Endocannabinoid CB1 antagonists inhibit hepatitis C virus production, providing a novel class of antiviral host-targeting agents

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Direct-acting antivirals have significantly improved treatment outcomes in chronic hepatitis C (CHC), but side effects, drug resistance and cost mean that better treatments are still needed. Lipid metabolism is closely linked with hepatitis C virus (HCV) replication, and endocannabinoids are major regulators of lipid homeostasis. The cannabinoid 1 (CB1) receptor mediates these effects in the liver. We have previously shown upregulation of CB1 receptors in the livers of patients with CHC, and in a HCV cell-culture model. Here, we investigated whether CB1 blockade inhibited HCV replication. The antiviral effect of a CB1 antagonist, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251), was examined in HCV strain JFH1 cell-culture and subgenomic replicon models. The effects on the expression of genes involved in lipid metabolism were also measured. CB1 short hairpin RNA (shRNA) was used to confirm that the effects were specific for the cannabinoid receptor. Treatment with AM251 strongly inhibited HCV RNA (~70 %), viral protein (~80 %), the production of new virus particles (~70 %) and virus infectivity (~90 %). As expected, AM251 reduced the expression of pro-lipogenic genes (SREBP-1c, FASN, SCD1 and ACC1) and stimulated genes promoting lipid oxidation (CPT1 and PPARα). This effect was mediated by AMP-activated protein kinase (AMPK). Stable CB1 knockdown of cells infected with HCV showed reduced levels of HCV RNA compared with controls. Thus, reduced CB1 signalling inhibits HCV replication using either pharmacological inhibitors or CB1 shRNA. This may be due, at least in part, to reduced lipogenesis, mediated by AMPK activation. We suggest that CB1 antagonists may represent an entirely new class of drug with activity against HCV.

INTRODUCTION

Globally, 200 million people have chronic hepatitis C (CHC), a leading cause of cirrhosis, liver failure and liver cancer. Until recently, the standard therapy for CHC was pegylated IFN-α in combination with ribavirin (Fried et al., 2002; Manns et al., 2001). During the last 5 years, direct-acting antiviral drugs have been developed that specifically target hepatitis C virus (HCV) proteins to interrupt virus replication. The first-generation protease inhibitors boceprevir and telaprevir are now licensed for use in the USA, Europe and Australia (Poordad et al., 2011; Zeuzem et al., 2011). However, these drugs have significant side effects and only target HCV genotype 1, and drug-resistant variants emerge in patients failing therapy (Pawlotsky, 2011). The second-generation protease inhibitor simeprevir (Zeuzem et al., 2014) and the NS5B inhibitor sofosbuvir (Jacobson et al., 2013; Lawitz & Gane, 2013) were recently licensed in the USA, but their high cost (approx. US$1000 per pill) is likely to delay their widespread use in many countries. Therefore, there remains a need for alternative, cost-effective treatments, with broad activity and a high genetic barrier to resistance.

Drugs targeting host proteins should be active against all HCV genotypes, and drug resistance is much less likely to occur, making them ideal backbones for combination
all-oral antiviral therapy (Pawlotsky, 2014; Pereira & Jacobson, 2009). Due to the importance of cellular lipids in the HCV life cycle (Bassendine et al., 2013), targeting lipid metabolism can inhibit HCV replication (Gastaminza et al., 2008; Olmstead et al., 2012), making them attractive therapeutic candidates (Negro, 2010). Indeed, there is a growing body of clinical evidence to support this approach, including in combination with direct-acting antiviral drugs (Cheng et al., 2014).

The endocannabinoid system is an important regulator of hepatic lipid metabolism; signalling through the hepatic cannabinoid 1 (CB1) receptor induces hepatic steatosis in both clinical and experimental studies (Engeli et al., 2005; Osei-Hyiaman et al., 2005, 2008). We recently showed that hepatic CB1 expression is upregulated in patients with CHC and correlates with steatosis grade and the expression of the pro-lipogenic genes sterol regulatory element-binding protein 1 (SREBP-1c) and fatty acid synthase (FASN) (van der Poorten et al., 2010). This suggests that HCV-induced steatosis may be mediated by increased CB1 expression, further supporting virus replication. Consistent with our findings, patients with CHC who smoke cannabis have more severe steatosis than those who do not (Hézode et al., 2008; van der Poorten et al., 2010).

