to 100% mobile phase B over 13 min and held at 100% B for a further 2 min at a flow rate of 1 mL/min. Mobile phase A was water containing 0.1% formic acid; mobile phase B was acetonitrile containing 0.1% formic acid. The HPLC system described above was coupled to a Bruker Daltonics Esquire 3000+ electrospray mass spectrometer, scanning from 50 to 1500 amu in switching mode. High-resolution MS were measured on a Bruker BioApex II 4.7e Fourier Transform Ion Cyclotron Resonance spectrometer fitted with an electrospray source and operating in positive ion mode. Final compounds were shown to be greater than 95% pure by NMR and the LC-MS method described above on two solid phases (C_{18} and phenyl-hexyl). The major confirmer of each sangamide was elucidated using HRMS and 1D and 2D NMR experiments (^{1}H, ^{13}C, APT, COSY, HMBC and HMQC).

Genetic engineering of *Streptomyces* sp. A92-308110

The ~7kb EcoRV-StuI fragment of cosmid TL3006 encompassing *sfaA* (nucleotide position 14396-21362, NCBI sequence accession number FJ809786) was excised by digestion with EcoRV and StuI and the resulting isolated fragment ligated directly into pKC1139 that had previously been digested with EcoRV and treated with shrimp alkaline phosphatase (Roche). This plasmid was designated pSGK268.

An in frame deletion of the *sfaA* gene contained within this clone was performed using Red/ET recombination (GeneBridges) as described below.

Supplementary Information

SfaA17161f 5'-
CGCTCTGTGGCGCCTGGTTTCCAAGCGGCTCGCGGACCGGCACCGGCACATGCATAATTAACCCTCACTAAAG
GGCG-3'

SfaA17825r 5'-
TGGATGTATCGTCGCAGGACGCCCAGAATTCACCTGCGACGTCCTCCAGATGCATTAATACGACTCACTATAG
GGCTC-3'

Two oligonucleotides, SfaA17161f and SfaA17825r were used to amplify the neomycin marker from the FRT-PGK-gb2-neo-FRT template DNA supplied in the kit using KOD DNA polymerase. The resulting ~1.7kb amplified product was isolated by gel electrophoresis and purified from the gel with QiaEX resin.

Plasmid pSGK268 was transformed into *E. coli* DH10B using standard techniques and selected on plates containing apramycin (50 μg/ml). A single colony was grown overnight in 2TY apramycin (50 μg/ml) and transformed with the pRedET (tet) plasmid and selected on apramycin (50 μg/ml) and tetracycline (3 μg/ml) at 30 °C. A single colony was used to prepare an overnight culture of this strain in 3ml 2TY apramycin (50 μg/ml) and tetracycline (3 μg/ml) at 30 °C. 0.5 ml of this culture was used to inoculate 10 ml 2TY apramycin (50 μg/ml) and tetracycline (3 μg/ml) at 30 °C and grown to an OD 600nm ~0.5. 1.4 ml of this culture was transferred to each of two Eppendorf tubes and 50 μl 10 % arabinose added to one tube to induce expression of the Red/ET recombination proteins. Tubes were shaken for ~1 hour at 37 °C. Induced and non-induced cells were pelleted in a bench top centrifuge and washed twice with chilled sterile water; resuspending and centrifuging to pellet the cells each time. The resulting pellets were suspended in about 30-40 μl of water and kept on ice. The 1.7 kb disruption fragment isolated previously was added to the induced and non-induced tubes and transferred to 1mm Biorad electrocuvettes on ice. The samples were electroporated (Biorad Micropulser at 1.8kV, resulting time constant ~4ms) and 1 ml 2TY (no antibiotics) added and mixed to remove the cells from the cuvette. Cells were incubated for ~3 h at 37 °C with shaking (1100rpm, Eppendorf thermomixer compact) before plating onto 2TY plates containing apramycin (50 μg/ml and kanamycin 25 μg/ml and incubating over night at 37 °C. Colonies from the induced sample plates were streaked onto 2TY plates containing kanamycin at 50 μg/ml to purify and confirm introduction of the kanamycin resistance cassette. PCR on individual bacterial colonies was used to confirm the introduction of the cassette. Plasmids were prepared from these cultures and digested to confirm the expected plasmid pSGK270. Plasmids were then digested with NsiI to remove the marker fragment, and the remainder religated to produce the *sfaA* in-frame deletion construct pSGK271.

