PI4KIII inhibitor enviroxime impedes the replication of the hepatitis C virus by inhibiting PI3-kinases

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Running title: Enviroxime inhibits HCV replication by PI3K inhibition

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SYNOPSIS

Objectives: Many positive-stranded RNA viruses, including the hepatitis C virus (HCV), drastically remodel intracellular membranes to generate specialized environments for RNA replication. The phosphatidylinositol kinase-4 α (PI4KIIIα) plays an essential role in the formation of HCV replication complexes and has therefore been explored as a potential drug target. Here, we characterized the anti-HCV activity of the PI4KIII inhibitors enviroxime and BF738735 and elucidated the mechanism of action.

Methods: Antiviral assays were performed using HCV subgenomic replicons and infectious HCV. Enviroxime- and BF738735-resistant HCV replicons were generated by long-term culturing with increasing compound concentrations. Intracellular localization of PI4P lipids was analyzed by confocal microscopy.

Results: HCV subgenomic replicons resistant to either enviroxime or BF738735 proved cross-resistant and carried mutations in the NS3, NS4B and NS5A genes. Knockdown of PI4KIIIβ by siRNA did not affect the replication of the HCV subgenomic replicon in this study. Furthermore, the compounds did not affect PI4P lipid levels at the replication complexes nor the phosphorylation status of NS5A, activities attributed to PI4KIIIα. Interestingly, the broad-spectrum PI3K inhibitor LY294002 proved to be 10-fold less effective against the resistant replicons. In addition, enviroxime and BF738735 inhibited several PI3-kinases in enzymatic assays.

Conclusions: Unlike assumed, our data indicate that PI4KIIIα and PI4KIIIβ are not the main targets for the anti-HCV activity of enviroxime and BF738735. Instead, we demonstrate that both molecules impede HCV replication at least partially by an inhibitory effect on PI3-kinases. Moreover, HCV is able to by-pass PI3K inhibition by acquiring mutations in its genome.

INTRODUCTION

Worldwide approximately 71 million people are chronically infected with the hepatitis C virus (HCV). Chronically infected patients are at increased risk of developing liver fibrosis, liver cirrhosis and hepatocellular carcinoma making HCV infections one of the leading causes for liver transplantations in the western world. In recent years, a number of highly potent direct-acting antivirals in different combinations have been approved for treatment of patients. These combination regimens are highly effective in most patients and result in >95% of HCV-infected patients in a sustained virological response after just a few weeks of treatment for all genotypes. However, only 20% of all HCV infections were diagnosed in 2015 and only 7% of those diagnosed started treatment during that year.[1]

HCV belongs to the *Flaviviridae* family and has a single-stranded RNA genome of positive polarity. Many positive-stranded RNA viruses drastically remodel intracellular membranes to generate specialized environments for RNA replication. HCV appears to replicate on modified intracellular membranes that are derived of the endoplasmic reticulum (ER). An essential host factor that plays an important role in the formation of HCV replication complexes is the lipid kinase phosphatidylinositol kinase-4 α (PI4KIIIα).[2] Knockdown of PI4KIIIα expression drastically changes the membranous web morphology and disturbs viral RNA replication.[3] The HCV NS5A protein was found to recruit PI4KIIIα to the replication complexes and to stimulate its kinase activity resulting in elevated PI4P lipid levels.[3] The enzymatic activity of PI4KIIIα was also shown to modulate NS5A phosphorylation.[4] In addition to PI4KIIIα, PI4KIIIβ also emerged as an indispensable host factor for HCV replication in some, but not all siRNA screens,[5,6] giving conflicting data about the involvement of PI4KIIIβ in HCV replication. It has been suggested that the proviral role of PI4KIIIβ on HCV replication

is genotype-dependent, because NS5A competes with PI4KIIIβ for association with the acyl-coenzyme A binding domain containing protein 3 (ACBD3) in a genotype-dependent manner, with ACBD3 binding with a higher affinity to GT1b NS5A than to GT2a NS5A.[7] In this study, NS5A was shown to deprive existing ACBD3/NS5A complexes to form NS5A/ACBD3 complexes, enabling the relocation of PI4KIIIβ to the HCV replication complexes.

Inhibitors of PI4Ks exhibit antiviral activities, especially against enteroviruses.[8] One such molecule is enviroxime [2-amino-1-(isopropylsulfonyl)-6-benzimidazole phenyl ketone oxime]. This compound is long known as an inhibitor of the *in vitro* replication of rhinoviruses and enteroviruses. Enteroviruses that carry mutations in the 3A protein are resistant to enviroxime.[9] Picornavirus inhibitors that have little or no structural similarity to enviroxime but select for the same resistance mutations in the 3A-coding region have been described, including among others BF738735 (2-fluoro-4-(2-methyl-8-(3-(methylsulfonyl)benzylamino)imidazo[1,2-a]pyrazin-3-yl)phenol).[10] These molecules are referred to as enviroxime-like inhibitors, despite bearing little structural similarity. Interestingly, enviroxime and enviroxime-like inhibitors were shown to inhibit enterovirus RNA replication by inhibiting PI4KIIIβ.[11]

As enviroxime and BF738735 are able to inhibit PI4KIIIβ and (to a certain extent) PI4KIIIα *in vitro*, we wondered whether these compounds may exert *in vitro* antiviral activity against HCV. Here we report that enviroxime and BF738735 elicit potent *in vitro* anti-HCV activity against several HCV genotypes. Enviroxime-resistant and BF738735-resistant HCV replicons were generated and characterized. Surprisingly, both PI4KIIIα and PI4KIIIβ were not the major targets of the anti-HCV activity of these molecules in the HCV subgenomic replicon cultures used. In contrast, we here provide evidence that these compounds inhibit HCV replicon replication at least

partially by blocking PI3 kinases. In addition, our data indicate that HCV replicons could overcome the selective pressure of a PI3K inhibitor by the acquisition of specific mutations.

MATERIALS AND METHODS

Cells, virus and replicon constructs

Huh-7 cells containing subgenomic HCV replicons I_{389}luc-ubi-neo/NS3-3'/5.1 (Huh 5-2) and I_{377}/NS3-3'/wt (Huh 9-13) have been described before.[12] A plasmid encoding a genotype 1a (H77 strain) HCV subgenomic replicon, pH/SG-neo(L + I) was obtained from Apath, LLC (St. Louis, MO). GT2a and 4a replicon plasmids were kindly provided by Gilead Sciences. HCV JFH-1/CS-N6 was a generous gift from Prof. F. Zoulim (University of Lyon, France).[13] The Huh 7.5.1 cell line was a generous gift from Dr. F. Chisari (The Scripps Research Institute, La Jolla, USA). Huh-7 Lunet cells are derived from a cell clone that was generated by "curing" Huh-7 replicon cells with a selective drug.[14] Cells were cultured as described before.[15] The U2OS cell line with inducible HCV expression (UHCVcon57.3) was kindly provided by Prof. D. Moradpour (University of Lausanne, Switzerland).

Compounds

Enviroxime was kindly provided by Prof. G. Pürstinger (University of Innsbruck, Austria). BF738735 was provided by Galapagos NV. IFN-α 2a was purchased from Roche.[16] VX-222, BMS-790052 and LY294002 were purchased from Selleck Chemicals (Nuclilab, The Netherlands). VX-950 and 2'-*C*-methylcytidine were synthesized as reported.[15] PIK93 was purchased from Sigma and itraconazole and 25-hydroxycholesterol were purchased from Sanbio. AL-9 was a generous gift from Prof. R. de Francesco (University of Milan, Italy). Compound stock solutions were prepared in DMSO.

Antiviral assay with HCV subgenomic replicon cells of different genotypes

Antiviral assays were performed as described previously.[15] Briefly, cells were seeded at a density of $5x10^3$ cells per well in a 96-well cell culture plate in complete DMEM.

Following incubation of 24h at 37°C, serial dilutions of the test compounds in complete DMEM were added in a total volume of 100 µL. For Huh 9-13 replicon cells and GT4a replicon cells, replicon RNA levels were determined by a reverse transcription quantitative polymerase chain reaction (RT-qPCR). For pH/SG-neo(L + I) replicon cells, luciferase activity was determined by the Renilla Luciferase assay system (Promega, The Netherlands) according to manufacturer's instructions. For the GT2a replicon cells and Huh 5-2 replicon cells, luciferase activity was determined after 3 days of incubation by using the Steady-Glo luciferase assay system (Promega). The 50% effective concentration (EC_{50}) was determined using logarithmic interpolation. To determine the cytotoxicity of the compounds, cytostatic assays were performed as described previously.[15] Briefly, cells were seeded at a density of $5x10^3$ cells per well in a 96-well cell culture plate in complete DMEM. After 24h of incubation at 37°C serial dilutions of the test compounds in complete DMEM were added. After 3 days of incubation at 37°C, cell viability was determined using the MTS/PMS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine methosulfate] method (Promega). The 50% cytotoxic concentration (CC_{50}) was calculated using logarithmic interpolation.

Resistance selection

The selection of drug-resistant subgenomic HCV replicons was performed as described previously.[17] In brief, approximately $3x10^5$ Huh 9-13 replicon containing cells were seeded in a T25 tissue culture flask in complete DMEM with G418 and under constant antiviral pressure of enviroxime (5 µM) or BF738735 (4 µM). The cells were subcultured when 80% confluence was reached. When replicon containing cells suffered from compound pressure (as a result of a too low level of replicon content),

144 G418 and compound pressure were removed until cells recovered. Sanger
145 sequencing was performed as described previously.[15]
146 **Additional material and methods are posted as Supplemental Information.**

RESULTS

Enviroxime and BF738735 inhibit HCV replication *in vitro*.

We first evaluated the effect of enviroxime and a panel of enviroxime-like inhibitors on *in vitro* HCV replication in HCV subgenomic replicons of different genotypes (GT). BF738735, enviroxime and PIK93 (a PI4KIII inhibitor[18]) inhibited the replication of HCV GT1a, 1b and 4a replicons in a dose-dependent manner (Figure 1A, Table 1). All three molecules proved roughly equipotent with EC_{50} values ranging between 0.1-0.7 µM. At concentrations of 10 µM and lower, enviroxime and BF738735 did not cause notable changes to cell viability. The inhibition of GT2a replicons and of the infectious JFH-1/CS-N6 HCVcc by these compounds was on average 10- to 100-fold less efficient than that of GT1b (Figure 1B). The antiviral activity of BF738735 was also confirmed on primary human hepatocytes infected with HCV GT1b or GT3a serum (EC_{50} of 0.15 and 0.20 µM, respectively) (Figure 1C). Compound treatment had no adverse effect on the cells (Figure 1D).