We hypothesized that inhibiting CB1 signalling would impair HCV replication, and tested the effect of CB1 antagonists in an HCV cell-culture model. We demonstrated that CB1 antagonists inhibited the virus replication cycle, particularly the production of infectious particles. These data further support a role for antiviral agents that target the host, and suggest that CB1 antagonists may be a useful class of antiviral drug for the treatment of hepatitis C.

RESULTS

A CB1 antagonist reduces intracellular HCV protein and RNA in a time- and dose-dependent manner

To investigate the effect of CB1 inhibition on HCV replication, Huh7 cells were electroporated with HCV (strain JFH1) RNA and cultured until >90% of cells were infected (1–2 weeks), then treated with the CB1 antagonist N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251) (Tocris Bioscience) at doses of 1, 5, 7.5 or 10 μM, or with DMSO alone as a control (stock solutions of AM251 were prepared in DMSO). Cells were lysed after 12, 24 or 48 h and total RNA was extracted. HCV RNA was measured by quantitative real-time PCR (qPCR) using specific primer/probes targeting a conserved region of the HCV 5′-UTR. As shown in Fig. 1(a), HCV RNA was reduced in cells treated with AM251 in a dose-dependent manner, with a 60–70% reduction at a dose of 10 μM (P=0.003) after 48 h.

To investigate the effect of AM251 on viral protein, Western blot analysis was performed using antibodies directed against the HCV core and NS5A proteins. The amount of HCV core and NS5A protein was reduced in cells treated with AM251 in a time-dependent manner (Fig. 1b). Core protein concentration decreased from 24 h, with a significant 70–80% reduction after 48 h. NS5A protein concentration was significantly reduced in AM251 treated cells after 48 h compared with untreated controls.

To confirm a dose-dependent inhibitory effect of AM251 on virus protein production, JFH1-infected Huh7 cells were treated with AM251 at doses of 0.1, 1, 5, 7.5 or 10 μM. After 48 h, Western blot analysis confirmed a dose-dependent reduction in HCV core protein (Fig. 1c). A cell viability assay (WST-1; Roche) confirmed minimal toxicity of AM251 at doses up to 10 μM (Fig. 1d).

CB1 receptor knockdown using short hairpin RNA (shRNA) suppresses HCV RNA and protein expression

To confirm that the inhibitory effect of AM251 on HCV was due to reduced CB1 signalling, the CB1 receptor (CNR1) was specifically targeted using lentiviral shRNA. For this, Huh7 cells were transduced with a lentivirus expressing shRNA-CNR1 or a negative control (scrambled shRNA) at an m.o.i. of 10. Generation of stable cell lines took approximately 2 weeks and successful knockdown of CB1 mRNA and protein was confirmed by qPCR and Western blotting, respectively. As shown in Fig. 2(a, b), CB1 mRNA expression was reduced by ~60% in CB1 shRNA-transduced cells and CB1 protein was reduced by ~50% (P=0.02 and P=0.04, respectively).

To determine whether reducing CB1 expression inhibited HCV replication, CB1 knockdown cells and controls were infected with supernatant from JFH1-infected Huh7 cells. One week after infection, HCV RNA and core protein expression were measured by qPCR and Western blotting. HCV RNA and core protein were reduced significantly (30–32%; P=0.01 and P=0.04, respectively) in shRNA-CNR1-transduced cells, compared with controls, despite the modest CB1 knockdown (Fig. 2c, d).