Plasmid pSGK271 was transformed into *E. coli* ET12567 pUZ8002 using standard techniques and selected on 2TY plates containing apramycin (50 μg/ml), kanamycin (25 μg/ml) and chloroamphenicol (10 μg/ml). The resulting strain was inoculated into 3ml liquid 2TY containing apramycin (50 μg/ml), kanamycin (25 μg/ml) and chloroamphenicol (10 μg/ml) and incubated overnight at 37 °C, 250rpm. 0.8 ml of this culture was used to inoculate 10 ml liquid 2TY containing apramycin (50 μg/ml), kanamycin (25 μg/ml) and chloroamphenicol (10 μg/ml) in a 50 ml Falcon tube and incubated at 37 °C 250 rpm until OD600nm ~0.5 was reached. The resulting culture was centrifuged at 3500 rpm for 10 min at 4 °C, washed twice with 10 ml 2TY media using centrifugation to pellet the cells after each wash. The resulting pellet was resuspended in 0.5ml 2TY and kept on ice

before use. This process was timed to coincide with the complete preparation of *Streptomyces* spores described below.

Spores of *Streptomyces* sp. A92-308110 (DSM9954) (Biot-4370) were harvested from a 1-2 week old confluent plate by resuspending in ~3 ml 20 % glycerol. Spores were centrifuged (5000 rpm, 10 min room temperature) and washed twice with 50 mM TES buffer before resuspending in 1ml 50 mM TES buffer and splitting between 2 Eppendorf tubes. These tubes were heat shocked at 50 °C for 10 min in a water bath before adding 0.5 ml 2TY and incubating in an Eppendorf Thermomixer compact at 37 °C for 4-5 h.

The prepared *E. coli* ET12567 pUZ8002 pSGK271 and BIOT-4370 were mixed at ratios 1:1 (250 µL each strain) and 1:3 (100 µL *E. coli*) and immediately spread on R6 plates and transferred to a 37 °C incubator. After approximately 2 h incubation these plates were overlaid with 2 ml of sterile water containing nalidixic acid to give a final in-plate concentration of 25 µg/L. Plates were returned to the 37 °C incubator overnight before overlaying with 2ml of sterile water containing apramycin to give a final in-plate concentration of 20-25 µg/L. Ex-conjugant colonies appearing after 4-7 days were patched to ISP4 media containing apramycin (25 µg/L) and nalidixic acid (25 µg/L) and incubated at 37 °C. Once adequate mycelial growth was observed strains were repatched to ISP4 media containing apramycin (25 µg/L) at 37 °C and allowed to sporulate. Strains were then subcultured three times (to promote removal of the temperature sensitive plasmid) by patching to ISP4 (without antibiotic) and incubating at 37 °C for 3-4 days. Strains were finally patched to ISP4 and incubated at 28 °C to allow full sporulation (5-7 days). Spores were harvested and serially diluted onto ISP4 plates at 28 °C to allow selection of single colonies. Sporulated single colonies were doubly patched to ISP4 plates with or without apramycin (25 µg/L) to confirm loss of plasmid and allowed to grow ~ 7 d before testing for production of sanglifehrins.

A single ~7 mm agar plug of a well sporulated strain was used to inoculate 7 ml of sterile SM25-3 media and incubated at 27 °C 200 rpm in a 5 cm throw shaker. After 48 h of growth 0.7 ml of this culture was transferred to a sterilised falcon tube containing 7 ml of SGP2 media with 5 % HP20 resin. Cultures were grown at 24 °C 300 rpm on a 2.5 cm throw shaking incubator for 5 days before harvest. Bacterial culture (0.8 ml) was removed and aliquoted into a 2 ml Eppendorf tube ensuring adequate dispersal of the resin in throughout the culture prior to sampling. Acetonitrile (0.8 ml) and formic acid (15 µl) were added and the tube mixed for 30 minutes. The mixture was cleared by centrifugation and 170 µl of the extract removed into a HPLC vial and analyzed by HPLC.