Resistant replicons acquired mutations in NS4B and NS5A.

To characterize the mechanism of action of enviroxime and BF738735, drug-resistant HCV replicons were selected. These cultures proved to be cross-resistant: the enviroxime^{res} replicon was markedly less sensitive to both enviroxime and BF738735 when compared to wild-type (WT) with EC_{50} values of respectively 15 ± 5 µM and 15 ± 3 µM (Figures 2A and 2C). Likewise, the BF738735^{res} replicon proved markedly less sensitive to both BF738735 (EC_{50} of 8.3 ± 2.7 µM) and enviroxime (EC_{50} of 35 ± 9 µM) (Figures 2B and 2C). Furthermore, both resistant replicons were less susceptible to PIK93 and AL-9, a PI4KIIIα inhibitor,[19] albeit at a lesser extent. HCV direct-acting antivirals of different classes such as polymerase inhibitors (2'-*C*-methylcytidine and VX-222), a protease inhibitor (VX-950) and a NS5A inhibitor

(BMS-790052) retained WT antiviral activity against enviroxime^{res} and BF738735^{res} replicons (Figure 2C).

To study whether the *in vitro* resistance to enviroxime and BF738735 was mediated by the viral genome, RNA isolated from enviroxime^{res}, BF738735^{res} and WT replicon cultures was stably transfected into naïve Huh-7 Lunet cells. As expected, enviroxime proved less effective in the enviroxime^{res} transfected Huh-7 Lunet cultures when compared to the WT transfected cultures (Figure 2D). The protease inhibitor VX-950 was equipotent in both WT and enviroxime^{res} cell lines. The resistance to enviroxime and BF738735 is thus at least partially associated with the viral genome.

To evaluate the contribution of changes of the host cells to the resistant phenotype, several attempts were undertaken to stably transfect enviroxime^{res} replicon cells that were cleared from the resistant replicon with WT HCV replicon RNA, but these attempts were not successful. Sanger sequencing of both drug-resistant replicons identified two mutations in the NS4B gene (V38M and D167E) and one to two mutations in the NS3 gene (Table S1). Only 2 predominant mutations were shared by both resistant replicons, i.e. the two NS4B mutations. Interestingly, we also observed a large diversity in the NS5A genes of these resistant replicons (Table S2); most of these mutations were however not predominant.

Contribution of the identified mutations to the resistant phenotype

Since the majority of identified mutations clustered in the NS5A region, the entire NS5A gene of the BF738735^{res} genome was swapped into a WT replicon backbone (Table S2). BF738735 and enviroxime proved equally potent in replicons that contained a BF738735^{res} NS5A sequence compared to replicons that contained the WT NS5A sequence (Figure 2E).

Single mutations were introduced into a WT genotype 1b replicon backbone by site-directed mutagenesis. None of the introduced mutations altered the sensitivity to enviroxime or BF738735, nor to the reference HCV inhibitors (Figure 2E). Of note, antiviral assays with the NS4B mutant D167E and all double and triple mutants containing the D167E mutation could not be performed because the luciferase signal obtained with these mutants was too low (equal to background luciferase signal). Next, the replication fitness of each mutant was studied and compared to that of WT replicon (Figure 2F). The replicative capacity of mutants M581L (NS3), K583E (NS3), V38M (NS4B), and D389N (NS5A) was comparable or better than that of WT replicon. In contrast, replicon carrying the D167E mutation in NS4B had a pronounced reduction in replication fitness as compared to the WT (12%). The replication fitness of all double and triple mutants containing the D167E mutation was decreased as well (≤11%, Figure 2F), explaining the lack of luciferase signal in the antiviral assays. The luciferase signal of the D167E mutant could not be improved by swapping the BF738735^{res} NS5A gene into its backbone. Resistance to enviroxime and BF738735 thus seemed to rely on a complex combination of mutations conferring resistance and probably rescuing fitness costs, which could not be reconstituted by single mutations or combinations of two or three individual mutations.

A resistant PI4KIIIβ mutant cannot rescue HCV replication in the presence of enviroxime, BF738735 or PIK93

It was previously shown that both enviroxime and BF738735 inhibit the kinase activity of PI4KIIIα and PI4KIIIβ *in vitro*, with the β isoform being 30 to 300-fold more potently inhibited than the α isoform.[10] We therefore studied the dependence of the replication of the HCV Con1 replicon on PI4KIIIβ by siRNA knockdown. The knockdown

efficiency of PI4KIIIα and PI4KIIIβ at mRNA level was ~70-85% (Figure 3A). Knockdown was not toxic to the cells as verified by microscopy. WT HCV replicon replication was reduced by 75% upon PI4KIIIα knockdown, whereas knockdown of PI4KIIIβ did not significantly decrease HCV RNA levels (Figure 3A).

For the enterovirus Coxsackievirus B3 (CVB3) it was shown that overexpression of a PI4KIIIβ mutant (Y583M) resistant to PI4KIIIβ inhibitors resulted in almost complete protection of CVB3 replication against the inhibitory effect of BF738735.[10] To study whether this was also the case for HCV replication, a similar replication rescue assay was performed. The overexpression of WT or resistant PI4KIIIβ did not affect the replication of the subgenomic replicon in untreated conditions (data not shown). For none of the concentrations and compounds studied (Figure 3B, Figure S1), the inhibitory effect on HCV replication could be negated by the overexpression of the resistant PI4KIIIβ-Y583M mutant, which is in contrast to what has been observed for enteroviruses.[10,20] These data suggest that PI4KIIIβ is not the major target of enviroxime and BF738735 for the inhibition of HCV replication and that this is also the case for the PI4KIIIβ inhibitor PIK93.

Enviroxime and BF738735 do not affect HCV-induced PI4P pools

As PI4KIIIβ is likely not the major target for the anti-HCV activity of the enviroxime and BF738735, we investigated PI4KIIIα as a secondary target. It was shown earlier that PI4KIIIα is responsible for the increased PI4P pools at the HCV replication complexes.[3] Therefore, the effect of both molecules on PI4P pools inside the cell and at the plasma membrane was assessed in U2OS cells expressing HCV proteins. This approach allowed us to study the effect of molecules on PI4P production without having an effect on viral replication. When the expression of HCV proteins in U2OS cells was induced, the intracellular level of PI4P lipids markedly increased (Figure

4A). A portion of the PI4P foci colocalized with NS5A, as reported previously[2]. Treatment with enviroxime or BF738735 did not result in an inhibitory effect on these intracellular PI4P pools (Figure 4A). In HCV Con1 replicon cells treated for 4h, only a slight decrease was observed in concentrations that exceeded the EC_{50} values by 25- to 50-fold (Figure S2). In contrast, both compounds inhibited the production of PI4P pools present at the perinuclear Golgi region in U2OS cells not expressing HCV proteins (Figure 4A). This steady state pool of PI4P in the Golgi complex is partially maintained by PI4KIIIβ.[21]

The PI4P lipids present on the plasma membrane are also generated by PI4KIIIα.[22] The PI4P levels in the plasma membrane in the untreated cells were increased when HCV proteins were expressed (- tetracycline condition) (Figures 4B-4C). Treatment of the HCV-U2OS cells with enviroxime or BF738735 did not alter the plasma membrane PI4P pools when compared to the untreated cells, thus suggesting that the molecules do not inhibit PI4KIIIα at the concentrations used in this experiment (25- to 50-fold EC_{50}) (Figure 4C). In contrast, when the expression of HCV proteins was suppressed by tetracycline, treatment with enviroxime and BF738735 resulted in a very moderate increase in PI4P levels (1.5-2 fold increase).

NS5A phosphorylation is inhibited only by high concentrations of enviroxime

PI4KIIIα was also shown to regulate the phosphorylation status of NS5A.[4] The effect of enviroxime and BF738735 on NS5A phosphorylation was therefore studied upon HCV JFH-1 or Con1 expression. For enviroxime, modest dose-dependent effects on NS5A phosphorylation were observed for both JFH-1 and Con1; however, these effects were only significant at high concentrations (5-10 μM) (Figure 4D, Figure S3). Treatment with BF738735 did not markedly affect the phosphorylation status of

NS5A. These data, together with the PI4P staining results, suggest that PI4KIIIα is not the main target for the anti-HCV activity of enviroxime and BF738735.

OSBP is not the target of the anti-HCV activity of enviroxime and BF738735

Some enviroxime-like inhibitors inhibit enterovirus replication by targeting oxysterol-binding protein (OSBP) instead of PI4KIIIβ. These molecules are cross-resistant with PI4KIIIβ inhibitors.[23] As the replication of HCV also requires OSBP,[24] we explored whether this protein could be the target of the anti-HCV effect of enviroxime and BF738735. To this end, we evaluated the anti-HCV activity of 25-hydroxycholeterstol (25-HC) and itraconazole. 25-HC is a ligand of OSBP and was shown to inhibit enterovirus replication *in vitro.*[23] 25-HC and itraconazole were able to inhibit HCV replication in a dose-dependent manner (Figures 5A-B). Both OSBP-targeting molecules were not cross-resistant with enviroxime and BF738735, as they retained WT activity against the resistant replicons.

The broad-spectrum PI3K inhibitor LY294002 is cross-resistant with enviroxime

Most kinase inhibitors target a broad range of cellular kinases. For example PIK93, commonly used as a PI4KIIIβ inhibitor, also inhibits several PI3Ks including class I PI3Ks, PI3KC2β and hsVPS34.[18] Targets other than PI4KIIIα/β could thus contribute to the inhibitory effect of enviroxime and BF738735 on HCV replication. Therefore, we studied the anti-HCV effect of a broad-spectrum PI3K inhibitor, LY294002. LY294002 inhibited *in vitro* HCV replication in a dose-dependent manner (Figure S4, EC_{50} = 2.2 ± 0.3 µM). The cytotoxic effect of this molecule was more pronounced in our replicon model than previously described.[25] Interestingly, the enviroxime- and BF738735-resistant replicons were markedly less sensitive to the effect of LY294002 (fold resistance of 10 ± 0.4, Figure 6A). Next, the effect of enviroxime and BF738735 in *in vitro* PI3K enzyme assays was evaluated (Figure 6B). PI4KIIIβ was included as

a positive control. Both compounds inhibited a wide range of PI3Ks at the tested concentration (10 µM), but none of these PI3Ks were completely inhibited. Furthermore, the profile of PI3K inhibition was not completely overlapping between enviroxime and BF738735, which could suggest off-target inhibition by the compounds.

