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Soraphen A: A broad-spectrum antiviral natural product with potent anti-hepatitis C virus activity

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Soraphen A: a broad-spectrum antiviral natural product with potent anti-hepatitis C virus activity

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Abbreviations used in this manuscript:

HCV, hepatitis C virus; HIV, human immunodeficiency virus; VLDL, very low density lipoprotein; LDL, low density lipoprotein; ACC, acetyl CoA carboxylase; SorA, Soraphen A; DMVs, double membrane vesicles; HCVcc, cell-culture-derived HCV; TOFA, 5-(Tetradecyloxy)-2-furoic acid; MOI, multiplicity of infection; ; EC_{50}, half maximal effective concentration; CC_{50}, half maximal cytotoxic concentration; SI, selectivity index; 2'-*C*-Met, 2'-*C*-methyladenosine; PHHs, primary human hepatocytes; $TCID_{50}$, Tissue culture infectious dose 50; SGR, subgenomic replicon; HCVpp, HCV pseudoparticles; IRES, internal ribosomal entry site; EM, electron microscopy; DMVs,

double-membrane vesicles; FDA, Food and Drug Administration; DAAs, direct-acting antivirals; Sol, Solubility; JFH1, Japanese fulminant hepatitis 1; EMCV, encephalomyocarditis virus; DMSO, dimethyl sulfoxide; UTR, untranslated region.

Keywords: HCV/HIV co-infection, HCV replication inhibitor, lipid metabolism, broad-spectrum antiviral, natural product.

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Authors Contributions:

GK designed the study and prepared the manuscript. GK, GPV, IRB, CB, PMP, FWRV, KH performed the experiments and analysed the data. JM, TP, RB, RM, MK, MB and AM supervised parts of this study. JD supervised all aspects of this study and contributed to manuscript preparation. All authors contributed to the interpretation and discussion of the results, as well as to the revision of the manuscript.

Abstract

Background & Aims: Co-infections by the hepatitis C virus (HCV) and human immunodeficiency virus (HIV) represent a significant challenge for treatment due to the necessity of combining diverse antiviral compounds that often leads to complex drug-drug interactions. Soraphen A (SorA) is a myxobacterial metabolite that inhibits the acetyl-CoA carboxylase, a key enzyme in lipid biosynthesis. We have previously identified SorA to efficiently inhibit HIV. The aim of the present study was to evaluate the capacity of SorA and analogues to inhibit HCV infection.

Methods: SorA inhibition capacity was evaluated *in vitro* using cell-culture derived HCV, HCV pseudoparticles and subgenomic replicons. Infection studies were performed in the hepatoma cell line Huh7/Scr and in primary human hepatocytes. The effects of SorA on membranous web formation were analyzed by electron microscopy.

Results: SorA potently inhibits HCV infection at nanomolar concentrations. Obtained EC_{50} values were 0.70 nM with a HCV reporter genome, 2.30 nM with wild-type HCV and 2.52 nM with subgenomic HCV replicons. SorA neither inhibited HCV RNA translation nor HCV entry, as demonstrated with subgenomic HCV replicons and HCV pseudoparticles, suggesting an effect on HCV replication. Consistent with this, evidence was obtained that SorA interferes with formation of the membranous web, the site of HCV replication. Finally, a series of natural and synthetic SorA analogues helped to establish a first structure-activity relationship.

Conclusions: SorA has a very potent anti-HCV activity. Since it also inhibits HIV, SorA is a promising candidate for the development of simplified treatments of HCV/HIV co-infection.

Introduction

The hepatitis C virus (HCV) and the human immunodeficiency virus (HIV) have infected around 170 million and 35 million individuals worldwide, respectively. Due to overlapping acquisition modes, the number of individuals co-infected with HCV and HIV has risen up to 7 million [1]. In developed countries, HIV-infected individuals who inject drugs and HIV-infected men who have sex with men comprise the majority of HCV/HIV co-infected patients. Amongst the latter, multiple HCV outbreaks and reinfections have recently been described [2].