Extracts of strains were analyzed by HPLC. Strains that produced sanglifehrin A and B were not analyzed further as these had reverted to wild type. Strains lacking sanglifehrin A and B production showed small levels (~1-2 mg/L) of a peak retention time 6.5 minutes that displayed a sanglifehrin like chromophore. Analysis by LCMS indicated this peak revealed a m/z 1073, 16 mass units fewer than that of sanglifehrin A. It was postulated this peak was due to incorporation of phenylalanine in absence of *meta*-tyrosine.

Eight strains showing loss of sanglifeherin production were subsequently regrown to assess whether the potential *sfaA* mutation could be complemented chemically allowing a mutasynthetic process to novel sanglifehrins. Strains were grown in SM25-3 seed media for 48 h before transferring to SGP2 production media with 5 % resin. After a further 24 hours growth strains were fed in triplicate with 2 mM DL *meta*-tyrosine (addition of 100 µl of a 0.16 M solution in 1M HCL) or 2 mM L-phenylalanine

with an unfed strain used as control. Strains were harvested after a further 4 days growth and extracted and analysed by HPLC. *meta*-tyrosine was shown to completely complement the *sfaA* mutation and addition of L-phenylalanine increased levels of the -16 amu compound. Strain BIOT-4585 was chosen for further study as the *sfaA* deletion mutant.

Fermentation of BIOT-4585 and isolation of BC457

Cryopreserved spore stocks of BIOT-4585 were thawed at room temperature. Vegetative cultures (seed cultures) were prepared by transferring 4.0 mL of spore stock into 400 mL medium SM25 in 2 L Erlenmeyer flasks with foam plug. Cultivation was carried out for 48 h at 27 °C and 250 rpm (5.0 cm throw). From the seed culture 25 mL was transferred into 250 mL production medium SGP2 + 5 % HP20 in 2 L Erlenmeyer flasks with foam plug. After 24 hours cultivation at 24 °C and 250 rpm (2.5 cm throw), a solution of (*S*)-methyl 2-amino-3-(3-fluoro-5-hydroxyphenyl) propanoate [L-*meta*-tyrosine methyl ester] hydrochloride salt in 1 M hydrochloric acid (2 ml) and DL-piperazic acid in methanol (2 ml) was added to each production flask at 26 h to give a final 1 mM concentration of the individual enantiomers of the precursors. Cultivation was continued for further four days at 24 °C and 250 rpm (2.5 cm throw).