DISCUSSION

Enviroxime and enviroxime-like inhibitors such as BF738735 were previously identified as inhibitors of rhinoviruses and enteroviruses in cell culture. We here report that enviroxime and BF738735 inhibited the *in vitro* replication of HCV subgenomic replicons of different genotypes (GT1a, 1b, 4a). The sensitivity of the GT2a replicon to the compounds was markedly less pronounced and so was the sensitivity of the infectious HCV JFH-1 strain. This is in line with a previous report, in which PIK93 was described to be devoid of antiviral activity against HCV GT2a subgenomic replicons and against the infectious GT2a virus.[26]

Resistant HCV GT1b replicons against enviroxime and BF738735 were found to be cross-resistant. Mutations were identified in NS3, NS4B and NS5A. These mutations were not identical to the mutations identified in HCV replicons resistant to PI4KIIIα inhibitors (4-anilino quinazoline and thiazolyldihydroquinazoline), but the complexity of mutations is comparable.[19,27] By transfecting the isolated RNA from resistant replicon cultures into naïve hepatoma cells, we could demonstrate that the resistant phenotype was indeed associated with the viral genome. Single mutations or combinations of two or three individual mutations could however not reconstitute the resistant phenotype. A similar observation was reported with the PI4KIIIα inhibitor compound A, for which the replication of the NS4B and NS5A mutants could not be rescued in WT Huh-7 cells.[27] It is thus possible that adaptions of the host cells contributed as well to the resistant phenotype. This was previously reported for another class of host-targeting antivirals, i.e. statins.[28] Unfortunately, we did not succeed to transfect wild-type HCV replicon RNA into enviroxime^{res} replicon cells that were cleared from the resistant replicon.

The cellular target of the anti-enterovirus activity of enviroxime and BF738735 has been demonstrated to be the lipid kinase PI4KIIIβ.[11] BF735738 and enviroxime also inhibit PI4KIIIα, albeit roughly 10-fold less potently than PI4KIIIβ.[10] As PI4KIIIα is an essential host factor for HCV replication, it was therefore assumed that the *in vitro* inhibition of HCV replication by these compounds was caused by the inhibitory effect on PI4KIIIα. However, no decrease in PI4P levels was observed following compound treatment in a cell line with inducible expression of the HCV polyprotein (HCV-U20S). In addition, the phosphorylation status of NS5A was not markedly affected by these compounds, providing evidence that PI4KIIIα is likely not the main target for the anti-HCV activity of enviroxime and BF738735. Likewise, PI4KIIIβ did not seem to be the main target either, as indicated by siRNA knockdown experiments and replication rescue experiments with a resistant PI4KIIIβ mutant. This is in clear contrast to what has been described for the anti-enterovirus activity of these compounds.[10] On the other hand, some enviroxime-like inhibitors do not target PI4KIIIβ, but inhibit the oxysterol-binding protein (OSBP) instead.[23] These inhibitors are cross-resistant with PI4KIIIβ-targeting inhibitors as to what concerns their anti-enterovirus activity. However, our data showed that OSBP is not involved in the mechanism of action of the anti-HCV activity of enviroxime and BF738735.

Most kinase inhibitors target a broad range of cellular kinases, which may also be the case for enviroxime and BF738735. PIK93 for example, which is designated as a selective PI4KIIIβ inhibitor, also inhibits several PI3 kinases.[18] AL-9, a PI4KIIIα inhibitor, also inhibits PI3K p110α *in vitro* at concentrations comparable to those needed to inhibit type III PI4-kinases.[19] We found that LY294002, a broad-spectrum PI3K inhibitor, inhibited HCV replicon replication and that this compound was cross-resistant with enviroxime and BF738735. LY294002 has no inhibitory effect on

PI4Ks; however, it does inhibit other kinases. In addition, enviroxime and BF738735 were able to inhibit a wide range of PI3Ks in *in vitro* kinase assays. None of these PI3Ks was completely inhibited and the inhibition profiles of enviroxime and BF73875 were not completely overlapping. Together, these data provide evidence that PI3Ks may be the target of the anti-HCV activity of these compounds. The role of PI3K in HCV replication has been described previously. PI3K was shown to enhance HCV replication through the activation of SREBPs.[29] The transcriptional activities of SREBPs were shown to be increased by the HCV NS4B protein and this upregulation required PI3K activity.[30] Furthermore, LY294002 was shown to inhibit the transactivation of SREBP-2 in cells expressing NS4B.[30]

In summary, we here report on the *in vitro* anti-HCV activity of enviroxime and BF738735. Although these molecules were previously shown to inhibit enterovirus replication through the inhibition of PI4KIIIβ, our data indicate that this particular lipid kinase as well as the PI4KIIIα isoform are not the major targets for the anti-HCV activity of these molecules. Instead, we show that both molecules inhibit several PI3 kinases *in vitro*. HCV replicons resistant to the antiviral effect of enviroxime and BF738735 could bypass the PI3K inhibition by acquisition of mutations in its genome. Therefore, both the antiviral target as well as the resistance mechanism are different from those of enteroviruses, for which enviroxime-resistant variants can circumvent the need of PI4KIIIβ.

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TRANSPARENCY DECLARATIONS

None to declare.

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FIGURE LEGENDS

Figure 1. Antiviral activity of enviroxime and BF738735 in different *in vitro* HCV culture systems. (A) Effect of enviroxime and BF738735 on HCV replicon replication in Huh 5-2 GT1b replicon containing cells and on the proliferation of exponentially growing cells. Data are mean values ± SD for at least three independent experiments. AA: antiviral activity, Tox: toxicity. **(B)** Antiviral activity of enviroxime, BF738735 and VX-950 against JFH1/CS-N6 HCVcc infection on Huh 7.5.1 cells. Data shown are average values ± SD of at least three independent experiments. **(C)** Antiviral efficacy of BF738735 on primary human hepatocytes infected with HCV GT1b or GT3 isolates. Data shown are average values ± SD of 2 independent experiments. **(D)** Effect of BF738735 on the total RNA content of HCV infected primary human hepatocytes. Data shown are average values ± SD of 2 independent experiments.

Figure 2. Phenotype of HCV replicons resistant to enviroxime and BF738735. (A) Dose-response curves for the inhibition of HCV replication by enviroxime in WT replicon or in enviroxime-resistant replicon cells. Data are mean values ± SD for at least three independent experiments. **(B)** Dose-response curves for the inhibition of HCV replication by BF738735 in WT replicon or in BF738735-resistant replicon cells. Data are mean values ± SD for at least three independent experiments. **(C)** Fold resistance values for BF738735, enviroxime and a panel of reference compounds in enviroxime^{res} replicon cells and in BF738735^{res} replicon cells. Fold resistance values were calculated as the ratio of the EC_{50} in resistant replicon to the EC_{50} in WT replicon. **(D)** Dose-response curves for inhibition of HCV replication by enviroxime or VX-950 in Huh-7 Lunet cells stably transfected with RNA isolated from WT or enviroxime^{res} replicon cells. Data are mean values ± SD for at least three

494 independent experiments. **(E)** Fold resistance values for enviroxime, BF738735, VX-
495 950 and BMS-790052 in Huh-7 Lunet cells transiently transfected with mutant
496 replicon RNA. Fold resistance values were calculated as the ratio of the EC_{50} in
497 resistant replicon to the EC_{50} in WT replicon. Data are mean values ± SD for at least
498 three independent experiments. **(F)** Replication fitness of the different mutant
499 replicons. Values shown are expressed as a percentage of WT value at 72h post
500 transfection and are mean values ± SD for at least two independent experiments. *,
501 $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (unpaired Student's t-test).

502 **Figure 3. HCV replication depends on PI4KIIIα in both WT and resistant**
503 **replicon cells. (A)** Ratio of mRNA levels of HCV, PI4KIIIα and PI4KIIIβ as compared
504 to untreated control cells 48h post siRNA transfection in WT Huh 9-13 cells replicon
505 cells following transfection with scrambled siRNA, siPI4K3A or siPI4K3B. Data shown
506 are average values ± SD of at least three independent experiments. Data were
507 analyzed with the two-tailed Student's t-test. **, $p<0.005$; ***, $p<0.001$. **(B)** Huh-7
508 Lunet cells were electroporated with HCV replicon RNA and seeded in a 96-well
509 plate in the presence of 0.05 µM of enviroxime, BF738735 or PIK93. The cells were
510 transfected with PI4KIIIβ-WT, PI4KIIIβ- Y583M, or as negative controls the kinase-
511 dead PI4KIIIβ-D656A or EGFP. Luciferase activity was determined 72h post
512 electroporation. Data shown are mean values ± SD of three independent
513 experiments.

514 **Figure 4. Enviroxime and BF738735 do not affect PI4P pools in cells expressing**
515 **HCV proteins nor NS5A phosphorylation. (A-C)** Osteosarcoma cells with inducible
516 HCV expression were treated with enviroxime or BF738735 in the presence or
517 absence of tetracycline for 48h. PI4P pools (in red) were visualized by
518 immunofluorescence intracellularly (A) or at the plasma membrane (B). HCV NS5A

expression is shown in green, cell nuclei in blue. (C) Quantification of the PI4P fluorescence measured in the plasma membrane with Cell Profiler software. Values shown are integrated intensity values per individual cell. Lines represent the mean value ± SEM. Data shown are integrated intensity values per individual cell. Lines represent the mean value ± SEM. Data were analyzed with the Kruskal-Wallis test using the Graphpad Prism software. **, $p<0.005$; ****, $p<0.0001$; ns, not significant. **(D)** Naïve Huh-7 Lunet cells were transfected with plasmids encoding the NS3 to NS5B polyprotein of JFH-1 WT or Con1 ET. Starting at 7h post-transfection, cells were incubated with indicated concentrations of enviroxime or BF738735. Cell lysates were analyzed by Western blotting using a NS5A specific antibody. Data shown are average values ± SD of 4 independent experiments. Data were analyzed with the unpaired Student's t-test. *, $p<0.05$; **, $p<0.01$.