HCV mono-infection and HCV/HIV co-infection treatment(s) have changed dramatically in the era of Direct-Acting Antivirals (DAAs) [3]. Currently approved DAAs include NS3-4A protease inhibitors (telaprevir, boceprevir and simeprevir) NS5A inhibitors (daclatasvir and ledipasvir) and the NS5B polymerase inhibitor sofosbuvir. While regimens containing these DAAs have brought major progress, several limitations remained. First, the therapeutic efficiency towards the different HCV genotypes varies. Second, although some DAAs present high barrier to resistance (i.e. sofosbuvir), in general this property is low. Third, the deleterious interactions between some anti-HCV and anti-HIV drugs and the associated liver toxicities [4]. Forth, therapy costs are extremely high and represent a significant financial burden even for countries with developed economies .Treatment simplifications that may include broad-spectrum antiviral drugs targeting both viruses could help in solving some of these issues.

Due to their genome simplicity, viruses rely on a network of host-cell factors to multiply.

Host-targeting drugs inhibiting cellular factors required for HCV to replicate are already in clinical trials. These include the cyclophilin inhibitors alisporivir and SCY-635, the miR-122 inhibitor miravirsen and the SR-B1 receptor blocker ITX-5061 [5, 6]. Since some host factors are exploited by different viruses, conceptually, it would be possible to inhibit several viruses by targeting shared host factors. Given the genetic stability of the host, the development of such broad-spectrum antivirals would have the additional advantage of rendering selection of drug resistant virus mutants less likely, a major problem of conventional drugs that target viral proteins [7]. Initial efforts in this direction are showing promising results [8, 9]. Predicted good targets for the development of antiviral drugs that act on HCV and HIV are the proteins that modulate the lipid metabolism. HCV particles that circulate in the blood of infected patients are associated with very low and low density lipoproteins (VLDL and LDL), resembling the so-called lipo-viral particle. Lipids have been shown to play an important role during entry (apolipoproteins), replication (fatty acids) and during budding and egress (VLDLs and LDLs) [10]. Thus, the HCV life cycle is intimately linked to intracellular membranes and host-cell lipid synthesis. The role of lipids in the HIV life cycle is also quite profound. HIV virion assembly, encapsidation and budding from the cell surface of the infected cell require a lipid milieu enriched in cholesterol and phosphatidylinositol-4,5-bisphosphate [11]. In addition, modifiers of the cellular lipid metabolism have been shown to affect HIV propagation [12].

By screening a library of secondary metabolites produced by *Myxobacteria*, we discovered recently that soraphens possess anti-HIV activities [13]. Soraphens are a group of polyketide natural products that have been first identified due to their potent antifungal activity [14] and more recently received much attention due to their anti-

cancer [15], insulin-sensitizing [16] and immunoregulatory functions [17]. Soraphens mediate their function via inhibition of acetyl-CoA carboxylase enzyme (ACC) [18] that plays a key role in the cellular homeostasis of fatty acids. In humans exist two ACC isoforms (ACC1 and ACC2, a cytosolic and mitochondrial isoform, respectively) that are encoded by separate genes [19]. As soraphens are known to alter the lipid metabolism of treated cells [20], and the HCV life cycle critically depends on lipids [10], we tested soraphen A (SorA) for its anti-HCV activity. Here, we show that SorA is a highly potent HCV inhibitor that is active in the low nanomolar range. It exerted its activity, at least in part, via interfering with the formation of double membrane vesicles (DMVs) that are the sites of HCV replication. A structure/activity analysis conducted in this study suggested options for structural modifications to optimize the pharmacological properties of SorA towards a strategy for a potent anti-HCV/HIV clinical drug development.

Materials and Methods

A detailed description of the methods used in this manuscript is provided in the *Supplementary Material* section.

