J Antimicrob Chemother
doi:10.1093/jac/dky327

Journal of Antimicrobial Chemotherapy

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

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Received 5 March 2018; returned 15 May 2018; revised 26 June 2018; accepted 17 July 2018

Objectives: Many positive-stranded RNA viruses, including HCV, drastically remodel intracellular membranes to generate specialized environments for RNA replication. Phosphatidylinositol 4-kinase III (PI4KIII)α 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 their 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 culture with increasing compound concentrations. Intracellular localization of phosphatidylinositol 4-phosphate (PI4P) lipids was analysed 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 small interfering RNA (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 phosphoinositide 3-kinase (PI3K) inhibitor LY294002 proved to be 10-fold less effective against the resistant replicons. In addition, enviroxime and BF738735 inhibited several PI3Ks in enzymatic assays.

Conclusions: Contrary to assumptions, our data indicate that PI4KIIIα and PI4KIIIβ are not the main targets for the anti-HCV activity of enviroxime and BF738735. Instead, we demonstrated that both molecules impede HCV replication at least partially by an inhibitory effect on PI3Ks. Moreover, HCV is able to bypass PI3K inhibition by acquiring mutations in its genome.

Introduction

Worldwide ∼71 million people are chronically infected with 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 achieving 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 from 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 4-kinase III (PI4KIII)α.[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 phosphatidylinositol 4-phosphate (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, small interfering RNA (siRNA) screens,[5,6] giving conflicting data about the involvement of PI4KIIIβ in HCV replication. It has been suggested that the role of PI4KIIIβ in 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/PI4KIIIβ complexes to form NS5A/ACBD3 complexes, enabling 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-(isopropylsulphonyl)-6-benzimidazole phenyl ketone oxime]. This compound has been 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-(methylsulphonyl)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, 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 phosphoinositide 3-kinases (PI3Ks). 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, USA). 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-α2a 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 (25-HC) 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 5×10^3 cells per well in a 96-well cell culture plate in complete DMEM. Following incubation for 24 h at 37°C, serial dilutions of the test compounds in complete DMEM were added for 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 PCR (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 the manufacturer's instructions. For the GT2a replicon cells and Huh 5-2 replicon cells, luciferase activity was determined after 3 days of incubation using the Steady-Glo Luciferase Assay System (Promega). The 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 5×10^3 cells per well in a 96-well cell culture plate in complete DMEM. After 24 h 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 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium/phenazine methosulphate (MTS/PMS) method (Promega). The 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, $\sim3\times10^5$ 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 too low a level of replicon content), G418 and compound pressure were removed until cells recovered. Sanger sequencing was performed as described previously.[15]

Additional material and methods are available as Supplementary data at *JAC* Online.

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 (GTs). 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 and 0.7 μM. At concentrations of ≤10 μM, 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

