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Inhibition of the Subgenomic Hepatitis C Virus Replicon in Huh-7 Cells by 2′-Deoxy-2′-Fluorocytidine

Lieven J. Stuyver,[1]† Tamara R. McBrayer,[1] Tony Whitaker,[1] Phillip M. Tharnish,[1] Mangala Ramesh,[1] Stefania Lostia,[1] Leanne Cartee,[1] Junxing Shi,[1] Ann Hobbs,[1] Raymond F. Schinazi,[2] Kyoichi A. Watanabe,[1] and Michael J. Otto[1]*

Pharmasset, Inc., Tucker, Georgia 30084[1]; and Department of Pediatrics, Emory School of Medicine/Veterans Affairs Medical Center, Decatur, Georgia 30033[2]

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2′-Deoxy-2′-fluorocytidine (FdC) is a potent inhibitor of the hepatitis C virus RNA replicon in culture, and FdC-5′-triphosphate is an effective inhibitor of the NS5B polymerase. Dynamic profiling of cell growth in an antiviral assay showed that FdC caused cytostasis due to an S-phase arrest. These observations demonstrate that FdC treatment is affecting both a viral target and a cellular target.

Hepatitis C virus (HCV) infection is the leading cause of liver transplantation in the United States, with sequelae including fibrosis, cirrhosis, and hepatocellular carcinoma (1). In vivo, HCV replication occurs mainly in the cytoplasm of infected hepatocytes, but it has been difficult to demonstrate replication in vitro. Replicon-based systems have now been developed that sustain efficient replication of HCV RNA in cell culture. Initially, subgenomic replicons that expressed only nonstructural proteins were constructed; however, recent reports described replicons that can express the entire HCV polyprotein (5, 7).

In addition to the currently approved standard treatment options for HCV infections that use interferon and ribavirin, several new antiviral agents are in preclinical or clinical development. Similar to the case with human immunodeficiency virus type 1 treatment, multiple drug targets (e.g., protease, helicase, polymerase, and entry) may be needed to limit the emergence of drug-resistant variants. The HCV subgenomic replicon provides an excellent system for evaluating HCV antiviral agents in cell culture (3, 5, 6, 10, 16, 18). We report here the antiviral activity of 2′-deoxy-2′-fluorocytidine (FdC) (Fig. 1) measured in the HCV subgenomic replicon system and in the bovine viral diarrhea virus (BVDV)-Madin-Darby bovine kidney (MDBK) cell system.

HCV-replicon RNA-containing Huh-7 cells (Clone A cells; Apath, LLC, St. Louis, Mo.) were kept in exponential growth as described previously (16). Antiviral assays were performed in medium without G418. Cells were seeded in a 96-well plate at 1,000 cells per well, and test compounds were added immediately after seeding. After 96 h of incubation, total cellular RNA was isolated (Rneasy 96 kit; Qiagen, Valencia, Ca.), and HCV replicon RNA and an internal control (TaqMan rRNA Control Reagents; Applied Biosystems, Foster City, Ca.) were amplified in a single-step multiplex reverse transcription-PCR protocol. FdC (obtained from the Pharmasset compound library) was tested in a concentration range of 0.1 to 200 μM, and a 90% effective concentration (EC_{90}) for reducing the intracellular HCV replicon RNA levels of 5.0 μM was found (Fig. 2A). FdC was found to be more potent than ribavirin (EC_{90}, ~100 μM) and comparable in potency to β-D-N^4-hydroxycytidine (NHC) (EC_{90} = 5 μM) (16). The cellular toxicity against Huh-7 and HepG2 cells was measured after 96 h of incubation by using the CellTiter 96 AQ_{ueous} One solution cell proliferation assay (Promega, Madison, Wis.), and the concentration resulting in 50% reduction in cell growth (CC_{50}) was found to be greater than 100 μM. This resulted in a therapeutic index (CC_{50}/EC_{90}) of greater than 20.