Results

SorA inhibits HCVcc infection

We have recently identified SorA as HIV inhibitor [13]. To assess the inhibitory effects of SorA (Fig. 1A) on HCV infection we used the HCV cell-culture (HCVcc) system [23-25]. Unless otherwise stated, we used the Huh7/Scr and the Huh7/Lunet [26] cells, which are highly permissive for HCV propagation *in vitro*. Our experiments were performed in the context of genotype 2a (Jc1 chimera [27]) HCVcc virus. To facilitate the quantification of infection, we used the bicistronic Jc1 luciferase reporter construct, designated Luc-Jc1 [28]. Cell viability was monitored in parallel by a commercial ATP assay [29]. The commercial ACC inhibitor 5-(Tetradecyloxy)-2-furoic acid (TOFA), which has been previously described to inhibit HCV replication [30], was used for comparison. The NS3-4A serine protease inhibitor VX-950 [31], a known HCV replication inhibitor, was used as positive control. As shown in Fig. 1B, Luc-Jc1 virus infection in Huh7/Scr cells (at an MOI of 0.03 $TCID_{50}$/cell) was strongly inhibited by SorA at concentrations in the 1-10 nM range. Viability assays show no toxicity of the compounds up to 10 µM. The half maximal effective concentration (EC_{50}) for SorA was estimated 0.70 nM [the half maximal cytotoxic concentration (CC_{50}): ~98.52 µM, and the selectivity index (SI): 140,742], as compared with an EC_{50} of 1.01 µM for TOFA [CC_{50}: ~33.87 µM, SI: 33.53] and an EC_{50} of 18.92 nM for VX-950 [CC_{50}: ~94.51 µM, SI: 4,995] in this assay. To demonstrate the specific inhibitory activity of SorA against HCV at a higher MOI, Jc1 virus (which does not carry a reporter gene) was challenged in similar experiments with increasing doses of SorA. As shown in Fig. 1C, SorA also inhibited Jc1 infection at an MOI of 1 $TCID_{50}$/cell in an equipotent manner. The EC_{50}

value was 2.30 nM [$CC_{50} \geq 10$ μM, SI ≥ 4,357]. A 2'-modified nucleoside analog (2'-*C*-methyladenosine, 2'-*C*-Met) [32] was used as a positive control [EC_{50}: 0.101 μM, CC_{50}: 12.42 μM, SI: 122.97].

Although Huh7 cells are a well-accepted cell culture model for HCV infection studies, these cells might present differences in lipid metabolism compared to primary human hepatocytes (PHHs). To further corroborate our findings in a more natural system, PHHs were inoculated with Jc1 virus (at an MOI of 0.2 $TCID_{50}$/cell) in the presence of SorA or VX-950. 24 and 48h post-infection cell culture supernatants were harvested and the amount of virus production by the PHHs was quantified by an endpoint dilution assay ($TCID_{50}$). As shown in Fig. 1D, Jc1 infection of PHHs was also inhibited by SorA. Similar to Huh7/Scr cells, SorA was not toxic to PHHs up to 10 μM (data not shown). Conclusively, our *in vitro* results using human hepatoma carcinoma-derived cell line Huh7/Scr as well as PHHs demonstrate that SorA is a very potent non-toxic inhibitor of HCV infection.

SorA inhibits HCV subgenomic replicons

To investigate the impact of SorA in HCV RNA translation and/or replication, we transfected Huh7/Scr cells with a subgenomic JFH1 luciferase replicon (SGR-JFH1, Fig. 2A, top) [33]. These HCV subgenomes do not contain structural proteins and therefore cannot sustain HCV particle production. However, they possess the ability to replicate autonomously in cell culture, rendering them powerful tools for HCV translation and replication studies. As shown in Fig. 2A, SorA inhibits HCV RNA translation and/or replication as deduced by the reduced luciferase values. The EC_{50} for

SorA was estimated to be 2.52 nM [CC_{50}: 75.78 μM, SI: 30,071], while EC_{50} values for the control compounds TOFA and VX-950 were estimated to be 72.55 nM [CC_{50}: 27.2, SI: 374.91] and 1.55 nM [CC_{50}: 43.6 μM, SI: 28,129], respectively. To expand our analyses in other genotypes we treated with SorA a stable Huh7 cell line which harbors the Con1 subgenomic replicon that belongs to the 1b genotype (I_{389}/NS3-3′/LucUbiNeo-ET [34], Supplementary Fig. 1A). As shown in Supplementary Fig. 1B, SorA inhibited potently the replication of the Con1 subgenomic replicon in a nanomolar range (0.01-1.0 nM), confirming an inhibitory capacity of SorA against the 1b genotype as well.

SorA does not inhibit HCV entry

Infection of hepatocellular carcinoma-derived cell lines with HCV pseudoparticles (HCVpp) is a well-established system to investigate HCV entry and neutralization [35]. HCVpp are formed by incorporation of the full-length HCV glycoproteins E1 and E2 into the envelope of lenti- or retroviral core particles. To test the effect of SorA on HCV entry we used HCVpp which carry glycoproteins (genotype 2a, isolate J6CH) identical to those of the Luc-Jc1 virus. The results are presented in Fig. 2B and show that SorA does not inhibit HCVpp infection at 5 μM concentration while dasatinib [36], a known HCV entry inhibitor, showed potent inhibitory HCVpp capacity at the same concentration. This demonstrates that SorA does not exert its inhibitory capacity in the entry step and in combination with previous results it narrows down its inhibitory activity to translation and/or replication. Similar to the HCVcc experiments, the compounds were found to be non-toxic at a concentration of 5 μM in Huh7/Scr cells.