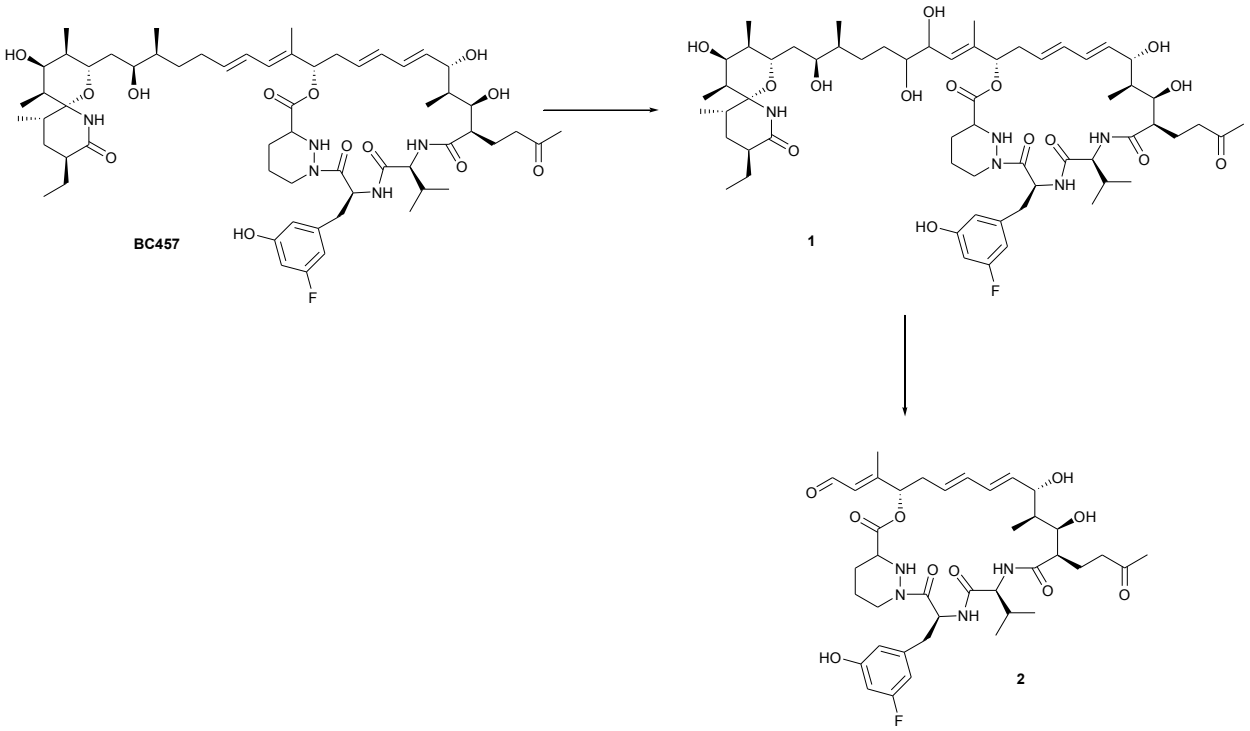
After harvesting, the culture broths were pooled and centrifuged (3300 g) for 25 minutes to separate the cells and resin from the clarified broth. The clarified broth was discarded after assay having confirmed less than 5% of target compound present. The cells and resin were stirred with 2 volumes of acetonitrile for 1 hour using a magnetic stirrer. The acetonitrile extract was recovered either by centrifugation or by allowing it to settle under gravity. A second acetonitrile extraction of the cells and resin was then performed under the same conditions. The combined acetonitrile extracts were concentrated to a residual aqueous volume under reduced pressure. This was extracted twice with ethyl acetate and the combined organics taken to dryness under reduced pressure to give the final crude (1.3 g).

The crude extract (1.3 g) was dissolved in ethyl acetate (2 ml) and loaded onto a silica gel column (10 x 2 cm) conditioned with ethyl acetate (500 ml). The column was eluted with ethyl acetate and then with stepwise increases in acetone (10%, 20%, 30%, etc. in ethyl acetate). Approx. 250 mL fractions were collected and the target compound identified by analytical LC, combined and taken to dryness. This material (278 mg) was dissolved in methanol (1.8 ml) and purified by preparative HPLC. A Waters Xterra MSC18 column (10 micron, 19 cm x 250 mm) was used with solvent pumped at 21 mL/min. Solvent A was water and solvent B was acetonitrile. The column was eluted isocratically at 50 % B for 6 min following the injection followed by a linear gradient to 100 % B at 30 min. Pure fractions were identified by HPLC-UV and combined. These fractions were taken to dryness under reduced pressure to yield BC457 as an off-white amorphous solid (20 mg).

HRMS: $C_{60}H_{91}O_{13}N_5F_1$ requires 1108.6592, found 1108.6572 (Δ -1.78 ppm).