Since FdC is an analogue of cytidine and fluorine is isosteric with a hydroxyl group, it is anticipated that its 5′-triphosphate would inhibit the viral NS5B RNA-dependent RNA polymerase. FdC was chemically converted to the corresponding FdC-triphosphate (FdCTP), and an in vitro NS5B polymerase assay was performed (16). In brief, 400 ng of RNA template was incubated with 400 ng of NS5B protein in a buffer containing 125 μM GTP, 5 μM ATP and CTP, 2.5 μM UTP, and 10 μCi of [α-^{32}P]UTP (800 Ci/mmol, 20 mCi/ml; Amersham Biosciences, Piscataway, N.J.) and incubated for 1 h at 27°C. De novo-synthesized RNA was precipitated on a Hybond N+ membrane (Amersham), unincorporated nucleotides were washed away using 4× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), the total amount of incorporated UTP was measured in a liquid scintillation counter, and the 50% inhibitory concentration was calculated. β-D-N^4-hydroxycytidine–5′-triphosphate was included as a control. FdCTP had a 50% inhibitory concentration of 14.9 μM, compared to 11.0 μM for β-D-N^4-hydroxycytidine–5′-triphosphate (Fig. 2B). These results suggest that at least part of the antiviral effect observed with FdC in the replicon system is derived from inhibition of the NS5B enzyme.

Maintenance of the steady-state replicon level requires logarithmic cell growth, and any candidate antiviral agent that influences cell growth rates might indirectly alter replicon levels (8, 15, 16). The EC_{90} determined on day 4 is a single static

* Corresponding author. Mailing address: Pharmasset Inc., 1860 Montreal Rd., Tucker, GA 30084. Phone: (678) 395-0050. Fax: (678) 395-0030. E-mail: motto@pharmasset.com.
† Present address: Virco BVBA, Mechelen, Belgium.

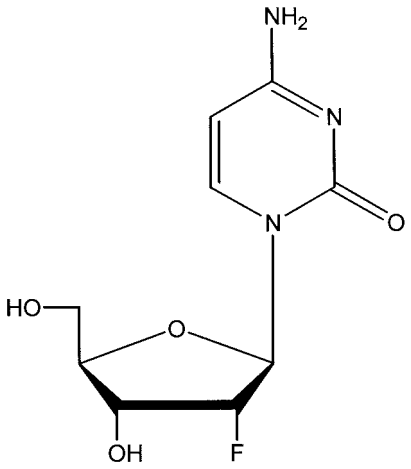

FIG. 1. Chemical structure of FdC.

efficacy measurement that does not account for compound-related changes in the obligatory requirement for logarithmic cell growth. Therefore, experiments were conducted to monitor HCV RNA levels and cell growth dynamics over a 7-day period. A total of 10^4 cells per well were seeded in a 24-well plate in the presence or absence of FdC, and at the end of the incubation, cells were counted using the trypan-blue exclusion method, followed by total cellular RNA isolation and quantification of replicon RNA. Alpha 2a interferon (IFN-α-2a) at a concentration of 100 IU/ml was used as a control. FdC was added at an 8 μM concentration. Compared to the untreated control, IFN-α-2a at a 100-IU/ml concentration slowed the cell proliferation minimally but caused a significant drop in HCV RNA levels (a drop of 0.53 ± 0.4 $\log_{10}$ for IFN versus an increase of 1.79 ± 0.4 $\log_{10}$ for the control; $P = 0.0005$) (Fig. 2C). The apparent rebound in replicon RNA from day 4 onwards was previously reported by Cheney et al. (4). The increases in cell number for the untreated control and for FdC were essentially identical on day 3. However, from day 4 on-