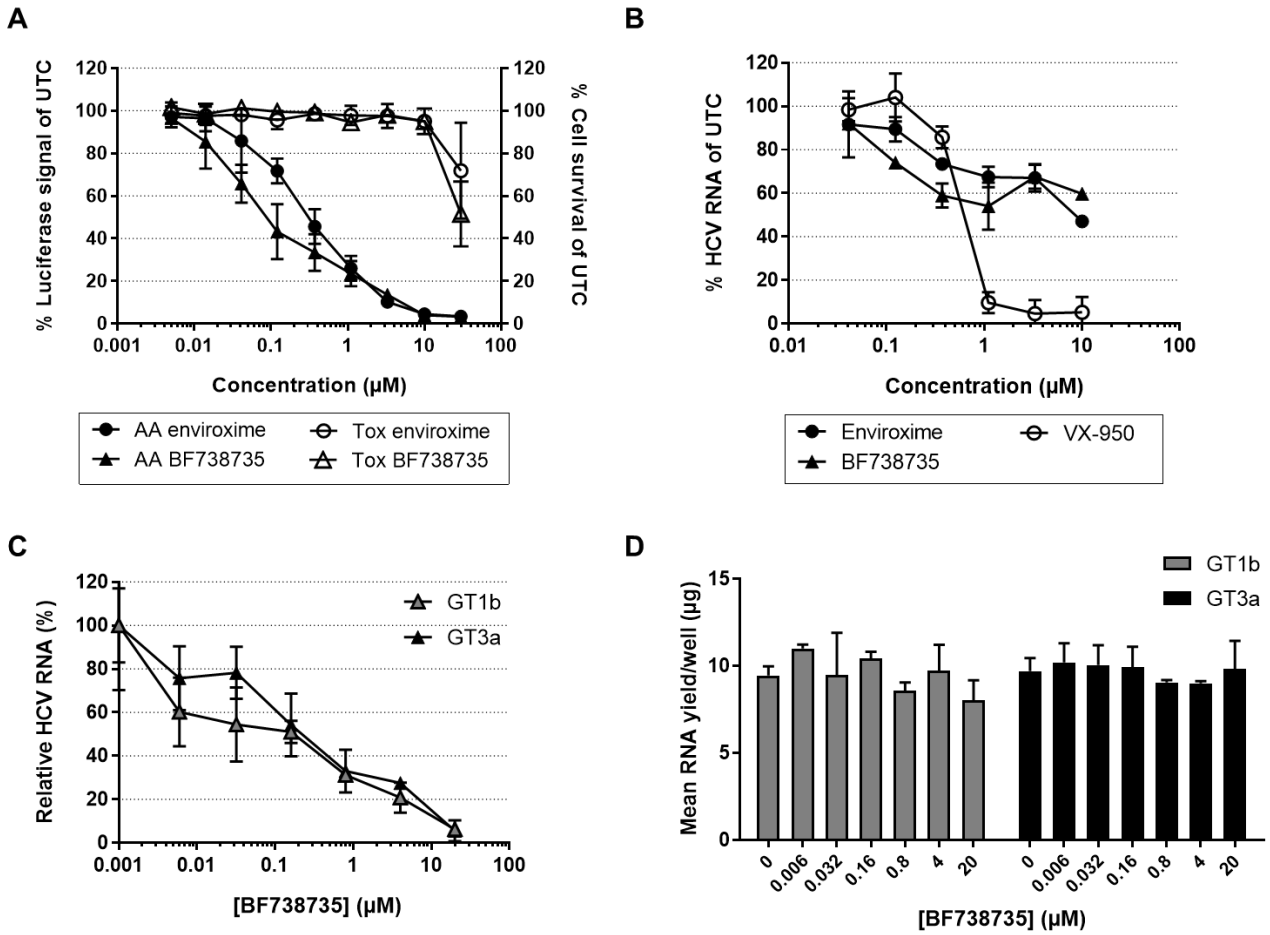
Figure 5. OSBP is not the target of the anti-HCV activity of enviroxime. (A-B) Dose-response curves for the inhibition of HCV replication by 25-hydroxycholesterol (A) and itraconazole (B) in WT replicon, enviroxime-resistant replicon or BF738735-resistant replicon cells. Data are mean values ± SD of at least three independent experiments. **(C)** Effect of 25HC and itraconazole on the proliferation of exponentially growing WT replicon cells. Data are mean values ± SD for two independent experiments.

Figure 6. The broad-spectrum PI3K inhibitor LY294002 is cross-resistant with enviroxime and BF738735. (A) Dose-response curves for the inhibition of HCV replication by LY294002 in WT replicon (black), enviroxime-resistant replicon or BF738735-resistant replicon containing cells. Data are mean values ± SD for at least three independent experiments. **(B)** Inhibition of the *in vitro* enzymatic activity of a

543 panel of PI3 and PI4 kinases by enviroxime and BF738735 at a concentration of 10

544 μM.

545 **Figure 1.**



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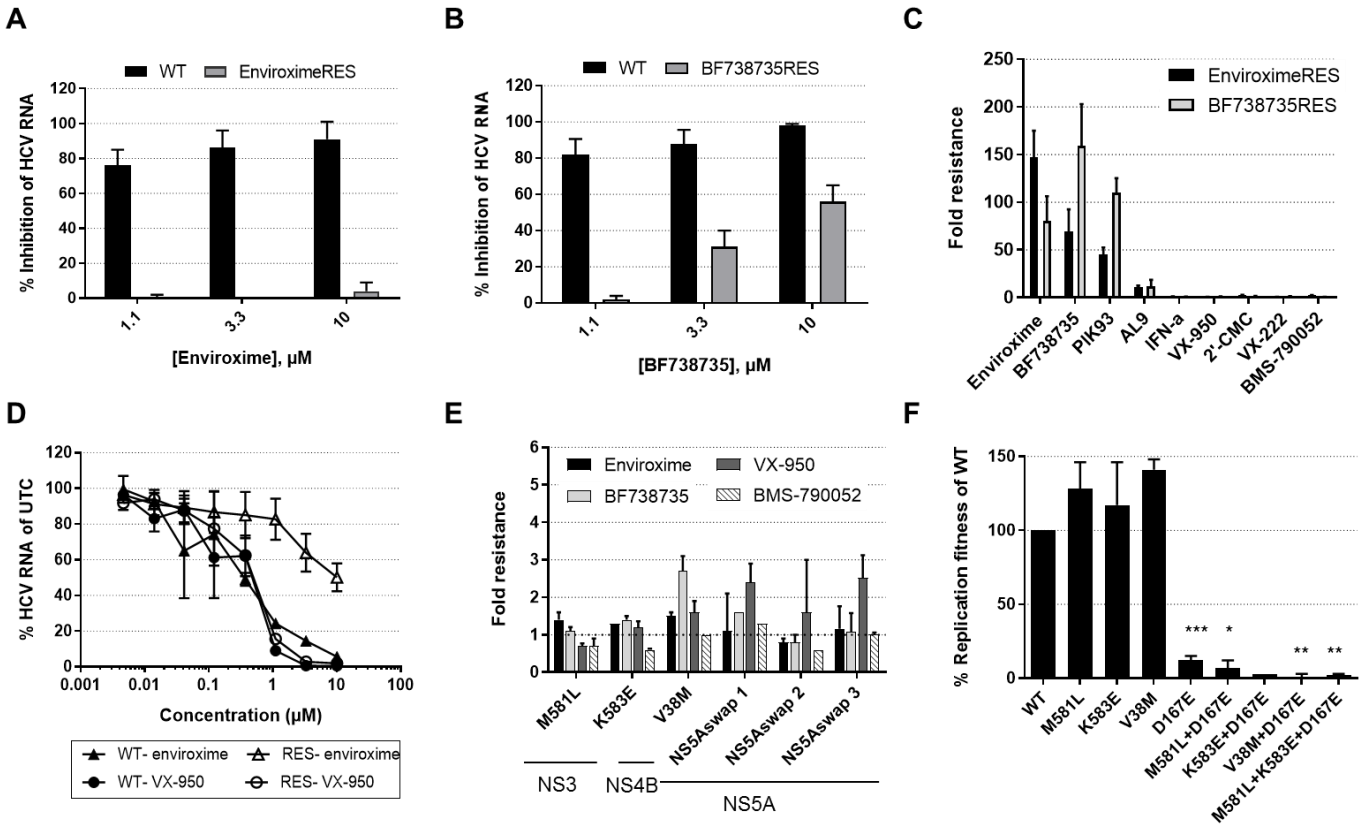
547 **Figure 2.**