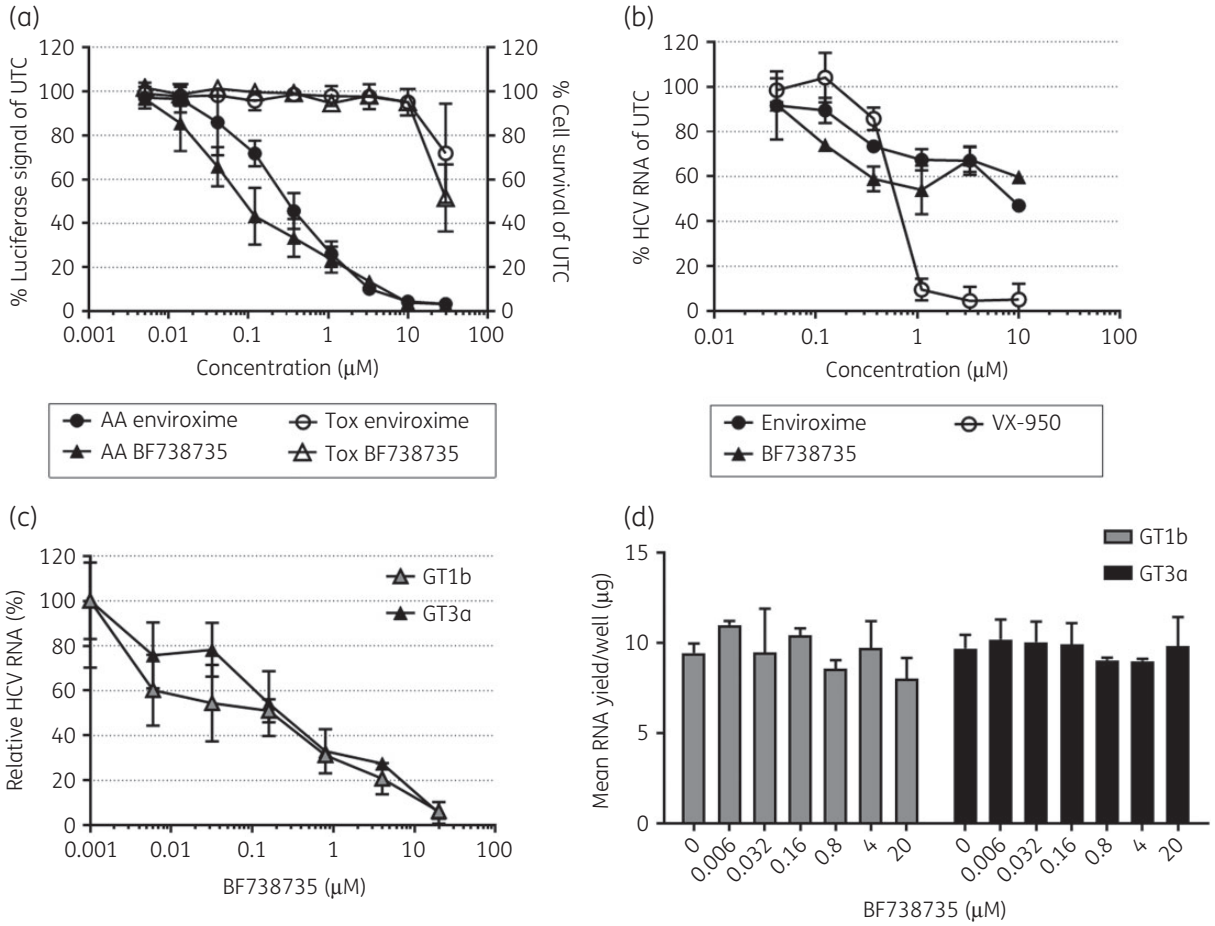

Figure 1. Antiviral activity of enviroxime and BF738735 in different *in vitro* HCV culture systems. (a) Effect of enviroxime and BF738735 on HCV replicon replication in Huh 5-2 GT1b replicon-containing cells and on the proliferation of exponentially growing cells. Data are mean values ± SD for at least three independent experiments. AA, antiviral activity; Tox, toxicity; UTC, untreated control. (b) AA of enviroxime, BF738735 and VX-950 against JFH-1/CS-N6 HCVcc infection in Huh 7.5.1 cells. Data shown are mean ± SD of at least three independent experiments. (c) Antiviral efficacy of BF738735 in primary human hepatocytes infected with HCV GT1b or GT3a isolates. Data shown are mean values ± SD of two independent experiments. (d) Effect of BF738735 on the total RNA content of HCV-infected primary human hepatocytes. Data shown are mean values ± SD of two independent experiments.

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 with WT, with EC_{50} values of 15±5 μM and 15±3 μM, respectively (Figure 2a and c). 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) (Figure 2b and c). Furthermore, both resistant replicons were less susceptible to PIK93 and AL-9, a PI4KIIIα inhibitor,[19] albeit to a lesser extent. HCV direct-acting antivirals of different classes such as polymerase inhibitors (2′-*C*-methylcytidine and VX-222), a PI (VX-950) and an 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 naive Huh-7 Lunet cells. As expected, enviroxime proved less effective in the enviroxime^{res}-transfected Huh-7 Lunet cultures when compared with the WT-transfected cultures (Figure 2d). The PI 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 in 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 two predominant mutations were shared by both resistant replicons,

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]					
	GT1a	GT1b (Huh 5-2)	GT1b (Huh 9-13)	GT2a	GT4a	CC_{50} (μM)[b]
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	±1.4	24±4

ND, not determined.
[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.