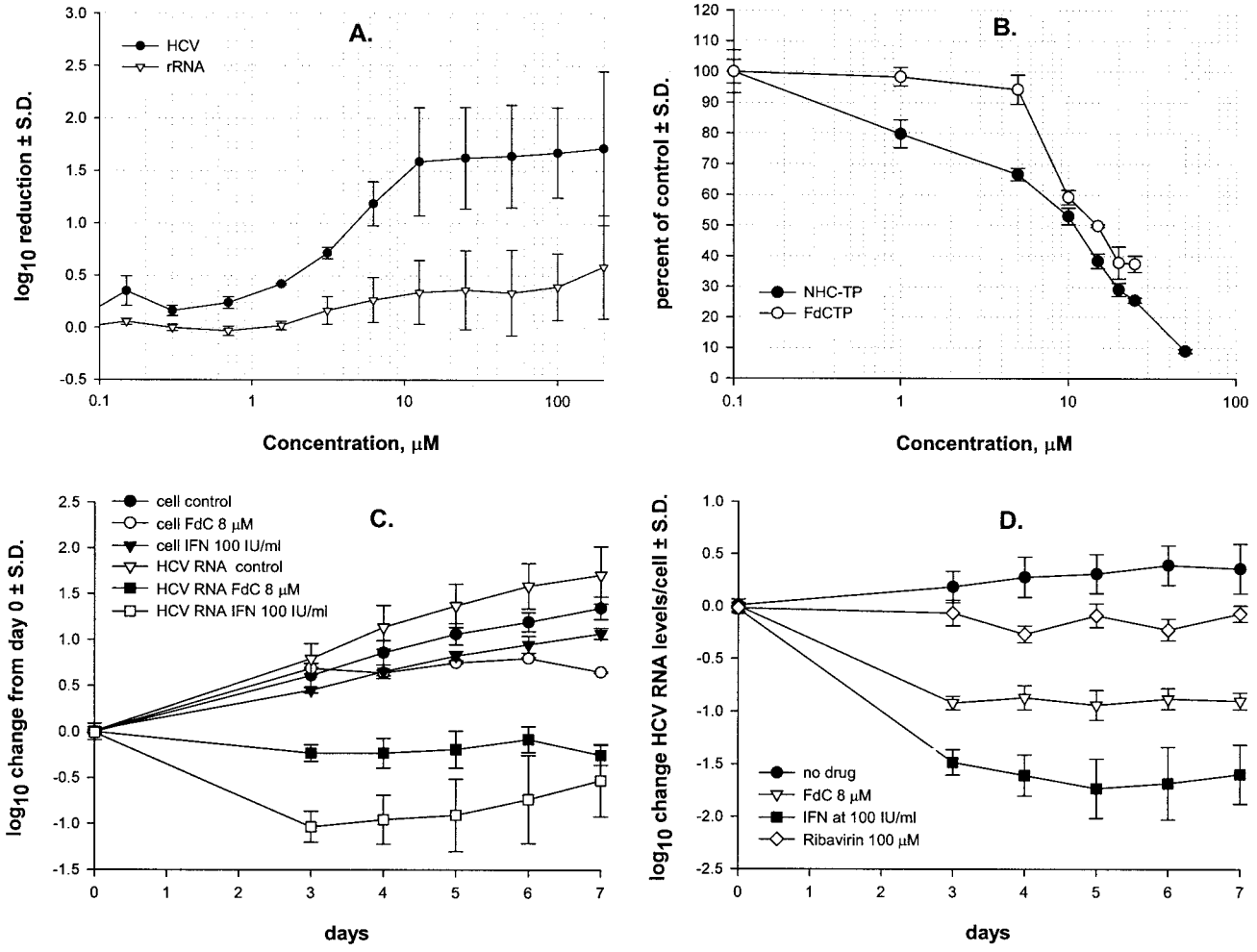

FIG. 2. Antiviral activity of FdC. (A) Dose-dependent antiviral effect on HCV replicon RNA containing Huh-7 cells. Cells were seeded at 1,000 cells per well in a 96-well plate in the presence of compound, and after 96 h of incubation, replicon HCV and rRNA levels were quantified by real time reverse transcription-PCR. (B) Dose-dependent inhibitory effect of FdCTP on the HCV RNA-dependent RNA polymerase in an in vitro assay system. (C) Comparison of the effects of FdC and IFN on cell growth and HCV replicon dynamics over 7 days. (D) Change in HCV replicon RNA copy number per cell over a 7-day incubation period.