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549 **Figure 3.**

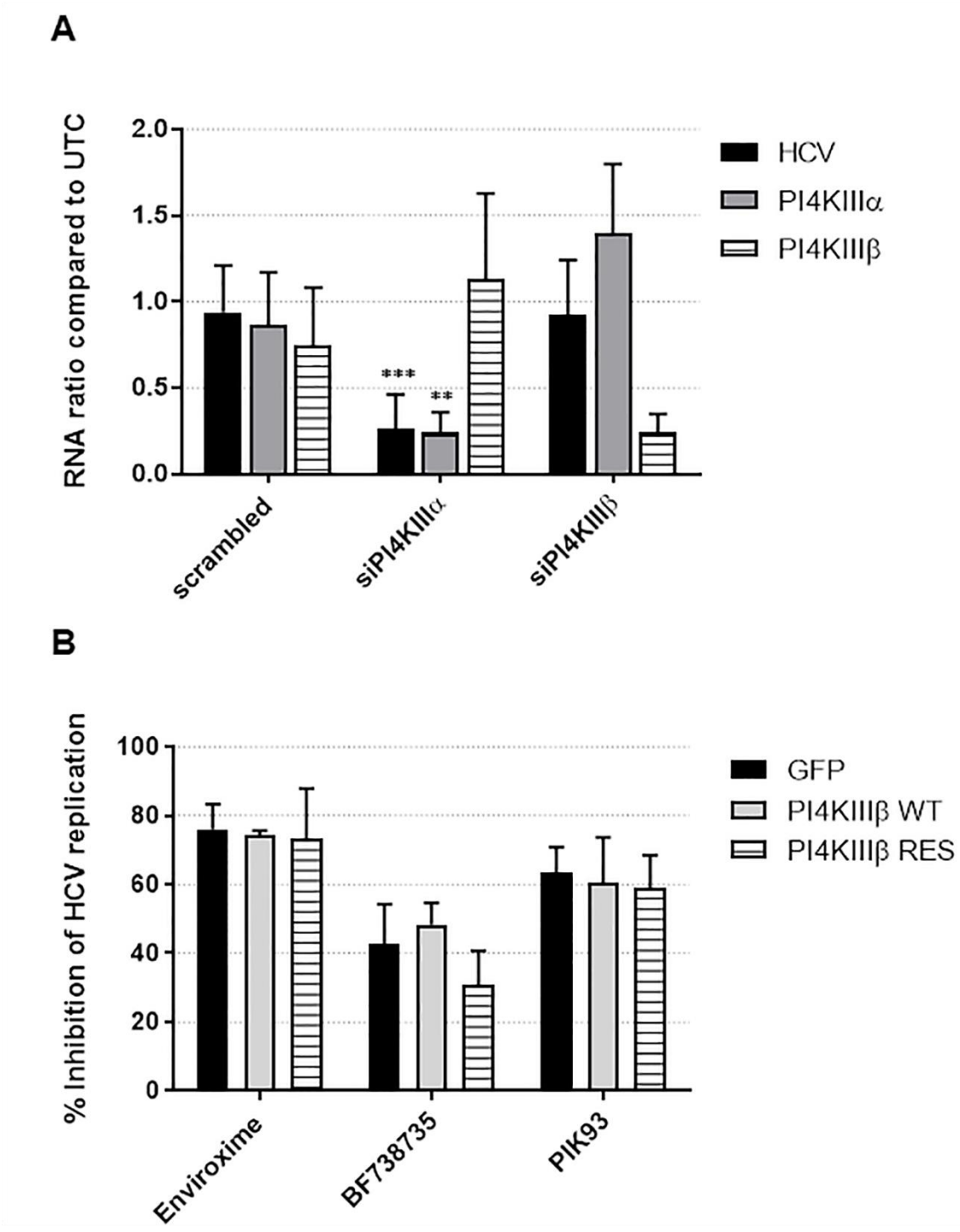

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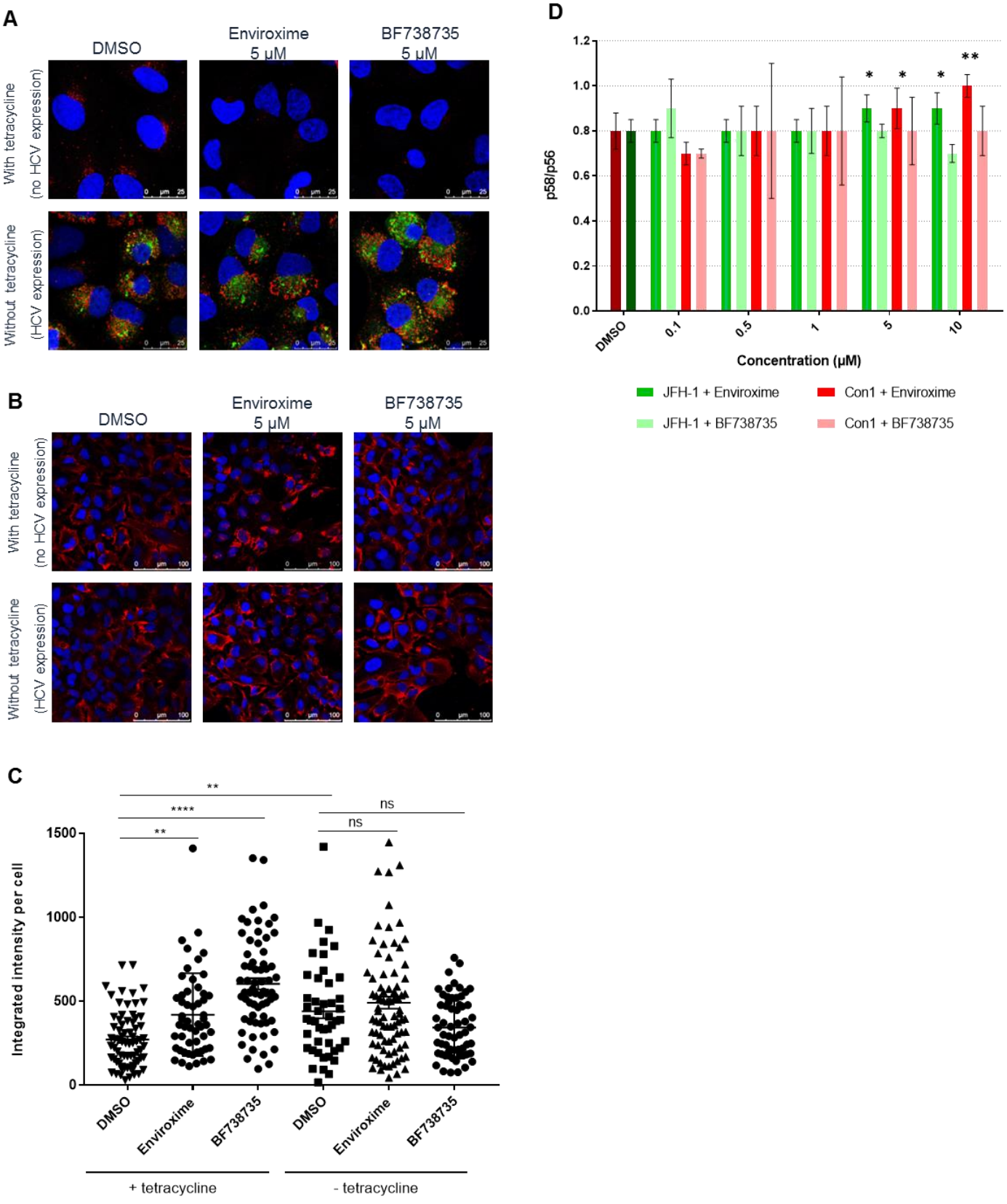
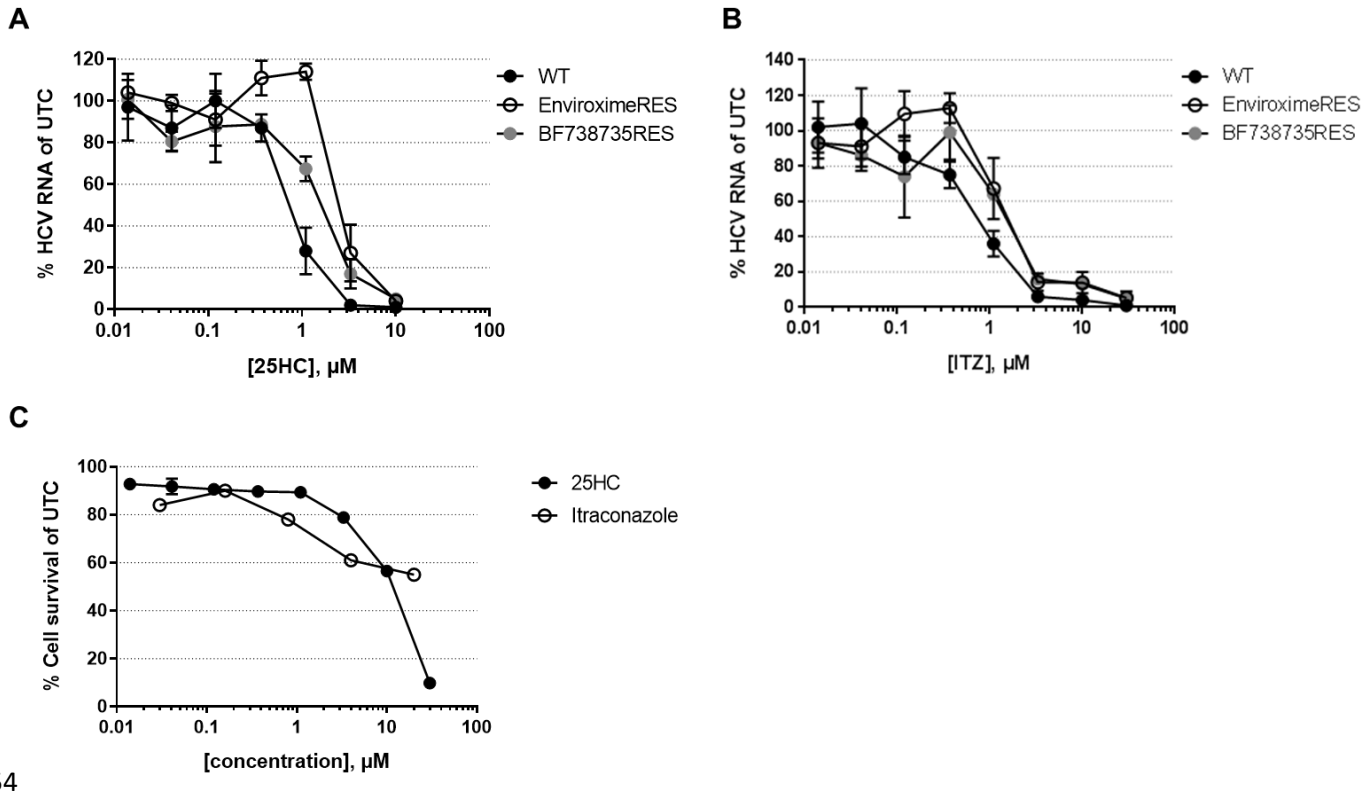
Figure 4.


A
DMSO
Enviroxime 5 µM
BF738735 5 µM
With tetracycline (no HCV expression)
Without tetracycline (HCV expression)
B
DMSO
Enviroxime 5 µM
BF738735 5 µM
With tetracycline (no HCV expression)
Without tetracycline (HCV expression)
C
Integrated intensity per cell
1500
1000
500
0
**
****
**
ns
ns
DMSO
Enviroxime
BF738735
DMSO
Enviroxime
BF738735
+ tetracycline
- tetracycline
D
p58/p56
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
**
DMSO
0.1
0.5
1
5
10
Concentration (µM)
JFH-1 + Enviroxime
Con1 + Enviroxime
JFH-1 + BF738735
Con1 + BF738735