SorA does not inhibit HCV RNA translation

To investigate a putative inhibitory effect of SorA on HCV RNA translation we used a replication incompetent SGR-JFH1 which harbors a ΔGDD deletion in the NS5B protein, thus abolishing its replication activity [37]. In this subgenomic reporter system the expression of the *Firefly luciferase* gene is driven by the internal ribosomal entry site (IRES) of HCV and thus, reporter gene expression is attributed solely to HCV-IRES mediated translation. As shown in Fig. 2C, neither SorA, nor TOFA, nor VX-950 presented an effect on HCV IRES-driven luciferase expression. As a positive control, puromycin, a drug that causes premature chain termination during translation, almost completely inhibited HCV RNA translation as expected (Supplementary Fig. 2). Taken together, these results point to a role of SorA in HCV RNA replication.

Fatty acids add-back rescues HCV replication in SorA treated cells

ACC is a biotin-dependent enzyme that catalyzes the formation of acetyl-CoA to malonyl-CoA, which is the rate-limiting step for the biosynthesis of fatty acids [38]. Since HCV possesses an intimate connection to fatty acids and lipids [10] we sought to rescue HCV replication in SorA treated cells by fatty acids or lipids add-back during infection. As shown in Fig. 3A, addition of palmitic, myristic or lauric acids to the culture medium simultaneously to infection partially restored HCV replication in a dose dependent-manner, while addition of sphingomyelin or phosphatidylserine did not exert any beneficial effect for HCV replication. Cell viability data for these assays are shown in Fig. 3B.

SorA affects the formation of double-membrane vesicles

HCV RNA replication occurs in close association with rearranged intracellular membranes forming a vesicular network that has been designated membranous web [39]. It consists predominantly of double-membrane vesicles (DMVs) [40], which contain viral proteins, replicating viral RNA and altered intracellular membranes [41]. To study the effect of SorA on DMVs' formation we used electron microscopy (EM) in two different experimental systems. First, we analyzed DMVs formation in stable T7 RNA polymerase-expressing Huh7-Lunet/T7 cells, transfected with a plasmid encoding the proteins NS3 to NS5B (NS3-5B) of the JFH1 isolate under control of the T7 promoter (Fig. 4A-C). As previously shown, expression of the NS3-5B polyprotein induces a membranous web that is morphologically indistinguishable from the one found in HCV-infected cells [40] (Fig. 4D-F). Importantly, treatment of transfected cells with SorA reduced in a statistically significant manner the DMVs number (Fig. 4G). This result is consistent with the observed inhibition of HCV replication by this compound. To validate this finding in a HCV infectious system, we repeated the analysis by using cells that had been transfected with replication-competent Jc1 RNA and treated with SorA for 96h (Fig. 5). Abundance of HCV proteins was not affected at 1 nM SorA whereas at 10 nM protein level was reduced by ~50% as determined by Western blot and immunofluorescence (Fig. 5A-D). By using these conditions we found that SorA treatment profoundly reduced both DMV size (Fig. 5H) and number (Fig. 5I), especially when using 10 nM SorA. Although at this concentration HCV protein amount was reduced, the degree of this reduction was much lower as compared to the very pronounced inhibition of DMV formation. In conclusion, these results provide strong

evidence that SorA inhibits HCV replication, at least in part, by interfering with formation of the membranous replication factory.