CB1 antagonists inhibit production of extracellular virus particles

As shown in Fig. 1, treating HCV-infected cells with a CB1 antagonist inhibited intracellular RNA replication and viral protein translation. Next, we examined whether AM251 inhibited production of extracellular infectious virus. For this, JFH1-infected cells were treated with AM251 (10 μM) or DMSO alone (control). After 48 h, the supernatant was collected from AM251- and mock-treated cells and the virus was precipitated as described above. RNA was extracted from the concentrated virus pellet and the HCV RNA concentration was determined by qPCR. As shown in Fig. 3(a), the amount of extracellular HCV RNA was reduced by 70% in the supernatant of cells treated with AM251 compared with untreated controls (P=0.001).
To determine whether there was reduced infectivity of the secreted virus following treatment with AM251, the supernatant was tested using a TCID\textsubscript{50} infectivity assay. For this, virus was precipitated from the supernatant of infected cells to remove any residual drug that might affect the assay. Virus from the supernatant of cells treated with AM251, or mock treated, was added to uninfected Huh7 cells in 1 : 5 serial dilutions. After 72 h, immunolabelling for HCV NS5A protein and TCID\textsubscript{50} calculations were performed. As shown in Fig. 3(b), the infectivity of supernatant from cells treated with AM251 was reduced by approximately 90% compared with controls (\(P<0.003\)).

Virus particles produced in the HCV cell-culture model vary in density, with the most infectious virus particles in the low-density fractions (Lindenbach \textit{et al.}, 2006). To determine the effect of AM251 on the density of secreted HCV particles, virus particles were precipitated from supernatants of infected cells treated with AM251, or from mock-treated cells, and purified using sucrose density centrifugation. The amount of infectious virus was measured in the lower-density fractions 1–6 (1.038–1.286 g ml\textsuperscript{-1}), as infectious particles are predominantly present in these fractions (Gastaminza \textit{et al.}, 2006). The total amount of HCV RNA present in each fraction was determined by qPCR and the infectivity determined by a TCID\textsubscript{50} assay.

As shown in Fig. 3(c), the total amount of HCV RNA was reduced (~56%) in the low-density fractions 1–6 from AM251-treated cells compared with controls. TCID\textsubscript{50} analysis confirmed that the CB1 antagonist AM251

\textbf{Fig. 1.} Time- and dose-dependent inhibitory effect of CB1 antagonist AM251 on HCV RNA and protein expression. (a) JFH1-infected cells were treated with increasing doses of AM251 (CB1 antagonist) or DMSO control (CTL). Intracellular HCV RNA was measured after 12, 24 and 48 h by qPCR. (b) JFH1-infected and mock-infected Huh7 cells were treated with AM251 (10 \(\mu\)M) for 12, 24 and 48 h. HCV core and NS5A proteins were measured by Western blotting. The relative concentration of both proteins was determined by densitometry using ImageJ software and normalized to \(\beta\)-actin. (c) JFH1-infected Huh7 cells were treated with increasing concentrations of AM251, and after 48 h, HCV core protein was measured by Western blotting and analysed by densitometry. \(\beta\)-Actin was used as a loading control in (b) and (c). (d) Cell viability was confirmed in cells treated with increasing doses of AM251 using the WST-1 cell viability assay. **Significant change from untreated control (\(P<0.01\)).
inhibited production of infectious virus by approximately 90% in these fractions (Fig. 3d). Notably, the reduction in infectivity was much greater than the reduction in total HCV RNA.

**CB1 antagonists delay the establishment of HCV RNA replication**

To understand better the inhibitory effect of CB1 antagonists on other parts of the virus life cycle, we used both transient and stable JFH1-based subgenomic replicons (SGRs) that support RNA replication and express non-structural proteins but do not produce infectious virus. These models allowed us to investigate whether CB1 antagonists directly affected virus replication and protein expression.

To look for the effects of AM251 on established HCV replication, Huh7 cells harbouring the stable HCV replicon were treated with AM251, and after 24 and 48 h, HCV RNA was measured by qPCR (Fig. 4a) and NS5A protein by Western blotting (Fig. 4b). There was a non-significant trend towards reduced HCV RNA and NS5A protein for up to 48 h following treatment with AM251.

To look for possible effects of AM251 on the early stages of establishing HCV RNA replication, a transient JFH1-based SGR containing a firefly luciferase reporter was used (Targett-Adams & McLauchlan, 2005). When cells are electroporated with HCV replicon RNA, this bypasses the requirements for virus particle binding and entry. Following transport of viral RNA to the endoplasmic reticulum, HCV non-structural proteins are produced and HCV RNA replication occurs, but no infectious virus is produced. For this experiment, Huh7 cells were pre-treated with AM251 for 24 h and then transfected with the replicon. Luciferase activity was measured after 2 h, then every 3 h up to 24 h, using a commercial luciferase assay.