553 **Figure 5.**



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555 **Figure 6.**

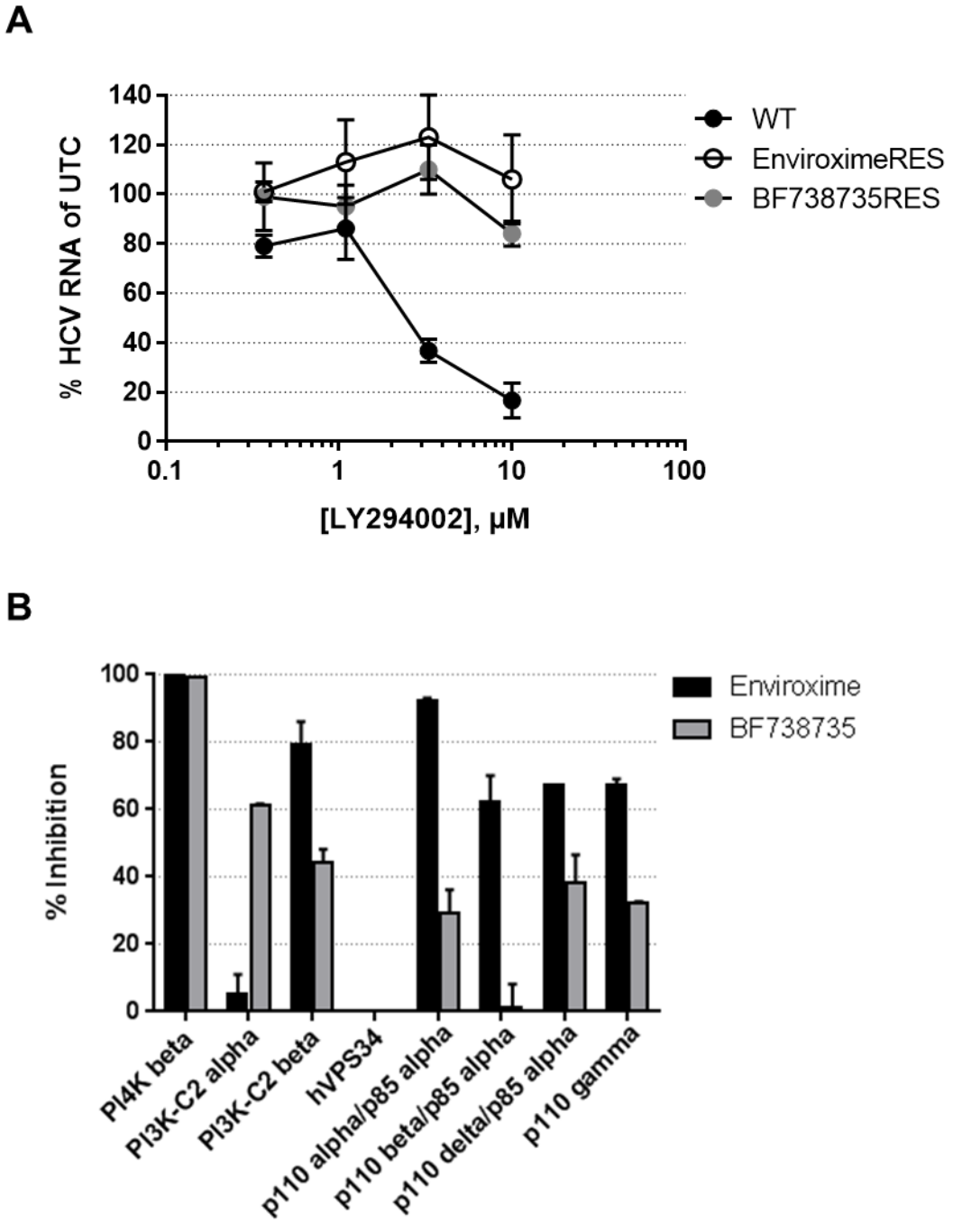

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Table 1. EC_{50} and CC_{50} values of PI4KIII inhibitors and VX-950 against HCV subgenomic replicons of different genotypes.

	EC_{50} (μM)[a]					CC_{50} (μM)[b]
	GT1a	**GT1b *(Huh 5-2)***	**GT1b *(Huh 9-13)***	**GT2a**	**GT4a**	
Enviroxime	0.49 ± 0.07	0.33 ± 0.1	0.22 ± 0.06	2.3 ± 0.8	0.20 ± 0.1	25 ± 2
BF738735	0.15 ± 0.01	0.11 ± 0.07	0.10 ± 0.06	9.3 ± 6	0.30 ± 0.3	> 30
PIK93	0.47 ± 0.1	0.28 ± 0.07	0.17 ± 0.1	5.8 ± 0.5	0.72 ± 0.01	8.1 ± 4
VX-950	nd	0.39 ± 0.1	0.10 ± 0.04	0.53 ± 0.1	4.2 ± 1.4	24 ± 4

[a]Determined by measuring the luciferase signal (GT1a, GT1b Huh 5-2, GT2a, GT4a) or by qRT-PCR (GT1b Huh 9-13).

[b]Determined on Huh 9-13 subgenomic replicon containing Huh-7 cells by the MTS/PMS colorimetric method.

Nd, not determined.

SUPPLEMENTAL INFORMATION

SUPPLEMENTAL MATERIAL AND METHODS

Antiviral assay with infectious HCV on Huh 7.5.1 cells

A total of 5×10^3 Huh 7.5.1 cells per well of a 96-well cell culture plate were incubated with the HCV JFH-1/CS-N6 virus at a MOI of 1.0 and with serial dilutions of compounds. Following 3 days of incubation, medium was removed and HCV RNA was quantified by means of RT-qPCR.

Antiviral assay with infectious HCV on primary human hepatocytes

Primary human hepatocytes (PHH) were isolated from an encapsulated liver sample (donor FT383, female, 37 years, liver resection for hepatic hemangioma) as previously described[1]. After liver perfusion, $1x10^6$ cells/mL per well were plated in 12-well plates pre-coated with type I collagen (Beckton Dickinson) and allowed to attach for 12h. Supernatant containing dead cells and debris was then carefully removed and replaced by 1 mL of serum-free long-term culture medium (Lanford medium, LNF). The number of confluent attached cells was estimated at $2x10^5$ cells/cm^2.

PHH were cultured in 12-well plates with LNF medium at 37°C in a humidified 5 % CO_2 atmosphere from day 1 to day 3 post-seeding. Infections were performed by incubating cells with 30 µL HCV-positive donors S1b and S3a sera in 1 mL LNF/well. Compound was added at the same time with final concentrations of 20 µM, 4 µM, 0.8 µM, 0.16 µM, 0.032 µM and 0.006 µM/ well. Final concentration of DMSO was 0.2 % (v/v). After 16h at 37°C, the inoculum was removed and cells were washed three times with William's E medium. The compound was then added in 1 mL/well of fresh LNF. Seventy-two hours post-inoculation, cells were washed 3 times with PBS and lysed in 800µL of (RNAble, Eurobio). Lysates were stored at -80°C until RNA extraction.

Total cell RNA was isolated using guanidinium isothiocyanate (RNAble, Eurobio) according to the manufacturer's instructions. The concentration of total RNA in cells was determined using a Nanodrop Spectrophotometer ND-1000 (Fisher Scientific SAS, Illkirch, France). RNA quality was determined by the ratios DO_{260}/DO_{280} and DO_{260}/DO_{230}. The quantity of RNA/well (in µg) was calculated and used to estimate the number of attached cells/well. Compounds cytotoxicity was evaluated by the ratio [quantity of RNA for treated cell]/[quantity of RNA for untreated cells], RNA_T/RNA_{UT}. Intracellular HCV RNA copy number was quantified using the SuperScript III Platinium One-Step quantitative RT-PCR system (Invitrogen). The 5′-AGYGTTGGGTYGCGAAAG-3′ and 5′-CACTCGCAAGCRCCCT-3′ primers were used to amplify HCV RNA, and 6-FAM-CCTTGTGGTACTGCCTGA-MGB (Applied Biosystems, Foster City, CA, USA) as internal probe.

Reverse transcription quantitative polymerase chain reaction

Reverse transcription polymerase chain reactions (RT-qPCR) were performed as described previously.[2] Primers used for detection of HCV replicon RNA were: 5'-CCGGCTACCTGCCCATTC-3' (forward primer), 5'-CCAGATCATCCTGATCGACAAG-3' (reverse primer) and 5'-FAM-ACATCGCATCGAGCGAGC ACGTAC-TAMRA-3' (probe). Primers used for JFH-1/CS-N6 virus were: 5′-TGG CGT TAG TAT GAG TGT CGT ACA GCC TCC A-3′ (HCV JFH-1 forward primer), 5′-AAA GGA CCC AGT CTT CCC GGC AAT T-3′ (JFH-1 reverse primer), and 5′-FAM-TGG TCT GCG GAA CCG GTG AGT ACA CC-TAMRA-3′ (JFH-1 probe). For the quantification of PI4KIIIα, PI4KIIIβ, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels, the PI4KIIIα, and PI4KIIIβ TaqMan gene expression assays (Hs01021073_m1, Hs00387380_m1, Life technologies, Ghent, Belgium), and the GAPDH Control kit (Eurogentec) were used, respectively.

The reverse transcription step was performed at 50°C for 30 min, 10 min at 95°C, and subsequent PCR amplification of 40 cycles of denaturation at 95°C for 15s and annealing and extension at 60°C for 45s.

Stable transfection of Huh-7 Lunet cells

Huh-7 Lunet cells were stably transfected with wild-type (WT) Huh 9-13 replicon RNA and enviroxime-resistant replicon RNA using DMRIE-C (Promega). In brief, RNA was extracted from Huh 9-13 cells and enviroxime-resistant replicon cells using the RNeasy mini kit (Qiagen). Huh-7 Lunet cells were seeded at a density of $2x10^5$ cells in a 6-well cell culture plate in complete DMEM. After incubation of 24h, cells were transfected with a mixture of 12 µL DMRIE-C, 1 ml OptiMEM (Gibco, Belgium) and 5 µg of total RNA. After 4 hours, the transfection mixture was removed and complete DMEM was added to the cells. The next day, the cell culture medium was removed and complete DMEM with 1 mg/mL G418 was added. Enviroxime was added at a concentration of 1 µM to the cultures transfected with the enviroxime-resistant HCV RNA. Following four weeks of G418 selective pressure, stable cell lines were obtained.

Swapping of the NS5A gene in a wild-type replicon backbone

RNA was isolated from either wild-type Huh 9-13 or $BF738735^{res}$ subgenomic replicon cells using the RNeasy mini kit (Qiagen). First strand cDNA was synthesized by using the High-Fidelity cDNA transcription kit (Roche Applied Science). Total cellular RNA (4 µg) was gently mixed with 1.2 nmole of random hexamer primer, heated for 10 min at 65°C to denature RNA secondary structures and placed on ice to allow the primer to anneal to the template RNA. Next, the RNA/primer mixture was added to 8.6 µL of reaction mix, which contained 1x transcriptor high fidelity reverse transcriptase reaction buffer, 1 mM deoxynucleotide mix, 5 mM DTT, 20 U Protector RNase inhibitor and 10 U transcriptor high fidelity reverse transcriptase. Following incubation at 55°C for 30

min, the RT reaction was terminated at 85°C for 5 min. Subsequently, the NS5A-containing region was amplified by the Accuprime Pfx polymerase (Invitrogen) using the following primers: forward NS5A primer: 5'-GCGACTTCATTAATGAGGACTGCTCCACGCCA-3' and reverse NS5A primer: 5'-TCAGTGCATTGATGGG CAGCTTGGTTTCCT-3'. The forward NS5A primer induces a recognition site for AseI in front of the NS5A gene. This RE site is necessary for subsequent cloning in the WT Con1 swap plasmid (PI Luc Ase 4-14), kindly provided by Gilead Sciences. The PCR product was purified with the PCR purification kit (Promega). The amplified NS5A gene was digested with AseI and BclI. Following removal of the restriction enzymes by using the PCR purification kit (Promega), the NS5A PCR product was ligated into the WT vector that had been cut with the same restriction enzymes. All constructs were sequenced to verify correct manipulation.