wards, cell growth was inhibited (cytostasis), and a small reduction in cell number occurred on day 7 that was attributable to cell death. This indicates that apart from inhibition of the NS5B enzyme, FdC or one of its metabolites inhibits one or more cellular targets. In previous studies, it was shown that FdCTP was incorporated into cellular DNA and RNA, resulting in weak cytotoxic activity. Moreover, this effect was reversed upon compound removal, and therefore, FdC was described as a cytostatic rather than cytotoxic agent (13, 19). Other studies showed that FdC–5′-triphosphate is a substrate for incorporation by human polymerase α and polymerase γ, as well as for several nonhuman DNA polymerases (11). It was reported that FdC (10 μM) also inhibits the growth of Raji human lymphoblastic cells, resulting in cell viability reduction to 25% of the control after 72 h of incubation (2). Based on these findings, it is reasonable to assume that the observed growth arrest in HCV replicon cells from day 4 onwards was a consequence of incorporation of FdC into cellular nucleic acids.

Compared to the untreated control, replicon RNA levels were significantly reduced over the 7-day incubation period (Fig. 2C; compare the no-drug control line with the 8 μM FdC line). When the $\log_{10}$ change in replicon RNA copy number per cell was calculated for the 7-day assay, IFN-α-2a reduced the copy number per cell by approximately 1.6 $\log_{10}$ at day 3, after which a new steady-state level was achieved on days 4 to 7 (Fig. 2D). Interestingly, FdC (8 μM) reduced the replicon copy number per cell by 1.104 $\log_{10}$ at day 3; however, no additional significant antiviral effect was observed between days 4 (1.147 $\log_{10}$ reduction) and 7 (1.259 $\log_{10}$ reduction). Apparently, a consequence of FdC-induced cytostasis was that the de novo synthesis of replicative intermediates, the half-life of the replicon, and the inhibitory effect of FdCTP on the NS5B enzyme reached a new-steady state level. By comparison, ribavirin at a 100 μM concentration did not significantly reduce the replicon RNA copy number per cell.

Because FdC induced cytostasis in HCV RNA containing replicon cells (Fig. 2C), flow-cytometric analysis of DNA content following propidium iodide staining was used to determine whether incubation with FdC caused changes in cell cycle distribution. Exponentially growing Huh-7 replicon cells were exposed to 10 μM FdC for 0, 24, or 48 h. After trypsinization, the cells were washed with ice-cold phosphate-buffered saline, resuspended in 1 ml of phosphate-buffered saline, and fixed by the addition of 100% methanol (2 ml). The cells were incubated overnight in 67% methanol at 4°C and then stained for 3 h in 1 ml of ice-cold Na citrate (3.8 mM) solution containing propidium iodide (0.05 mg/ml) and RNase (0.5 mg/ml). DNA content was measured in untreated and FdC-treated samples using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, Calif.), and analyses of DNA histograms were performed using ModFit Verity software (Topsham, Maine). A significant accumulation of cells in S phase was observed after treatment with 10 μM FdC at 24 and 48 h (Fig. 3); moreover, the absence of a sub-G_1 peak indicated that FdC did not induce cell death (data not shown). These findings are consistent with the abovementioned cytostatic effect of FdC and concur with those of previous studies indicating that the triphosphate is a substrate for human cellular polymerases (11). The antiviral activity and cellular toxicity of FdC could be prevented by addition of

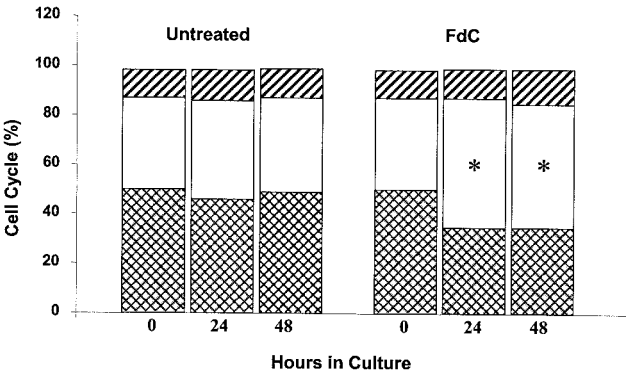

FIG. 3. Effect of FdC on cell cycle distribution in HCV replicon RNA containing Huh-7 cells. Outlined diamond, fraction of cells in G_0/G1 phase; white box, fraction of cells in S phase; wide upward diagonal, fraction of cells in G_2/M phase. *, significantly different from control ($P < 0.05$).