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**Fig. 2.** HCV RNA and core protein expression are inhibited in cells with reduced CB1 expression. Huh7 cells were stably transduced with lentiviruses expressing CB1 shRNA or scrambled shRNA controls. Two weeks later, the cells were infected with HCV (JFH1). One week after HCV infection, the relative expression of CB1 mRNA (a), CB1 protein (b), HCV RNA (c) and HCV core protein (d) was measured using qPCR and Western blotting. *Significant change from untreated control (P<0.05).
In contrast to its minimal effect in cells containing a stable HCV SGR (Fig. 4a, b), pre-treatment with AM251 inhibited the establishment of virus replication, with significantly reduced luciferase reporter activity after 24 h (Fig. 4c).

**CB1 antagonist AM251 inhibits HCV infection by impairing lipogenesis and promoting lipid oxidation**

It is now well established that HCV entry, replication, assembly and secretion are intimately associated with lipid metabolic pathways, and that HCV modulates the host lipid machinery to facilitate replication by simultaneously reducing fatty acid secretion and inducing lipogenesis (Syed et al., 2010). We confirmed in our model that HCV infection induced expression of key genes involved in lipogenesis, including SREBP-1c, FASN, stearoyl-CoA desaturase 1 (SCD1) and acetyl-CoA carboxylase (ACC1) (Fig. S1, available in the online Supplementary Material).

An important regulator of hepatic lipid metabolism is the endocannabinoid pathway, which promotes hepatic steatosis (Osei-Hyiaman et al., 2005), as well as SCD1 (Serrano et al., 2008). Animal studies have confirmed that specific CB1 antagonists decrease de novo fatty acid synthesis in the liver (Jeong et al., 2008, 2011; Tam et al., 2010) by reducing the expression of genes involved in lipogenesis. Moreover, in clinical studies, rimonabant (SR141716), the prototype CB1 antagonist, significantly improved dyslipidaemia in obese patients (Hollander et al., 2010). Based on these reports, we hypothesized that CB1 antagonists may inhibit virus infection by disrupting hepatocyte lipid metabolism.

To test our hypothesis, the effect of AM251 on lipogenic gene expression was investigated. For this, JFH1-infected cells were treated with 10 μM AM251 or DMSO control (CTL) for 48 h, and the culture supernatant was analysed for the presence of HCV RNA and infectious virus. (a) Relative HCV RNA concentration was determined using qPCR. (b) HCV infectivity was determined using a TCID₅₀ assay. To look for effects on the density of secreted virus particles, virus was precipitated from culture supernatant and separated on a sucrose density gradient. (c) HCV RNA was measured across the lower-density fractions (1–6) using qPCR. (d) Infectivity of the lower-density fractions (1–6) was determined by a TCID₅₀ assay. **Significant change from untreated control (P<0.01).
expression of proliferator-activated receptor type 1 (PPARα) and carnitine palmitoyltransferase 1 (CPT1) (Yamaguchi et al., 2005). CB1 activation has also been shown to reduce fatty acid oxidation in the liver by reducing CPT1 activity and expression (Osei-Hyiaman et al., 2008). Therefore, to investigate whether CB1 antagonists induce lipid oxidation in our model, expression of key genes involved in β-oxidation was examined. JFH1-infected cells were treated with the CB1 antagonist AM251 (10 μM) for 12–48 h, and levels of PPARα and CPT1 mRNA were measured by qPCR. Expression of PPARα started to increase 12 h after treatment, with a significant change by 48 h (Fig. 5e). CPT1 expression was significantly upregulated after 12 h, with further induction by 48 h (Fig. 5f). Once again there was no effect of AM251 on uninfected cells (data not shown).