Site-directed mutagenesis

Initially, the NS5B gene was excised from the pFK I389 Lucibineo EI NS3-3'ET construct by SpeI-XhoI restriction digestion and subcloned to construct pCRII-HCV5B, by MluI-NotI to construct pCRII-HCV3-4B and by MluI-XhoI to construct pCRII-HCV5A. Mutations were introduced into these constructs by using the Quickchange® XL II Site-Directed Mutagenesis Kit (Agilent, Stratagene Products, The Netherlands). Primers used for site-directed mutagenesis are shown in Supplementary Table 3. The 50 µL reaction mixture contained 1 x reaction buffer, 50 ng plasmid, 125 ng forward primer, 125 ng reverse primer, 1 µL dNTP mix, 3 µL Quik solution and 2.5 U PfuTurbo DNA polymerase. Thermal cycling was performed as follows: denaturation at 95°C 1 min, 18 cycles of 50s at 95°C, 50s at 60°C and 8 min at 68°C, followed by an elongation step at 70°C for 7 min. Following temperature cycling, the reaction mixture was digested with 10 U DpnI to remove the methylated, non-mutated parental supercoiled

dsDNA template. The mutated NS3, NS4B or NS5A fragment was ligated into pFK I389 Lucibineo EI NS3-3'ET. To confirm the presence of the desired mutations, the entire insert was sequenced.

In vitro transcription and transient transfection

Total DNA isolated with the Wizard Plus SV Miniprep kit from an overnight *E.coli* culture was linearized using *AseI* (New England Biolabs) and *ScaI* (Promega). The linearized plasmid was phenol/chloroform extracted, ethanol precipitated and dissolved in RNase free water. *In vitro* transcription was performed on 5 µg DNA by the RiboMAX™ Large scale RNA production system-T7 (Promega) according to the manufacturer's instructions. RNA was purified and collected by using the RNeasy Mini Protocol for RNA Cleanup (RNeasy Mini kit, Qiagen). The concentration and purity of RNA were measured spectrophotometrically.

Twenty micrograms of *in vitro* transcribed RNA was mixed with 400 µL of a suspension of $4x10^6$ Huh-Lunet cells in a cuvette with a gap width of 0.4 cm (VWR International, Leuven, Belgium). After one pulse at 1600V with an ECM 830 Electro Square Porator™ (BTX Harvard Apparatus), cells were immediately transferred into 20 ml of complete Dulbecco's modified Eagle medium. One hundred µL aliquots of the cell suspension were seeded in a 96-well plate (Falcon) previously filled with serial dilutions of the test compounds in complete Dulbecco's modified Eagle medium. Cells were allowed to proliferate for 3 days at 37°C, after which the luciferase activity was determined using the Steady-Glo luciferase assay system (Promega); the luciferase signal was measured using a Luminoskan Ascent (Thermo, Finland). The 50% effective concentration (EC_{50}) was defined as the concentration of inhibitor that reduced the luciferase signal by 50% when compared to non-treated transfected cells.

Replication fitness

Transfections were performed in Huh-7 Lunet cells as described above with the exception that cells were transfected with 5 µg RNA and 5 µg tRNA (Sigma Aldrich, Belgium). Transfected cells were immediately transferred to 24 mL of complete Dulbecco's modified Eagle medium and a 2 ml aliquot of the cell suspension was added to a 6-well plate (Iwaki). Cells were collected 4h (normalization point) and 3 days after transfection to compare luciferase values with wild-type (WT) values, after extraction of the GND background signal. Cells transfected with the GND replicon [a replication-deficient subgenomic replicon encoding a GDD to GND mutation in NS5B][3] were used as a negative control reflecting background activity from the residual input RNA.

Immunofluorescence microscopy

PI4P lipids were stained as described previously.[4] Briefly, cells were fixed in 4% PFA for 15 min and permeabilized with 20 µM digitonin for 5 min. For staining of PI4P pools in the plasma membrane, following fixation with PFA the cells were chilled on ice and permeabilized with 0.5% saponin. Primary antibodies were incubated in 1% BSA for 1h at RT. PI4P was stained using monoclonal mouse IgM anti-P4P antibody (Echelon, Z-P004) with a final concentration of 2.5 µg/mL. NS5A was detected by using a NS5A-specific monoclonal rabbit antibody (generous gift from Prof. Bartenschlager).

NS5A phosphorylation

NS5A phosphorylation experiments were performed as described previously.[5] In brief, naïve Huh7-Lunet cells were transfected with plasmids encoding the NS3 to NS5B polyprotein of JFH-1 WT or Con1 ET. Starting at 7h post-transfection, cells were incubated with indicated concentrations of enviroxime or BF738735. For Western blotting 1/10 cells of a T25 cell culture flask were denatured and heated in 2x Laemmli-

buffer and loaded onto an 8% polyacrylamide-SDS gel. After separation and transfer to a PVDF membrane, immunoblotting was performed detecting NS5A using a monoclonal mouse antibody (9E10) at a concentration of 0.1 µg/mL and β-actin with a monoclonal mouse antibody (Sigma, A5441) and a dilution of 0.5 µg/mL. LI-COR secondary antibodies conjugated to IRDye were used 1:10000. Bound antibodies were detected with the LI-COR Infrared Imaging System. NS5A p58 and p56 bands were analyzed by Integrated Density function of ImageJ using equally sized ROIs and background substraction for each lane.

siRNA knockdown of PI4KIIIα or PI4KIIIβ in HCV replicon cells

Cells were seeded at a density of $1x10^4$ cells per well in a 96-well cell culture plate in complete DMEM. Following incubation of 24h at 37°C, 10 nM of siRNA was transfected in the cells using siTran transfection reagent (Origene) according to manufacturer's instructions. 48h post-transfection, mRNA levels of the targeted genes and HCV replicon RNA levels were determined by qRT-PCR (*cf. supra*) and normalized to GAPDH mRNA levels. Scrambled siRNA was obtained from Invitrogen, siGenome Smartpool siRNAs for PI4KIIIα and PI4KIIIβ were purchased from ThermoScientific.

PI4KIIIβ rescue experiment

Wild-type PI4KIIIβ and wortmannin-resistant PI4KIIIβ-Y583M plasmids were kindly provided by Prof. F. van Kuppeveld (University of Utrecht, The Netherlands). Twenty micrograms of *in vitro* transcribed RNA was mixed with 400 µL of a suspension of $4x10^6$ Huh-7 Lunet cells in a cuvette with a gap width of 0.4 cm (VWR International, Leuven, Belgium). After one pulse at 1600V with an ECM 830 Electro Square Porator™ (BTX Harvard Apparatus), cells were immediately transferred into 10 mL of complete Dulbecco's modified Eagle medium. Fifty µL aliquots of the cell suspension

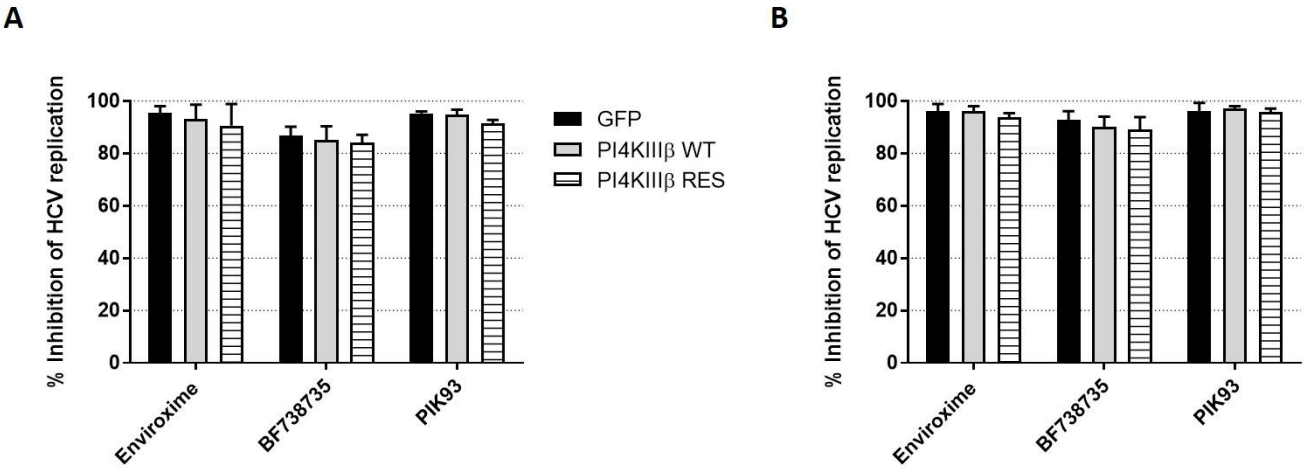
were seeded in a 96-well plate (Falcon) previously filled with serial dilutions of the test compounds in complete Dulbecco's modified Eagle medium. Cells were allowed to adhere for 4h at 37°C. Next, 200 ng of the PI4KIIIbeta plasmids was transfected in the cells using TransIt-LT1 transfection reagent (Mirus Bio) according to manufacturer's instructions. A plasmid expressing GFP was used as a control for transfection efficiency. Three days post-transfection, the GFP signal was checked with fluorescence microscopy. Cells were then lysed to measure the luciferase signal by using the Steady-Glo luciferase assay system (Promega).

PI3K and PI4K enzymatic assays

The inhibitory activity of enviroxime and BF738735 (10 µM) against PI4KIIIβ, PI3K-C2α, PI3K-C2β, hVPS34, p110α/p85α, p110β/p85α, p110δ/p85α and p110γ was evaluated in *in vitro* enzymatic assays by the Adapta technology (Life Technologies) at the apparent K_m value of ATP (with the exception of PI3K-C2β for which the ATP concentration was 10 µM). The assay was performed in duplicate.