2′-deoxycytidine to the culture media but not by other natural 2′-deoxynucleosides or ribonucleosides (data not shown). This suggests that cellular enzymes recognized the 2′-fluoro-sugar as a 2′-deoxyriboside. Previous in vitro studies showed that the enzyme responsible for the initial phosphorylation of FdC is probably deoxycytidine kinase (2). Indirect evidence came from the observation that the cell line L1210, which is resistant to β-D-arabinofuranosylcytosine because it lacks deoxycytidine kinase, did not phosphorylate FdC. In addition, FdC was found to be a good substrate for calf thymus deoxycytidine kinase (17). Prevention of FdC antiviral activities by 2′-deoxycytidine was also reported for herpes simplex virus type 1 (HSV-1) and influenza viruses (17, 19).

The 2′-modified nucleoside analogues are an interesting series of compounds as potential inhibitors of RNA viruses. Previously, 2′-fluororibonucleosides showed a high level of inhibitory activity against influenza A and B virus strains and against parainfluenza virus 1 (17). In addition, FdC has been reported to possess anti-HSV-1 and -HSV-2, anti-pseudorabies virus, and anti-equine abortion virus activity (19). It has also been shown that FdC could be phosphorylated by the HSV-1 viral thymidine kinase (19). However, when FdC was tested against the cytopathogenic BVDV strain NADL in MDBK cells (14), the compound was unable to inhibit the production of viral RNA in the culture supernatant (EC_{90}, >100 μM; data not shown) and was not cytotoxic (CC_{50}, >100 μM). The inactivity of FdC in the MDBK-cpBVDV model may have resulted from (i) poor initial activation to FdC-monophosphate by the bovine deoxycytidine kinase, (ii) rapid deamination by the bovine cytidine deaminase or deoxycytidine deaminase to the inactive 2′-deoxy-2′-fluorouridine (FdU), or (iii) an inability of BVDV polymerase to recognize FdCTP as a substrate. Suggestive evidence for the lack of initial FdC phosphorylation can be correlated with observations of a lack of potency for gemcitabine (2′-deoxy-2′-difluorocytidine [dFdC]) in this surrogate model system. dFdC is an extremely potent anticancer agent with a CC_{50} in the low nanomolar range for most human cell lines (9), including the HCV replicon cell line (unpublished observations). However, in MDBK cells, dFdC showed a CC_{50} of 100 μM for confluently growing cells. These

findings illustrate that whatever the cause for the lack of activation of these compounds might be, the MDBK-cpBVDV model may not always be a reliable predictive surrogate model for drug discovery for members of the *Flaviviridae* and especially for HCV in human cells.

As mentioned above, the related compound FdU was inactive against the HCV RNA replicons at concentrations up to 100 μM (data not shown). A previous report also found that FdU was inactive against influenza A and B viruses, while FdC was highly active (17). This lack of activity for FdU was ascribed in part to the absence of cellular phosphorylating enzymes. It is therefore possible that these phosphorylating enzymes are also missing or altered in Huh-7 HCV replicon cells, which would explain the inactivity of FdU in this in vitro model system.

In conclusion, although FdCTP acts as a viral polymerase inhibitor, the defined off-target activity referenced for cellular polymerases suggests that some of the activity in the replicon system may be the result of inhibition of cellular functions. The 2′-deoxy-2′-fluororibosides are interesting antiviral compounds, but because they are also recognized by cellular polymerases, most of them exhibit delayed toxicity only after prolonged exposure in 6- or 7-day assays; however, this toxicity is absent in 3-day assays. Interestingly, animal toxicology studies of FdC and FdU indicated that no adverse clinical effects were observed in rats and woodchucks after 90 days of treatment, and the no-observed-adverse-effect level for both compounds was determined to be 500 mg/kg of body weight/day for the male rat and 7.5 mg/kg/day for the woodchuck (12). Based on these observations, the anti-HCV therapeutic potential of these 2′-fluoronucleosides merits further investigation.

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