AMP-activated protein kinase (AMPK) activation mediates the effect of CB1 antagonist AM251 on lipid metabolism and HCV replication

As a fuel sensor, AMPK modulates lipogenesis by inhibiting the pro-lipogenic transcription factor SREBP-1c and inducing β-oxidation of fatty acids by phosphorylating ACC (Hardie & Pan, 2002; Li et al., 2011; Wu et al., 2011). To investigate whether the AMPK signalling pathway mediates the effect of CB1 antagonists on lipid metabolism and HCV replication, the phosphorylation of AMPK and its downstream target ACC was examined. JFH1-infected cells were treated with the CB1 antagonist AM251 for 0, 5, 10, 20 and 30 min, as well as for 1, 3, 6 and 12 h. Cells were lysed, the protein extracted and phosphorylation of AMPK and ACC detected by Western blotting. As shown in Fig. 6(a), following treatment with AM251, there was an increase in phosphorylated AMPK, peaking at 20–30 min, with a delayed but sustained increase in phosphorylated ACC (P<0.05).

To confirm that CB1 antagonists inhibit lipogenesis and HCV replication via AMPK activation, the AMPK inhibitor Compound C was used. JFH1-infected cells were pre-treated with AM251 (10 μM) for 1 h and then Compound C (10 μM) was added to the cells. After 48 h, SREBP-1c expression and HCV RNA levels were measured using qPCR. Inhibition of AMPK by Compound C reversed the effect of AM251 on SREBP-1c expression (Fig. 6b) and significantly reduced its effect on virus replication (Fig. 6c). Together, these results suggested that the inhibitory effects of the CB1 antagonist on lipogenesis and HCV replication are mediated by activation of AMPK.
DISCUSSION

The recent availability of direct-acting antiviral drugs has revolutionized the treatment of chronic hepatitis C. However, worldwide availability of the best IFN-free, all-oral direct-acting antiviral drug regimens will be limited in the intermediate term by their high cost, and also by their lack of pan-genotypic activity. Therefore, there remains a need to better understand HCV replication and to develop novel classes of antiviral agents. In this regard, drugs that target host rather than viral proteins have the dual advantages of efficacy against all HCV genotypes and a lower risk of drug resistance (Pawlotsky, 2014; Pereira & Jacobson, 2009). In this study, we demonstrated that targeting hepatocyte CB1 receptors with a specific antagonist inhibited HCV, thus identifying a novel class of host-targeting agents.

Using the JFH1 HCV cell-culture model, we showed that the CB1 antagonist AM251 inhibited HCV RNA replication and viral protein expression and impaired the production of infectious virus. We confirmed that these effects were specific to CB1 by knocking down the CB1 receptor using shRNA. Despite an almost total reduction in CB1 mRNA (90%) in uninfected cells, in virus-infected cells the knockdown was only partial for CB1 protein (~50%). Despite this more modest reduction in protein expression, HCV RNA replication and virus protein were reduced. The difficulty in suppressing CB1 in HCV-infected cells may be due to strong virus-induced upregulation of this receptor (up to sixfold), which we have reported previously (van der Poorten et al., 2010).

Density-gradient analysis confirmed that AM251 reduced the production of infectious, low-density HCV particles. The dramatic reduction in infectivity (90%) suggested that CB1 antagonists specifically inhibit the secretion of infectious viruses in the low-density fraction. This is consistent with clinical data showing that patients treated with the CB1 antagonist rimonabant had reduced serum very-low-density lipoprotein levels, but increased high-density lipoprotein (Christopoulou & Kiortsis, 2011).

Fig. 5. CB1 antagonist AM251 reduces lipogenesis and promotes lipid oxidation in HCV-infected hepatic cells. JFH1-infected Huh7 cells were treated with AM251 (10 μM) or DMSO control (CTL) for 0, 12, 24 and 48 h. Expression of genes involved in liver lipid metabolism was measured by qPCR. Expression of several genes involved in lipogenesis was reduced: SREBP-1c (a), FASN (b), SCD1 (c) and ACC1 (d). Expression of genes promoting fatty acid oxidation was increased: PPARα (e) and CPT1 (f). Significant change from the untreated control is indicated: *P<0.05; **P<0.01; ***P<0.001.
In contrast to cells infected with full-length virus, there was little effect of the CB1 antagonist on HCV RNA or protein in cells stably transfected with an SGR. We initially hypothesized that, as AM251 specifically reduced production of infectious virions, which are not produced by cells containing the SGR, it had no effect on virus replication per se. However, using a transient SGR model, we observed reduced HCV replication at early time points, suggesting that AM251 delayed the establishment of RNA replication. We subsequently confirmed that AM251 impaired lipogenesis in