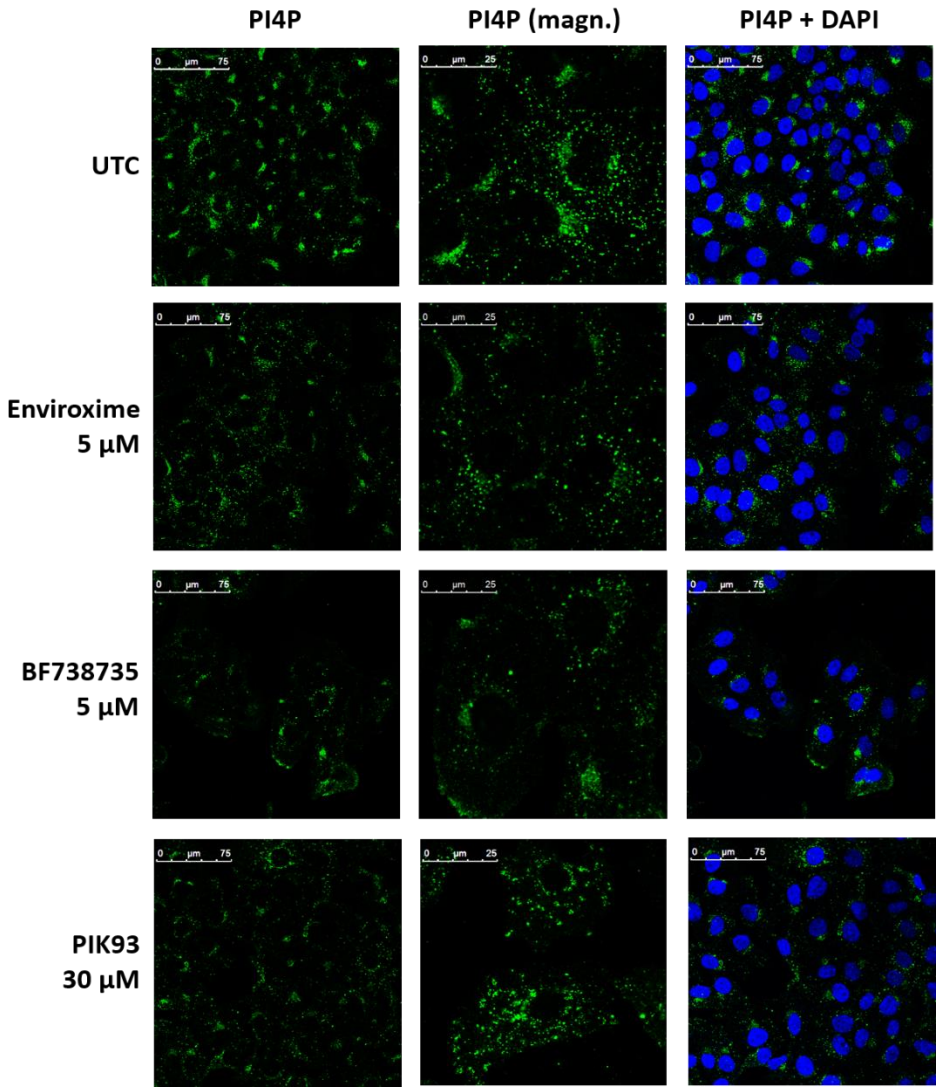
SUPPLEMENTAL FIGURES

Supplemental Figure 1. A resistant PI4KIIIβ mutant cannot rescue HCV replication in the presence of enviroxime, BF738735 or PIK93.



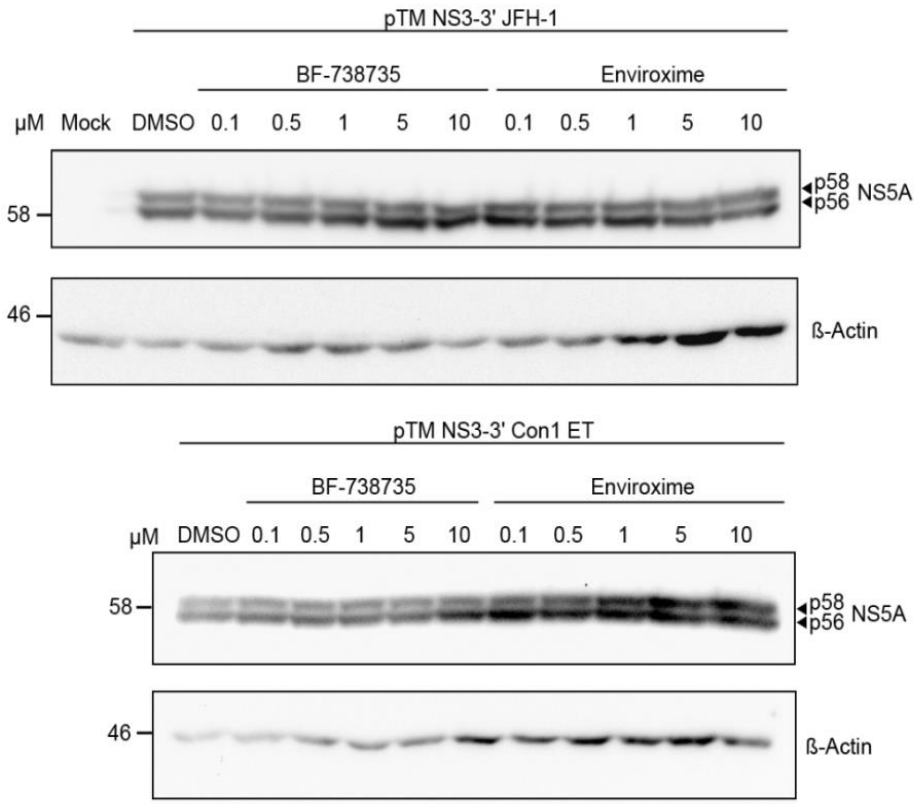
Huh-7 Lunet cells were electroporated with HCV replicon RNA and seeded in a 96-well plate in the presence of **(A)** 0.5 µM and **(B)** 5 µM of enviroxime, BF738735 or PIK93. Following 4h of incubation, the cells were transfected with PI4KIIIβ-WT (light grey), PI4KIIIβ-Y583M (striped), or as negative controls EGFP (black). Three days following electroporation, cells were lysed and the amount of luciferase activity was determined in the samples. Data shown are average values ± SD of three independent experiments.

Supplemental Figure 2. Enviroxime and BF738735 do not majorly affect PI4P pools in HCV subgenomic replicon cells.



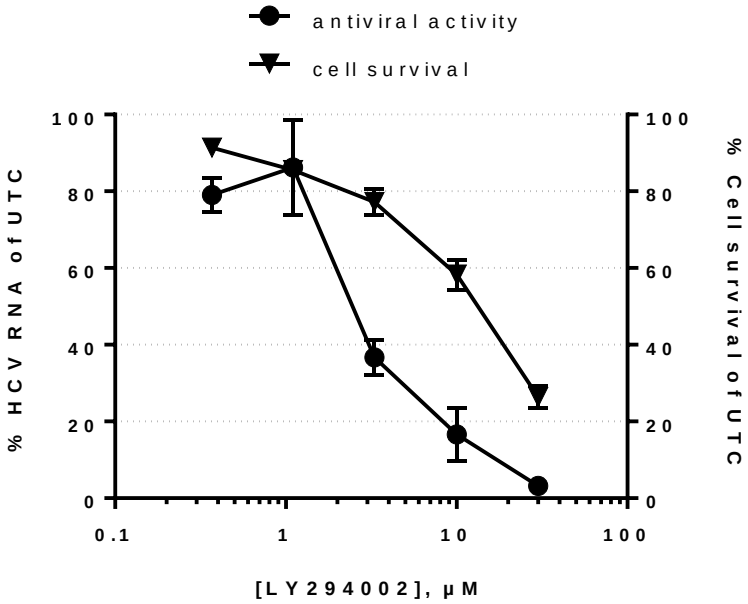
WT Con1 HCV replicon containing cells were treated with enviroxime (5 µM), BF738735 (5 µM) or PIK93 (30 µM) for 4h. Intracellular PI4P pools (in green) were visualized by immunofluorescence. Cell nuclei are shown in blue.

Supplemental Figure 3. NS5A phosphorylation is inhibited only by high concentrations of enviroxime



Naïve Huh-7 Lunet cells were transfected with plasmids encoding the NS3 to NS5B polyprotein of JFH-1 WT or Con1 ET. Starting at 7h post-transfection, cells were incubated with indicated concentrations of enviroxime or BF738735. Cell lysates subjected to immunoprecipitations using NS5A specific antibodies. Immuno-complexes were analyzed by Western blotting.

Supplemental Figure 4. Anti-HCV activity and cytotoxicity of LY294002 on HCV replicon containing cells



Effect of LY294002 on HCV replicon replication in Huh 9-13 GT1b replicon containing cells and on the proliferation of exponentially growing replicon cells. Data are mean values ± SD for at least two independent experiments.

SUPPLEMENTAL TABLES

Supplemental Table 1. Consensus genotype of HCV replicons resistant to enviroxime and BF738735 as determined by Sanger sequencing.

HCV gene	BF738735^{res}	Enviroxime^{res}
NS3	M581L	K583Q
	K583E	
NS4B	V38M	V38M
	D167E	D167E
NS5A	T17A/T	K215R
	N180D/N	I280V
	S306T/S	D292G
	R307G/R	F380F/S
	F380F/S	D389N
	D389N	
	P400S/P	

Supplemental Table 2. Genetic diversity of NS5A observed in HCV replicons resistant to enviroxime and BF738735 and in BF738735^{res} NS5A swap clones.

BF738735^{res}		Enviroxime^{res}		NS5A swap clone 1	NS5A swap clone 2	NS5A swap clone 3
Early passage[a]	*Late passage*[b]	*Early passage*[a]	*Late passage*[b]			
T17A/T	K215K/R	K215R	T56S	T56S	T17A	N180D
N180D/N	I280V/I	I280V	K240Q/K	K240Q	S306T	L183M
S306T/S	D292D/G	D292G	E273D/E	E273D	R307G	E273D
R307G/R	D389N/D	F380F/S	V361A/V	V361A	R318Y	S366P
F380F/S		D389N	F380S/F	F380S	Y321C	F380S
D389N			S382S/R	S382R	A387V	S382R
P400S/P				A440T	D389N	
					P400S	

[a]Sanger sequencing of the resistant replicon after the resistance selection protocol (when the resistant phenotype was confirmed).

[b]Sanger sequencing of the resistant replicon following passaging for approximately 4 months.

792 **Supplemental Table 3. Primers used for site-directed mutagenesis.**

HCV	Mutation	Forward primer	Reverse primer
NS3	M581L	5’-CGTGGGACCAACTGTGGAAGTGTCTCATACG-	5’-CGTATGAGACACTTCCACAGTTGGTCCCACG-3’
NS3	K583E	5’-CGTGGGACCAAATGTGGGAGTGTCTCATACG-	5’-CGTATGAGACACTCCCACATTTGGTCCCACG-3’
NS4B	V38M	5'-CTTGGATTCCACCATGGGAGCAGCAGC-3'	5’-GCTGCTGCTCCCATGGTGGAATCCAAG-3’
NS4B	D167E	5'-CCTCCACCGAGGAACTGGTTAACCTACTC-3'	5'-GAGTAGGTTAACCAGTTCCTCGGTGGAGG-3’

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