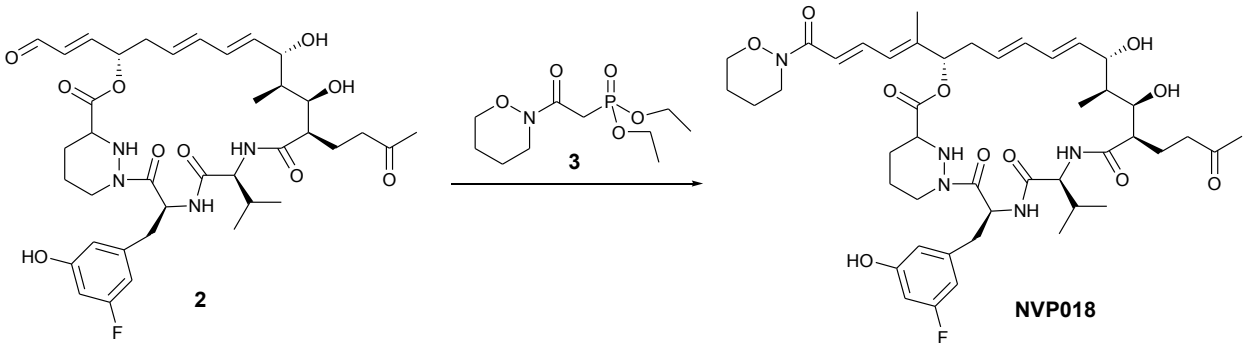
Semisynthesis of NVP018 from BC457

Supplementary Information



To a stirred solution of **BC457** (430 mg, 0.38 mmol), $(DHQ)_2PHAL$(18.6 mg, 0.024 mmol), osmium tetroxide (0.156 mL, 0.012 mmol) in *tert*-butyl alcohol (2.5 wt%, 0.079 mmol/ml), and methanesulfonamide (74 mg, 0.77 mmol) in 20 mL *tert*-butyl alcohol were added at room temperature, a solution of potassium ferricyanide (382 mg, 1.16 mmol) and potassium carbonate (160 mg, 1.16 mmol) in 20 mL water, resulting in a brown emulsion. After 2 h a solution of sodium sulfite was added, and stirring was continued for 20 min. The resulting mixture was extracted with ethyl acetate (3 x 50 ml). The combined organic layers were washed with brine, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure, purified by reverse-phase flash chromatography to yield **1** as a white solid (240 mg, 55%).

To a stirred solution of **1** (240 mg, 0.21 mmol) in 24 mL of a 2:1 mixture of THF and water was added sodium periodate (91 mg, 0.42 mmol). The resulting mixture was stirred at room temperature for 3 h, and then saturated aqueous sodium bicarbonate was added. This mixture was extracted with three portions of ethyl acetate. The combined organic layers were washed with one portion of water and two portions of saturated brine, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by reverse-phase flash chromatography to yield **2** ((130 mg, 81%).



To a solution of **3** (42 mg, 0.168 mmol) in THF (2.0 mL) was added NaH (1.2 mg, 0.05 mmol) in anhydrous THF (0.2 mL) at 0 °C with stirring. The synthesis of **3** was previously reported[29]. The solution was then stirred at 20 °C until it became clear. Then **2** (30 mg, 0.042 mmol) was added to the clear solution and the mixture stirred at 20 °C for 2 hours. The mixture was quenched with water (10 mL) and extracted with ethyl acetate (3 x 20 mL). The organic layer was washed with brine and dried over Na_2SO_4, filtered and reduced *in vacuo*. The residue was purified by preparative HPLC to obtained **NVP018** as an amorphous white solid (10.2 mg, 30%). This material and all future batches tested were >95% pure by HPLC-UV and LCMS methods (see general chemical methods) with no one impurity greater than 2%.

HRMS: $C_{45}H_{63}O_{11}N_5F_1$ requires 868.4503, found 868.4494 (Δ -0.99 ppm).

Solubility analysis

Solubility was measured by diluting test compounds in DMSO (10 mM) into PBS at pH 7.4 to a target concentration of 100 µM with a final DMSO concentration of 1%. Sample tubes were gently shaken for 4 h at room temperature, centrifuged and supernatants diluted into PBS. Diluted samples were mixed with the same volume (1:1) of methanol, then the same volume (1:1) of acetonitrile containing internal standard for LC-MS/MS analysis (see general methods).

***In vitro* assessment of inhibition of efflux transporters**

To assess the inhibition of the MRP2, MRP3 and BSEP efflux transporters, an in vitro vesicular transporter assay from Solvo Biotechnology Inc. was used. The Test Articles (TAs) (at 0.068, 0.2, 0.62, 1.8, 5.5, 16.7 and 50 µM) were incubated with efflux transporter membrane vesicles (Solvo Biotechnology Inc.) both in the absence and presence of 4 mM ATP to distinguish between transporter mediated uptake and passive diffusion of TA's into the vesicles. In the case of MRP2 and