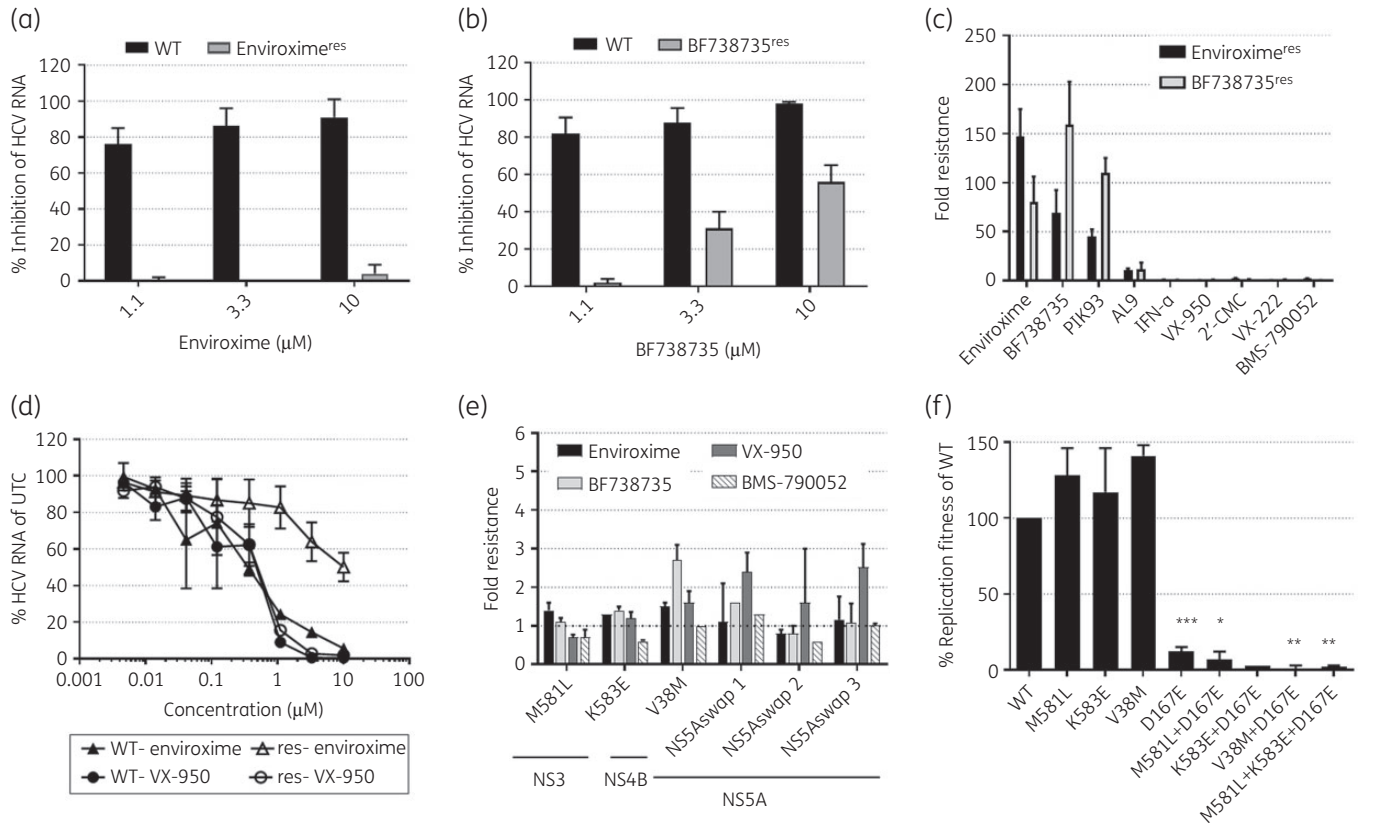

Figure 2. Phenotype of HCV replicons resistant to enviroxime and BF738735. (a) Inhibition of HCV replication by enviroxime in WT replicon or in enviroxime-resistant replicon cells. Data are mean values ± SD for at least three independent experiments. (b) Inhibition of HCV replication by BF738735 in WT 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 the resistant replicon to the EC_{50} in the 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 independent experiments. (e) Fold resistance values for enviroxime, BF738735, VX-950 and BMS-790052 in Huh-7 Lunet cells transiently transfected with mutant replicon RNA. Fold resistance values were calculated as the ratio of the EC_{50} in the resistant replicon to the EC_{50} in the WT replicon. Data are mean values ± SD for at least three independent experiments. (f) Replication fitness of the different mutant replicons. Values shown are expressed as a percentage of WT value at 72 h post-transfection and are mean values ± SD for at least two independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (unpaired Student's *t*-test).