SorA inhibits HCV replication in persistently infected Huh7/Scr cells and in a stable replicon cell line

To exclude the possibility that SorA inhibits HCV RNA replication only by inhibition of *de novo* replication complexes we sought to test the efficacy of SorA in a persistently infected Huh7/Scr cells. For this, we established over a period of four weeks cultures of Huh7/Scr cells persistently infected with Jc1 (>95% positive for NS5A by immunofluorescence, data not shown) and treated for 10 days with SorA or with the controls TOFA or VX-950. We observed that SorA reduced HCV RNA levels by ~80% and ~95% for the concentrations 1 and 10 μM, respectively (Fig. 6A). The EC_{50} for SorA was 0.152 μM, while for the controls TOFA and VX-950 EC50 values it was 0.976 and 0.110 μM, respectively. The resulting HCV RNA reduction was confirmed at the protein level by detection of NS5A protein by immunofluorescence (Fig. 6B). To further corroborate our results, we treated with SorA or controls a stable replicon cell line of Huh7 cells harboring the LucUbineo JFH1 replicon [42]. As shown in Supplementary Fig. 1C, SorA reduced HCV replication in this cell line as deduced by the luciferase activity.

SorA presents high barrier to resistance in cell culture

To explore the barrier to resistance of SorA in cell culture, Huh7/Scr cells were infected with the Jc1 virus (at an MOI 1 $TCID_{50}$/cell, corresponding to passage 0) and the

following day were treated with 100, 250 or 500 nM SorA (representing ~1x EC_{90}, ~2.5x EC_{90} and ~5x EC_{90}, respectively). Cells were passaged every 2-3 days and at each passage cell culture supernatant was frozen for further resistant viruses' analyses. The VX-950 inhibitor was used in parallel as a control at the same concentrations. As shown in Fig. 7, SorA presented high barrier to resistance for at least 6 cell culture passages and 3 supernatant passages (data not shown, in total 25 days). In contrary, VX-950 resistant viruses appeared as early as 3 passages post infection for the 100 nM concentration and 5 passages for the 500 nM concentration.

SorA analogues inhibit the Luc-Jc1 virus

Although we demonstrated that SorA potently inhibited HCV in cell culture, its limited water solubility (Sol) was reported to hamper its *in vivo* efficacy [17]. To address this obstacle, we explored the structural determinants of the anti-HCV activity of SorA in order to derive analogs with higher solubility without activity loss. To this end, a series of SorA analogues obtained from fermentation and total synthesis has been tested for their anti-HCV activity by using the Luc-Jc1 virus as described above (Fig. 8 and Supplementary Fig. 3). Various structural alterations of SorA were tolerated in terms of activity, in particular at the pyran ring: a derivatization of the alcohol function at C-5 with a long, solubilizing polyethylene-glycol chain (as in Sor-S1036), led to an approximately equipotent compound with an EC_{50} of 1.3 nM and Sol: ~2.0 mg/ml (vs. EC_{50}: 0.7 nM and Sol: ~0.2 mg/ml for SorA). Likewise, the methyl substituent at C-6 can be replaced by an ethyl group, yielding SorE1, without loss of activity. However, the so-called Paleo-soraphens A and B, featured by an α, β unsaturated double bond at C-2/C-3 and a lack of the pyran ring [43], were completely inactive. Also the extension

of the methyl group at C2 to an ethyl group (as in SorD1) was not tolerated. In the northern half of the molecule, the formal reduction of the double bond of SorA at C-9/C-10 led to the approximately equipotent analog SorF (EC_{50} of 1.8 nM and Sol: ~0.2 mg/ml). In contrast, the demethylation of the methoxy group at C-11 was associated with an at least 12-fold drop of activity, as demonstrated by SorA2β (EC_{50}: 36 nM and Sol: ~0.6 mg/ml) and SorC (EC_{50}: 8.8 nM and Sol: ~0.6 mg/ml). A double demethylation, as found in SorA4α, led to a sharp drop of activity (EC_{50} of 260 nM) compared to SorA. Finally, a formal hydroxylation at C14, yielding SorB1, led to a 10-fold loss of activity. For all compounds, a cellular cytotoxicity >10 μM was observed, thereby maintaining the very high (>1000) selectivity index of the active analogs.