MRP3 transporters reactions were carried out in the presence of 2 mM glutathione. Reaction mixtures were preincubated for 10 minutes at 37 °C. Reactions were started by the addition of 25 µl of 12 mM MgATP (4 mM final concentration in assay) or assay buffer for background controls. Reactions were stopped by adding 200 µl of ice-cold washing buffer and immediately followed by filtration on glass fiber filters in a 96-well format (filter plate). Scintillation buffer was added to the washed and dried filter plate and scintillation was counted subsequently. Probe substrates were taurocholate (2 µM) for BSEP vesicles and E217βG (1µM) for MRP2 and MRP3 vesicles. For all wells the translocated amount of the probe substrate was determined in cpm units. Relative activities were calculated with the following equation:

Activity % = 100x(A-B)/(C-D), where A= translocated amount of substrate in the presence of TA and ATP, B= translocated amount of substrate in the presence of TA, C= translocated amount of substrate in the presence of solvent and ATP and D= translocated amount of substrate in the presence of solvent. IC_{50} was defined as the TA concentration needed to inhibit transport of the probe substrate by 50%. IC_{50} was derived from the three-parameter logistic equation; a curve fitted onto the relative activity vs. TA concentration plot by non-linear regression.

***In vitro* assessment of inhibition of uptake transporters**

To assess the inhibition of the OAT1B1 and OAT1B3 uptake transporters, an *in vitro* uptake transporter assay from Solvo Biotechnology Inc. was used. Uptake experiments with Test Article (TA) at 0.068, 0.2, 0.62, 1.8, 5.5, 16.7 and 50 µM, were performed on CHO cells stably expressing human SLC transporters OATP1B1 and OATP1B3. Parental cell line CHO-K was used as negative control. Cells (1 × 105 in 200 µl 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 DMEM (F-12, Lonza, New Jersey, US) supplemented with 5 mM sodium butyrate) were plated on standard 96-well tissue culture plates and incubated 24 hours before the experiment at 37 °C in an atmosphere of 5% CO_2 and 95% air. Before experiments the medium was aspirated by vacuum suction, cells were washed with 2 × 100 µl of Krebs-Henseleit buffer pH 7.3 (prepared from Sigma chemicals, Sigma-Aldrich, St Louis, MO). Uptake experiments were carried out at 37 °C in 50 µl of Krebs-Henseleit buffer (pH 7.3) containing the probe substrate and the TA or solvent, respectively. The organic solvent concentration was equal in each well, and did not exceed 1 % v/v. The probe substrate for the OATP1B1 assay was E3S (0.1 µM) and for the OATP1B3 assay was Fluo-3 (10 µM). The translocated amount of probe substrate was determined for each well in cpm. Relative activities were calculated from the equation:

Activity % = 100x(A-B)/(C-D)

Where A= translocated amount of substrate in the presence of TA on transfected cells, B= translocated amount of substrate in the presence of TA on parental cells, C= translocated amount of substrate in the presence of solvent on transfected cells and D= translocated amount of substrate in the presence of solvent on parental cells. IC_{50} was defined as the TA concentration needed to inhibit

transport of the probe substrate by 50%. IC_{50} was derived from the three-parameter logistic equation; a curve fitted onto the relative activity vs. TA concentration plot by non-linear regression.

***In vitro* assessment of inhibition of Pgp transporters using MDCK cells**

To assess the inhibition of the P-glycoprotein (Pgp/MDR1) transporter, an in vitro ATPase assay from Cyprotex was used. MDR1-MDCK cells obtained from the NIH (Rockville, MD, USA) were used. Following culture, the monolayers were prepared by rinsing both basolateral and apical surfaces twice with buffer at pH 7.4 and 37 °C. Cells were then incubated with pH 7.4 buffer in both apical and basolateral compartments for 40 min at 37 °C and 5% CO_2 with a relative humidity of 95 % to stabilize physiological parameters. For the apical to basolateral study (A-B), buffer at pH 7.4 was removed from the apical compartment and replaced with loperamide dosing solutions before being placed in the 'companion' plates. The solutions were prepared by diluting loperamide in DMSO with buffer to give a final loperamide concentration of 5 µM (final DMSO concentration adjusted to 1 %). The fluorescent integrity marker Lucifer yellow was also included in the dosing solution. The experiment was performed in the presence and absence of the test compound (applied to both the apical and basolateral compartments). For basolateral to apical (B-A) study, the P-glycoprotein substrate, loperamide (final concentration = 5 µM) was placed in the basolateral compartment. The experiment was performed in the presence and absence of the test compound (applied to the apical and basolateral compartments). Incubations were carried out in an atmosphere of 5% CO_2 with a relative humidity of 95% at 37 °C for 60 min. After the incubation period, the companion plate was removed and apical and basolateral samples diluted for analysis by LC-MS/MS. A single determination of each test compound concentration was performed. On each plate, a positive control inhibitor was also screened. The test compound was assessed at 0.1, 0.3, 1, 3, 10, 30 and 50 µM. The integrity of the monolayers throughout the experiment was checked by monitoring Lucifer yellow permeation using fluorimetric analysis. After analysis, an IC_{50} was calculated (i.e., inhibitor concentration (test drug) achieving half maximal inhibition effect).