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

As 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 with 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 with that of the WT replicon (Figure 2f). The replicative capacity of mutants M581L (NS3), K583E (NS3), V38M (NS4B) and D389N (NS5A) was comparable to or better than that of the WT replicon. In contrast, the replicon carrying the D167E mutation in NS4B had a pronounced reduction in replication fitness as compared with 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

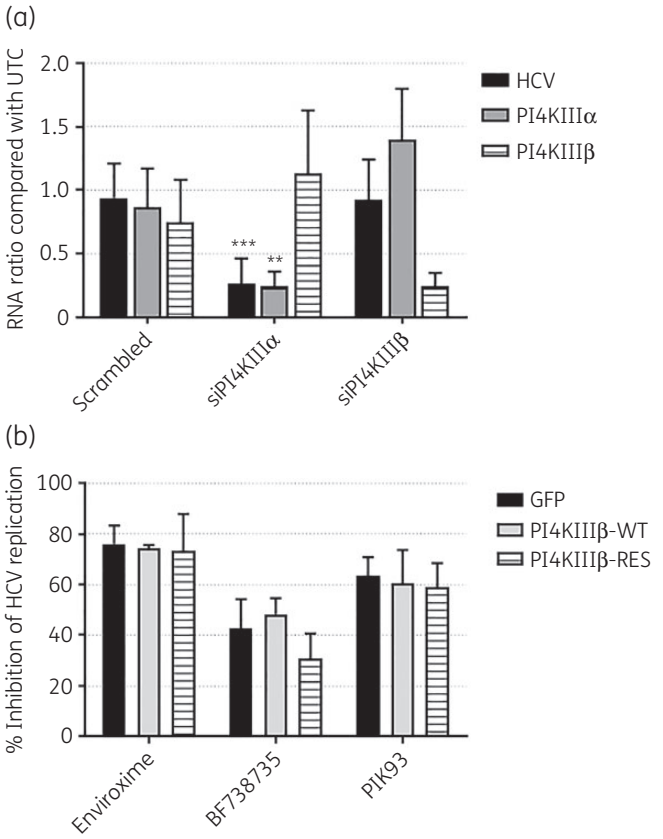

Figure 3. HCV replication depends on PI4KIIIα in both WT and resistant replicon cells. (a) Ratio of mRNA levels of HCV, PI4KIIIα and PI4KIIIβ as compared with untreated control cells 48 h post-siRNA transfection in WT Huh 9-13 replicon cells following transfection with scrambled siRNA, siPI4K3A or siPI4K3B. Data shown are mean values ± SD of at least three independent experiments. Data were analysed with the two-tailed Student's *t*-test. $^{**}P < 0.005$; $^{***}P < 0.001$. (b) Huh-7 Lunet cells were electroporated with HCV replicon RNA and seeded in a 96-well plate in the presence of 0.05 μM of enviroxime, BF738735 or PIK93. The cells were transfected with PI4KIIIβ-WT, PI4KIIIβ-RES, or as negative control GFP. Luciferase activity was determined 72 h post-electroporation. Data shown are mean values ± SD of three independent experiments.

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), could the inhibitory effect on HCV replication 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 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 co-localized 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 4 h, 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) (Figure 4b and c). Treatment of the HCV-U2OS cells with enviroxime or BF738735 did not alter the plasma membrane PI4P pools when compared with 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.

Oxysterol-binding protein (OSBP) is not the target of the anti-HCV activity of enviroxime and BF738735

Some enviroxime-like inhibitors inhibit enterovirus replication by targeting 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-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 (Figure 5a and b). Neither of the OSBP-targeting molecules was 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, $EC_{50} = 2.2 \pm 0.3$ 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 was completely inhibited. Furthermore, the profile of PI3K inhibition did not completely overlap 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]

HCV GT1b replicons resistant to enviroxime and BF738735, respectively, were found to be cross-resistant to the other compound. 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 naive hepatoma cells, we were able to demonstrate that the resistant phenotype was indeed associated with the viral genome. Single mutations or combinations of two or three individual mutations could not reconstitute the resistant phenotype however. 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 also contributed to the resistant phenotype. This was previously reported for another class of host-targeting antivirals, i.e. statins.[28] Unfortunately, we did not succeed in transfecting WT HCV replicon RNA into enviroxime^{res} replicon cells that were cleared from the resistant replicon.