Fig. 6. AMPK activation mediates the antiviral effect of CB1 antagonist AM251 on downstream targets. JFH1-infected Huh7 cells were treated with AM251 for 0, 5, 10, 20 and 30 min, and also for 1, 3, 6 and 12 h. (a) Phosphorylation of AMPK and ACC was measured by Western blotting, using phospho-specific antibodies (p-AMPK and p-ACC), and normalized against total protein (t-AMPK and t-ACC). Inhibiting AMPK activation partially reversed the effect of AM251 on HCV replication and SREBP-1c expression. (b, c) JFH1-infected Huh7 cells were pre-treated with AM251 for 1 h and were then treated with the AMPK inhibitor Compound C or mock treated. After 48 h, SREBP-1c mRNA (b) and HCV RNA (c) were measured by qPCR. *P<0.01; **P<0.05.

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CB1 antagonists inhibit HCV infection
HCV-infected cells, suggesting that the effect on early replication may be due to the reduced availability of lipids to form lipid rafts, an integral part of the virus replication complex (Aizaki et al., 2004; Amemiya et al., 2008). This hypothesis requires further validation.

One of the key regulators of lipid metabolism in hepatocytes is AMPK. AMPK activation inhibits de novo fatty acid synthesis and promotes fatty acid β-oxidation (Hardie & Pan, 2002; Li et al., 2011; Wu et al., 2011). Moreover, activation of AMPK by AICAR (an AMPK agonist) has been shown to block HCV-induced lipid accumulation and reduces HCV replication (Mankouri et al., 2010). An inhibitory effect of AMPK on virus replication has also been demonstrated for Rift Valley fever virus, another virus that depends on host de novo fatty acid synthesis (Moser et al., 2012).

Several studies have shown that cannabinoid receptor antagonists activate AMPK in mouse liver (Watanabe et al., 2009), in mouse adipocytes (Tedesco et al., 2008) and in cultured human hepatocytes (Wu et al., 2011). In human hepatocytes, the CB1 antagonist rimonabant induced phosphorylation of AMPK and ACC, and decreased lipogenesis in HepG2 cells (Wu et al., 2011). Interestingly, AMPK activation has also been shown to decrease HCV-induced lipid accumulation and inhibit HCV replication in vitro (Mankouri et al., 2010; Nakashima et al., 2011). Consistent with these reports, our data suggest that the antiviral effects of AM251 against HCV are caused by effects on lipid gene expression, mediated by AMPK signalling. Although we cannot exclude additional mechanisms, the effects of AM251 on cellular lipids in our model are clear. This is consistent with the known role of the CB1 receptor in regulating liver lipid metabolism, and the well-recognized importance of cellular lipid in the HCV life cycle. In our model, AM251 had no effect on expression of lipid genes in uninfected Huh7 cells. In contrast, AM251 reversed the increased expression of lipogenic genes in HCV-induced cells (Fig. 5) and in uninfected Huh7 cells treated with the CB1 agonist AEA (data not shown). The lack of AM251 effect in uninfected cells is most likely due to the very low basal level of CB1 receptor expression, which we have shown increases up to sixfold following HCV infection (van der Poorten et al., 2010), accounting for the increased effect on lipid gene expression in HCV-infected cells. Furthermore, in our HCV cell-culture model, the CB1 antagonist significantly reduced the lipid content of infected cells but had no effect on the lipid content of uninfected cells (data not shown). This is consistent with our observations of a minimal effect of AM251 on lipid gene expression in uninfected cells. As above, we hypothesize that CB1 antagonists are only effective when treating HCV-infected cells, due to their higher levels of CB1 receptor expression (van der Poorten et al., 2010).

Adding an AMPK inhibitor (Compound C) reversed the effects of AM251 on lipid gene expression and its antiviral effects against HCV, suggesting an important role for AMPK in mediating these effects. The reason why Compound C did not completely reverse the effects of AM251 on HCV replication is unclear. We hypothesize that AMPK may not be the only mediator of CB1 antagonist signalling in our model, so its residual effect, despite Compound C treatment, may be due to secondary signalling pathways.