Discussion

In this study we demonstrate that the *Myxobacteria*-derived polyketide natural product SorA and its analogues acted as highly potent HCV replication inhibitors. SorA is a nanomolar inhibitor of eukaryotic ACCs [18] and mediates its antiviral effect at least in part through interefering with membranous web formation, the sites of viral genome copying and amplification. These observations are remarkable for several reasons. First, when cells were pre-incubated with SorA or several of its analogues, the EC_{50} values were <10 nM for the JFH1-based assays (genotype 2a), regardless of the experimental system used (luciferase-carrying viruses, wild-type viruses or subgenomic replicons). While this manuscript was under review, Singaravelu *et. al.*, reported an EC_{50}: 5 nM of SorA in stable Huh7 cell lines harboring the Con1 subgenomic replicon (genotype 1b) [44]. We confirmed this result with a similar cell line harboring Con1 subgenomic

replicon as well (Supplementary Fig. 1B). Furthermore, there was no apparent toxicity up to 10 μM SorA. Notably, these values are comparable to those estimated for the Food and Drug Administration (FDA)-approved protease inhibitor VX-950. In contrast, the commercial ACC inhibitor TOFA has shown an EC_{50} in the range of 70-976 nM, depending on the experimental system used. Second, given the genetic stability of the host, the development of a broad-spectrum antivirals would have the advantage of rendering selection of drug resistant virus mutants less likely, a major problem of conventional drugs that target viral proteins [7]. Indeed, targeting a cellular host factor, ACC in this case, instead of a viral protein is an attractive strategy for anti-HCV intervention because this type of anti-viral drug, as shown in Fig.7, presents high barriers to resistance development. Third, SorA and F have previously been shown to inhibit HIV *in vitro* [13]. Thus, SorA and analogues represent very attractive candidates to be developed into single broad-spectrum antiviral agents for treating HCV/HIV co-infections.

SorA is a specific inhibitor of the first committed step of lipid biosynthesis catalyzed by ACCs. In the presence of SorA, HCV replication was partially restored when myristic, lauric or palmitic acids where added to the media, the latter having the highest effect. Palmitic acid, during fatty acid biosynthesis from acetyl-CoA, is the first fatty acid to be produced and the most common fatty acid in animals. In agreement with a SorA anti-HCV activity mediated by lipid alteration, EM studies show that in cells transfected with infectious HCV RNA, SorA significantly reduced the size and number of endoplasmic reticulum-derived DMVs, the main constituents of the membranous web. In the heterologous expression-based system we observed DMV inhibition only in numbers. This might be due to the higher expression level of HCV proteins achieved in

that system or to a coupling of inhibition of web formation by SorA with HCV RNA replication. These results emphasize the need to use systems that mimic HCV infections (i.e. replication-competent viruses).

Several SorA analogues were obtained from fermentation and total synthesis. Our results show that various structural alterations of SorA are tolerated in terms of activity. It was observed that the removal of hydroxy or methoxy groups at C-5, C-4 or C5/C-3 was associated with 5-fold, 10-fold and 300-fold drops of potency, respectively, while few substitutions at C-5 (e.g. by formyl) led to equipotent compounds [45-48]. An elongation at C-11 through ester or ether groups led to improved activities for some analogues, while all changes at C-12 or C-17 were associated with reduced activities. Our data provide first insights to structure activity relationships in a human cellular assay, suggesting that positions C-5 or the northern hemisphere (C-9 to C-10) are well-suited for the optimization of the pharmacologic and pharmacokinetic properties of SorA through structural modifications. The lack of activity of the Paleo-soraphens may explain why the costly biosynthesis of these variants has been eliminated by evolution. The solubility of SorA (0.2 mg/ml) was improved by replacing the methoxy group by a more polar hydroxyl group at C11, as demonstrated by the solubility of both derivatives SorA2β and SorC (0.6 mg/mL). However, this enhancement was associated with a drop of activity. In contrast, the derivative Sor-S1036 exhibited a higher aqueous solubility (2.0 mg/ml) while maintaining potency. Thus, it is possible to improve the physicochemical properties of SorA without loss of activity. Consequently, Sor-S1036 has demonstrated higher efficacy *in vivo* in a mouse model of EAE than SorA [17] . However, since the long solubilizing chain introduced in Sor-S1036 hampered the

permeability of the compound and prevented an oral administration, the search for Soraphen analogs with optimized pharmacological properties deserves continuation.

In conclusion, a new nanomolar inhibitor of HCV is described that blocks a cellular host factor with high affinity and mediates its antiviral effect via alterations of the lipid requirements of viral replication. Due to its interesting overlapping activity of inhibiting also HIV SorA may present a prototype of a new class of broad-spectrum antiviral compounds acting via lipid metabolic changes.