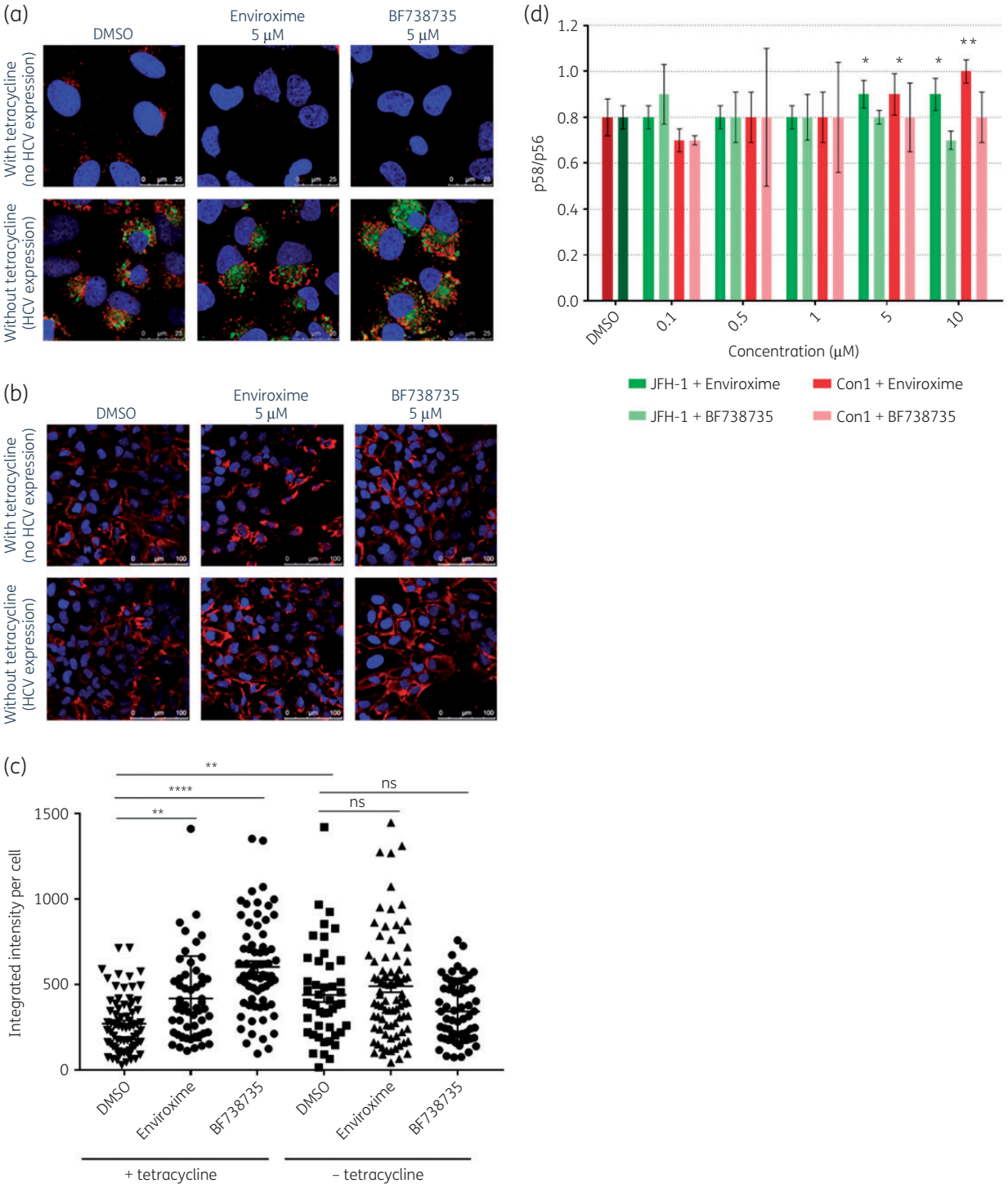

Figure 4. Enviroxime and BF738735 do not affect PI4P pools in cells expressing HCV proteins nor NS5A phosphorylation. (a–c) Osteosarcoma cells with inducible HCV expression were treated with enviroxime or BF738735 in the presence or absence of tetracycline for 48 h. PI4P pools (in red) were visualized by 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 analysed with the Kruskal–Wallis test using the GraphPad Prism software. $^{**}P < 0.005$; $^{****}P < 0.0001$; ns, not significant. (d) Naive Huh-7 Lunet cells were transfected with plasmids encoding the NS3 to NS5B polyprotein of JFH-1 WT or Con1 ET. Starting at 7 h post-transfection, cells were incubated with indicated concentrations of enviroxime or BF738735. Cell lysates were analysed by Western blotting using an NS5A-specific antibody. Data shown are mean values ± SD of four independent experiments. Data were analysed with the unpaired Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$.