In clinical studies, the CB1 antagonist rimonabant showed promising activity against obesity, type 2 diabetes, dyslipidaemia and the metabolic syndrome (Pacher et al., 2006; Van Gaal et al., 2008). Despite this, rimonabant was withdrawn from the market in 2007 because of an unacceptable safety profile, including adverse psychiatric effects (FDA, 2007; Moreira et al., 2009). It is now recognized that, in addition to effects of brain CB1 receptors on appetite, hepatic CB1 receptors are key regulators of lipid metabolism. In animal studies, liver-specific CB1 knockout mice (LCB1−/−) became obese when exposed to a high-fat diet but did not develop steatosis, dyslipidaemia or insulin resistance (Osei-Hyiaman et al., 2008). Therefore, second-generation CB1 antagonists have now been developed that do not cross the blood–brain barrier and so can improve lipid metabolism and induce weight loss without adverse central side effects (Cluny et al., 2010; Tam et al., 2010). These drugs show great promise for the treatment of obesity and metabolic syndrome, and several compounds are being considered for clinical trials (Serrano et al., 2012). In humans, it was recently confirmed that the peripherally selective CB1 antagonist TM38837 inhibits peripheral CB1 effects, with minimal central nervous system effects (Knunypers et al., 2013). Significantly, in this clinical study, serum drug levels of 5–20 μM were achieved, comparable to the 5–10 μM concentrations that showed potent antiviral activity in our model.

In summary, we have shown that inhibiting the CB1 receptor is an effective antiviral strategy against HCV, dramatically reducing the production of infectious virus and inhibiting the establishment of new replication complexes. Our results suggest that these effects are, at least in part, due to altered hepatocyte lipid metabolism mediated by AMPK (Fig. 7). As second-generation CB1 antagonists have now been developed and are undergoing clinical trials for the treatment of obesity and metabolic syndrome, we propose that these compounds may be effective adjuvant agents for the treatment of CHC, particularly in difficult-to-treat patients with steatosis and insulin resistance.

**METHODS**

**Cell and virus culture.** Plasmids containing pJFH1 HCV (Wakita et al., 2005) or the SGR based on JFH1 (pSGR-JFH1) (Kato et al., 2003) (kind gifts from Professor Takaji Wakita, National Institute of Infectious Disease, Tokyo, Japan) were used to generate JFH1 and SGR-JFH1 RNA as described previously (Wakita et al., 2005). HCV RNA was transfected into Huh7 hepatoma cells by electroporation to initiate infection. A stable cell line containing the HCV SGR was generated as described previously (Kato et al., 2005). A JFH1-based SGR containing a firefly luciferase reporter (pSGR-luc-JFH1) (Targett-Adams & McLauchlan, 2005) was a kind gift from Dr John...
McLauchlan (MRC-University of Glasgow Centre for Virus Research, UK). Lentivirus shRNA expression plasmids pGIPZ- shCNR1 [target set for NM_016083 (eight clones)], the packaging lentivirus helper plasmids pCMV-vesicular stomatitis virus (VSV)/G and pCMV-Gag/Pol were purchased from Open Biosystems.

Antibodies and reagents. The CB1 antagonist AM251 was purchased from Tocris Bioscience and In Solution AMPK Inhibitor, Compound C was from Calbiochem. HCV rabbit anti-core (308) antibody was kindly provided by Dr John McLauchlan. HCV sheep anti-NS5A was provided by Professor Mark Harris (University of Leeds, UK). AMPK, phospho-AMPK and phospho-ACC antibodies were supplied by Cell Signaling. N-terminal anti-cannabinoid receptor 1 (CB1) and β-actin were from Sigma-Aldrich.

RNA extraction, cDNA synthesis and qPCR. Total RNA was extracted from cell monolayers using an RNeasy kit (Qiagen) according to the manufacturer’s instructions. A QiAamp Viral RNA Mini kit (Qiagen) was used to extract viral RNA from the JFH1-infected cell-culture supernatant. cDNA was reverse transcribed from 500 ng total RNA using a Superscript III RT kit (Invitrogen) according to the manufacturer’s instructions. qPCR was performed using a Rotor-Gene 6000 (Corbett) with a SYBR Green or Taqman protocol. mRNA levels were normalized to 18S rRNA and glyceraldehyde 3-phosphate dehydrogenase levels, which gave comparable results. qPCR results were analysed using the Rotor-Gene 6000 Corbett software (for qPCR primer sequences and primer/probe information see Tables S1 and S2).