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FIGURE LEGENDS

Figure 1. **Soraphen A inhibits HCV genotype 2a infection in Huh7/Scr cells and PHHs.** (A) SorA structure. (B) Anti-HCV activity and cell viability, using Huh7/Scr cells infected with Luc-Jc1 reporter virus (depicted above) and treated with increasing concentrations of SorA, TOFA or VX-950. Data are expressed as mean values of three independent experiments (±SEM). (C) Anti-HCV activity and viability on Huh7/Scr cells infected with Jc1 virus depicted above and treated with increasing concentrations

of SorA or 2'-*C*-Met. Data were plotted as percentage relative to the DMSO control for both infectivity and cell viability. Data are expressed as mean values of four measurements of two biological replicates (±SEM). (D) Anti-HCV activity of SorA in PHHs. The inhibition of infection was assessed by an endpoint dilution assay ($TCID_{50}$) 24h and 48h post-infection and is expressed as the percentage of inhibition relative to DMSO-treated cells. Data are expressed as mean values of four measurements of two biological replicates (±SEM) and from a single experiment representative of three independent PHHs donors.

Figure 2. **Soraphen A inhibits HCV replication.** (A) Anti-viral activity of the indicated compounds using subgenomic HCV replicons (SGR-JFH1, depicted above). (B) Anti-HCVpp activity of SorA or dasatinib and cell viability using Huh7/Scr cells infected with HCVpp bearing J6 glycoproteins. Results were plotted as percentage relative to the DMSO control for both infectivity and cell viability. (C) Effect of SorA, TOFA or VX-950 on HCV translation. Results were plotted as percentage relative to the DMSO control and are expressed as mean RLU values of four measurements of two biological replicates (±SEM).

Figure 3. Fatty acids add-back rescues HCV replication in Soraphen A treated Huh7/Scr cells. The indicated fatty acids or lipids at final concentration of 10 (white bars) or 50 μM (black bars) were added simultaneously to cells infected with Luc-Jc1 and treated with SorA (10 nM). All data were plotted as percentage relative to solvent control for both (A) infectivity and (B) cell viability. Data are expressed as mean values of four measurements of two biological replicates (±SEM).

Figure 4. Effect of Soraphen A on the formation of HCV-induced DMVs in an expression-based system. (A) Western blot analysis of NS5A abundance in cells expressing the HCV non-structural proteins (NS3-5B) treated with increasing concentrations of SorA up to 100nM. (B-E) Representative IF and EM images of NS3-5B expressing cells. Cells were treated with (B, D) solvent only (DMSO) or (C, E) SorA (100 nM). (F) Comparison of DMVs diameter in DMSO vs. SorA treated cells. (G) Comparison of DMVs numbers in DMSO vs. SorA treated cells.

Figure 5. Effect of Soraphen A on the formation of HCV-induced DMVs in a replication system. (A) Western blot analysis of NS5A expression in cells electroporated with Jc1 RNA and treated with increasing concentrations of SorA. Numbers below indicate amount of NS5A normalized to the levels in DMSO-treated samples (set to 100%). (B - G) Representative IF and EM images of Jc1-transfected cells (B, E) treated with DMSO only, (C, F) 1 nM SorA or (D, G) 10 nM SorA. (H) Comparison of DMVs size in Jc1 RNA-transfected cells treated with DMSO or SorA (1 or 10 nM). (I) Quantification DMVs number per μm^2 in cells described in H.

Figure 6. Soraphen A inhibits HCV replication in persistently infected Huh7/Scr cells. (A) Anti-HCV activity using Huh7/Scr cells persistently infected with Jc1 viruses and treated with SorA, TOFA or VX-950 at the indicated concentrations. All data were plotted as percentage relative to the DMSO control and present the HCV RNA values as measured by RT-qPCR. Data are expressed as mean values of four measurements of two biological replicates (±SEM). (B) Immunofluoresence analysis of NS5A protein (green) in persistently infected cells treated with SorA (10 μM), TOFA (10 μM) or VX-950 (10 μM). Cell nuclei were counterstained with DAPI (blue), magnification 63x.

Figure 7. Soraphen A presents high barrier to resistance *in vitro.* The determination of resistant Jc1 viruses to SorA or VX-950 (at the indicated concentrations) in Huh7/Scr cells was assessed for 6 cell passages by a $TCID_{50}$ assay of the supernatant and is expressed plotted in relation to DMSO-treated cells.

Figure 8. Structures and antiviral activities, expressed as EC_{50}, of SorA and SorA analogues.

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