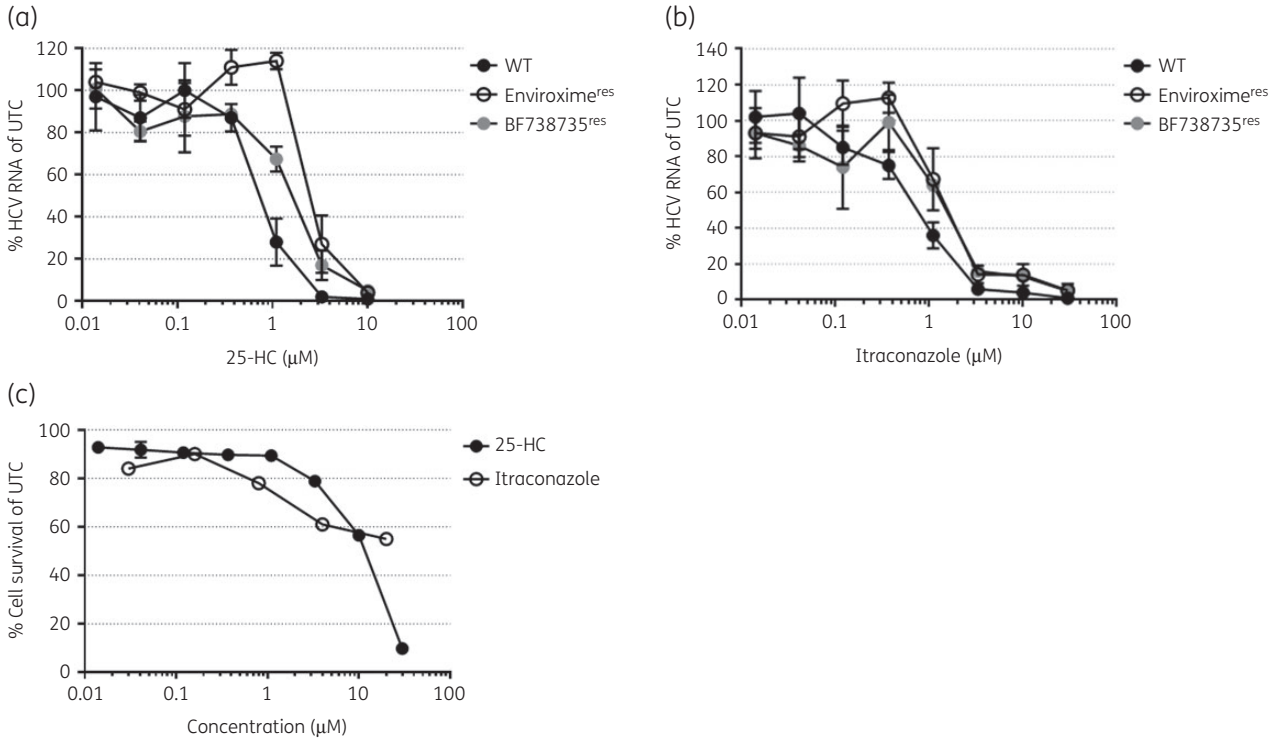

Figure 5. OSBP is not the target of the anti-HCV activity of enviroxime. (a, b) Dose–response curves for the inhibition of HCV replication by 25-HC (a) and itraconazole (b) in WT replicon, enviroxime-resistant replicon or BF738735-resistant replicon cells. Data are mean values ± SD of at least three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

The cellular target of the anti-enterovirus activity of enviroxime and BF738735 has been demonstrated to be the lipid kinase PI4KIIIβ.[11] BF738735 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 OSBP instead.[23] These inhibitors are cross-resistant with PI4KIIIβ-targeting inhibitors concerning 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, e.g. which is designated as a selective PI4KIIIβ inhibitor, also inhibits several PI3Ks.[18] AL-9, a PI4KIIIα inhibitor, also inhibits PI3K p110α *in vitro* at concentrations comparable to those needed to inhibit type III PI4Ks.[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 sterol regulatory element-binding proteins (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 and the PI4KIIIα isoform are not the major targets for the anti-HCV activity of these molecules. Instead, we show that both molecules inhibit several PI3Ks *in vitro*. HCV replicons resistant to the antiviral effect of enviroxime and BF738735 could bypass the PI3K inhibition by acquisition of mutations in their genome. Therefore, both