Western blot analysis. Cellular protein was extracted from cell-culture monolayers using RIPA buffer (Sigma-Aldrich) and protease inhibitor mix (Roche). After the addition of 1 × L mamml buffer, 20–50 μg reduced protein was loaded onto a 10 % SDS-PAGE gel, electrophoresed and transferred to nitrocellulose membranes. After blotting, the membranes were incubated with primary antibodies overnight at 4 °C followed by appropriate HRP-conjugated secondary antibodies for 1 h at room temperature. The probed membranes were visualized using SuperSignal West Pico Chemiluminescence (Thermo Scientific) and exposed to Kodak BioMax Films.

CB1 knockdown with lentiviral shRNA. CB1 shRNA lentiviral particles were generated using lentivirus shRNA expression vectors pGIPZ-shCNR1 [target set for NM_016083 (eight clones)]. Briefly, Hek293T cells were co-transfected with 6 μg gag/pol, 3 μg VSV and 9 μg lentiviral expression plasmids. After 48 h incubation at 37 °C, virus-containing supernatant was filtered with 0.45 μm MilliEX-HV filters (Millipore) and centrifuged using an SW-32 rotor and a Beckman Optima XL-100K Ultracentrifuge at 25 000 r.p.m. for 1 h 45 min at 4 °C. The virus pellet was resuspended in 10 % FBS/Dulbecco’s minimal essential medium and used to transduce Huh7 cells at an m.o.i. of ~10. To select for stable cell lines, 10 μg puromycin ml⁻¹ was added at 24 h post-transduction. Once antibiotic resistance was observed, the cells were infected with supernatant from JFH1-infected Huh7 cells and the efficiency of shRNA-mediated knockdown was evaluated by qPCR and Western blot.

Concentration of JFH1 viruses and sucrose density-gradient analysis. Viral supernatants were concentrated with polyethylene glycol 8000 as described previously (Lindenbach et al., 2005). Concentrated virus was then overlaid on a stepwise gradient of sucrose, prepared from 60 to 10 % (w/w) sucrose, and centrifuged at 35 000 r.p.m. for 16 h using an SW-41 rotor and a Beckman Optima
XL-100K Ultracentrifuge. Low-density fractions (1–6) were collected and analysed for HCV infectivity and HCV RNA.

**Determining virus infectivity by TCID<sub>50</sub> assay.** Uninfected Huh7 cells were grown in a 96-well plate at a concentration of 4 × 10<sup>4</sup> cells per well and incubated overnight. The next day, the supernatant from JFH1-infected Huh7 cells was added in 1:5 serial dilutions to 200 μl medium in each well and the cells were returned to the incubator for 72 h. The cells were then fixed with methanol for 20 min at −20 °C and blocked with PBS with 2% FCS for 15 min. To prevent endogenous peroxidase interference, the cells were incubated with 0.3% H<sub>2</sub>O<sub>2</sub> in PBS for 20 min. Cells were stained for HCV with anti-NS5A antibody at a 1:10,000 dilution for 1 h and washed twice with PBS for 5 min. Secondary HRP-conjugated anti-sheep antibody at a dilution of 1:1000 was added to the cells for 1 h, and infected cells were identified using ImmPACT DAB Peroxidase Substrate (Vector Laboratories). Cells were examined with an Olympus CK2 inverted microscope at ×20 magnification and a well was counted as positive if there was at least one infectious focus identified. The TCID<sub>50</sub> Enl<sup>−1</sup> was calculated using the formula described in the Spearman–Karber method (Carrières-Kremer et al., 2004).

**Statistical analysis.** Raw data were analysed using Microsoft Excel. Graphs were generated and statistical analysis was performed using GraphPad Prism 5 (GraphPad Software). Student’s t-test was used to determine significance at P<0.05, P<0.01 and P<0.001.

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