Plasma Protein Binding analysis

Human plasma protein binding was analyzed using a dialysis chamber methodology, with test article dosed at 1 µM. Equilibrium was allowed for 5 h at 37 °C, and levels of test article in each compartment were analyzed by LCMS/MS.

Rat and Mouse intravenous and oral pharmacokinetic analysis

Compounds were formulated in 5 % ethanol, 5 % cremophor EL and 90 % saline for both p.o. and i.v. administration. Groups of 3 male CD1 mice or SD rats were dosed with 1 mg/kg i.v. or 5 mg/kg p.o. Whole blood samples (40 µL) were taken *via* tail or saphenous vein, pre-dose and at 0.083, 0.25, 0.5, 1, 2, 8, and 24 hours, diluted with an equal amount of distilled H_20 and kept on dry ice immediately. Samples were stored at -70 °C until analysis. The concentration of the test article in the sample was then determined by LC-MS/MS. The time-course of blood concentrations was plotted and used to derive area under the whole blood concentration-time curve (AUC), which is directly proportional to the total amount of unchanged drug that reaches the systemic circulation). These values were used to generate PK parameters (with a non-compartmental model) using WinNonlin (version 5.2, Pharsight Corporation, California, USA) (see table S3).

Dog oral pharmacokinetic analysis

Compounds were formulated in 5 % DMSO, 5 % solutol HS15, 90 % 100nM Na_2CO_3 / $NaHCO_3$ (pH 8.39), for p.o. administration. Groups of 3 non-naïve beagle dogs were dosed with 5 mg/kg p.o. Whole blood samples (40 µL) were taken via cephalic or saphenous vein, pre-dose and at 0.083, 0.25, 0.25, 0.5, 1, 2, 8, 24 and 48 hours, diluted with an equal amount of distilled H_20 and kept on dry ice immediately. Samples were stored at -70 °C until analysis as described above.

7 day repeated dosing to CD-1 mice

Six to eight-week old male CD-1 mice were randomly assigned to 4 dose groups (0, 5, 50, 250 mg/kg), each containing 6 mice. The animals were treated with NVP018 via oral gavage at a dose volume of 10 mL/kg for 7 consecutive days. Serum bilirubin levels were measured in 3 mice per group 24 hours after dosing on day 7. NVP018 was dissolved in 5% Ethanol, 5 % Cremophor EL and 90 % Saline at 0.5 mg/mL prior to administration (see table S2).

Additional analysis of bilirubin after single doses of up to 500mg/kg to another group of three mice also showed no increase.

Pharmacokinetics in SD Rats

Three male SD Rats were fasted overnight and fed 4 hr post dosing, with free to access water. Rats were dosed PO via gavage needle, with serial blood samples taken from the tail vein into K2EDTA tubes at predose, 0.083, 0.25, 0.5, 1, 2, 4, 8 and 24 hours after dose. NVP018 was dissolved in 5% Ethanol, 5 % Cremophor EL and 90 % Saline at 0.5 mg/mL prior to administration. PK parameters were determined by the non-compartmental model of the non-compartmental analysis tool of Pharsight Phoenix WinNonlin® 5.3 software.

Effect of serum on HCV inhibition

The antiviral effect of NVP018 against Huh7-Con1 Luc cells was determined using the standard protocol as described above, but with increasing levels of either human or foetal bovine serum (see table S4).