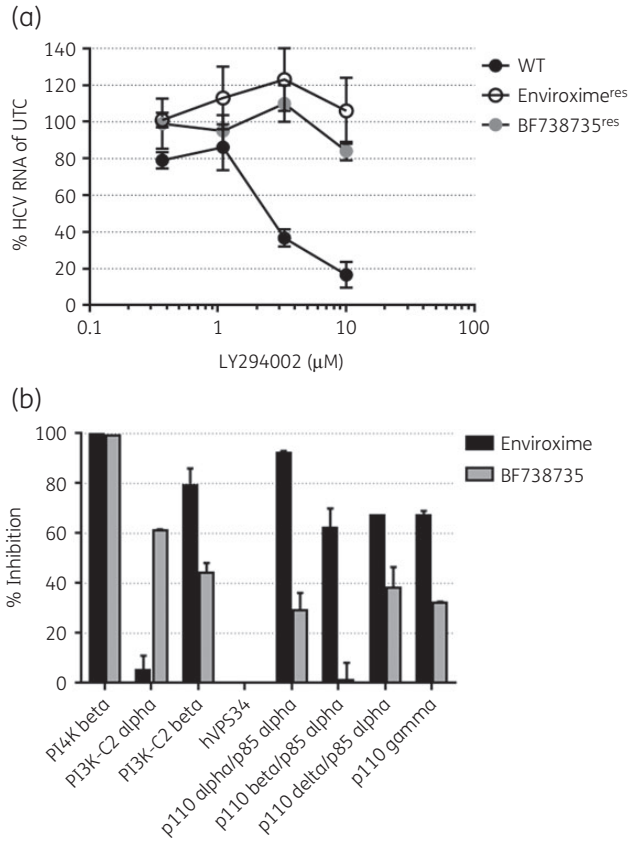

Figure 6. The broad-spectrum PI3K inhibitor LY294002 is cross-resistant with enviroxime and BF738735. (a) Dose–response curves for the inhibition of HCV replication by LY294002 in WT (black), enviroxime-resistant (white) or BF738735-resistant (grey) replicon-containing cells. Data are mean values ± SD for at least three independent experiments. (b) Inhibition of the *in vitro* enzymatic activity of a panel of PI3 and PI4 kinases by enviroxime and BF738735 at a concentration of 10 μM.

the antiviral target and the resistance mechanism are different from those of enteroviruses, for which enviroxime-resistant variants can circumvent the need for PI4KIIIβ.

Acknowledgements

The authors thank Katrien Geerts, Carolien De Keyzer, Els Vanstreels and Sandra Steensels for excellent technical assistance, Dr C. Gondeau (Hôpital Saint Eloi, Montpellier) for compound testing in primary human hepatocytes, Dr G. Cheng (Gilead Sciences) for providing the GT2a and 4a replicon plasmids and swap vector, Prof. D. Moradpour for the osteosarcoma cells with inducible HCV expression, Prof. G. Pürstinger for the synthesis of enviroxime, Prof. F. Zoulim for the JFH-1/CS-N6 HCVcc system, Prof. F. Chisari for the Huh 7.5.1 cell line, Prof. R. de Francesco for providing AL-9, Dr L. Coelmont for her help with the PI4P staining and Prof. F. van Kuppenveld for providing the PI4KIIIβ expression plasmids and helpful discussions.

Funding

This work was supported by a Vlaams Agentschap Innoveren & Ondernemen (VLAIO) O&O project from Instituut voor Innovatie door Wetenschap en Technologie (IWT) (100588) in collaboration with Galapagos and by a fellowship to L. D. from Fonds Wetenschappelijk Onderzoek (FWO) Vlaanderen (12A5713N). V. L. was supported by a grant from the Deutsche Forschungs-gemeinschaft (LO 1556/4-1).

Transparency declarations

None to declare.

Supplementary data

Supplementary Methods, Tables S1 to S3 and Figures S1 to S4 appear as Supplementary data at *JAC* Online.

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