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Activity of a Potent Hepatitis C Virus Polymerase Inhibitor in the Chimpanzee Model[▽]

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A-837093 is a potent and specific nonnucleoside inhibitor of the hepatitis C virus (HCV) nonstructural protein 5B (NS5B) RNA-dependent RNA polymerase. It possesses nanomolar potencies in both enzymatic and replicon-based cell culture assays. In rats and dogs this compound demonstrated an oral plasma half-life of greater than 7 h, and its bioavailability was >60%. In monkeys it had a half-life of 1.9 h and 15% bioavailability. Its antiviral efficacy was evaluated in two chimpanzees infected with HCV in a proof-of-concept study. The design included oral dosing of 30 mg per kg of body weight twice a day for 14 days, followed by a 14-day posttreatment observation. Maximum viral load reductions of 1.4 and 2.5 $\log_{10}$ copies RNA/ml for genotype 1a- and 1b-infected chimpanzees, respectively, were observed within 2 days after the initiation of treatment. After this initial drop in the viral load, a rebound of plasma HCV RNA was observed in the genotype 1b-infected chimpanzee, while the genotype 1a-infected chimpanzee experienced a partial rebound that lasted throughout the treatment period. Clonal analysis of NS5B gene sequences derived from the plasma of A-837093-treated chimpanzees revealed the presence of several mutations associated with resistance to A-837093, including Y448H, G554D, and D559G in the genotype 1a-infected chimpanzee and C316Y and G554D in the genotype 1b-infected chimpanzee. The identification of resistance-associated mutations in both chimpanzees is consistent with the findings of in vitro selection studies, in which many of the same mutations were selected. These findings validate the antiviral efficacy and resistance development of benzothiadiazine HCV polymerase inhibitors in vivo.

Hepatitis C virus (HCV) is a small, enveloped virus that contains a single-stranded, positive-sense RNA (2). The World Health Organization estimates that approximately 170 million people worldwide are infected with HCV (25). Of these, 130 million are chronic HCV carriers at risk for the development of liver cirrhosis and/or liver cancer. In the United States, HCV infection leading to liver failure is the major indication for liver transplantation (4, 12). The current standard of care, which consists of a combination of pegylated interferon and ribavirin, provides good clinical efficacy for patients infected with genotype 2 and 3 viruses but is less effective for patients infected with genotype 1 viruses. Subgenotypes 1a and 1b constitute the most commonly found isolates in the United States, Japan, and Western Europe; and thus, the development of effective treatments against genotype 1 viruses presents a pressing need.

HCV nonstructural protein 5B (NS5B) encodes a viral RNA-dependent RNA polymerase, an essential enzyme responsible for the replication of the viral genome. NS5B shares very limited sequence homology with cellular polymerases; hence, it is an attractive target for the development of antiviral therapy. Several classes of inhibitors, including nucleosides, nonnucleosides, and pyrophosphate mimics, that target this viral enzyme have been developed (3, 13). We have developed nonnucleoside inhibitors in the benzothiadiazine class and demonstrated their inhibitory activities in biochemical assays using purified polymerases as well as in vitro using the subgenomic replicon system (16, 19).

The chimpanzee remains the only recognized animal model susceptible to HCV infection for long periods of time (1, 9, 14). The chimpanzee model has been critical for the analysis of early events in viral infection. It is therefore a useful model system for investigation of the antiviral effects, pharmacokinetic (PK) properties, and resistance profiles of HCV polymerase inhibitors. Here we report on the results of a study in which HCV-infected chimpanzees were treated with the benzothiadiazine polymerase inhibitor A-837093.

MATERIALS AND METHODS

Inhibitors. The HCV polymerase inhibitor A-837093 (Fig. 1) and the HCV protease inhibitor BILN 2061 (8) were synthesized at Abbott.

Biochemical assay conditions. The activities of the inhibitors against HCV polymerases derived from genotypes 1a and 1b were determined in a standard [^{3}H]UTP incorporation assay with 50 nM purified enzyme and 20 nM template RNA containing the HCV 3′ untranslated region, as described previously (15).

Replicon assays. A genotype 1b strain N replicon and a chimeric genotype 1a strain H77-genotype 1b strain Con1 subgenomic replicon were licensed from

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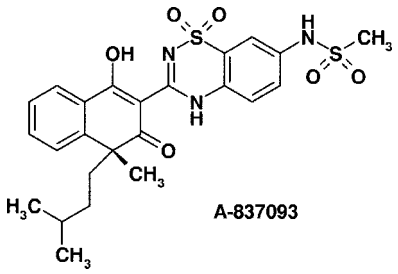

FIG. 1. Chemical structure of A-837093.

Stanley Lemon (UTMB, Galveston, TX) (26). In the chimeric replicon, the nonstructural genes NS3 (except for the N-terminal 73 amino acids), NS4A, and NS5B and the 3′ nontranslated region were derived from the genotype 1a strain H77 replicon; and the first 73 amino acids of NS3 along with all of NS4B and NS5A were from the genotype 1b strain Con1 replicon. The inhibitory potency of A-837093 against these HCV subgenomic replicons was measured on the basis of the reduction of the HCV RNA copy number in the presence of inhibitor. Cells containing subgenomic replicons were grown in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) and 400 μg/ml G418 (Invitrogen). Four thousand replicon-containing cells were seeded into each well of a 96-well plate. On the next day, the medium was removed and replaced with fresh medium containing 5% FBS plus inhibitor in a series of half-log dilutions. The cells were incubated for 4 days in the presence of inhibitor, after which total RNA was extracted with an RNeasy-96 kit (Qiagen) and the copy number of the HCV replicon RNA was determined by a TaqMan quantitative real-time reverse transcription-PCR (RT-PCR) assay. Cytotoxicity was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide colorimetric assay (21). The protein binding effect on inhibitor potency was assessed by including 40% human serum in culture medium containing 5% FBS during the 4-day incubation period with inhibitor.

PK profiles. A-837093 was formulated in polyethylene glycol 400 for single oral dosing of 5 mg/kg of body weight in rats, dogs, and monkeys. Serum samples were withdrawn at 0.3, 0.5, 1, 2, 4, 6, 9, 12, and 24 h postdosing. Plasma drug concentrations were determined by a liquid chromatography-mass spectrometry (LC-MS) assay, as follows. A-837093 was separated from plasma samples by protein precipitation with acetonitrile. A 250-μl aliquot of each sample or spiked standard was combined with 25 μl of internal standard and 500 μl of acetonitrile. The samples were vortexed vigorously for 1 min, followed by centrifugation for 10 min at 4°C. Each supernatant was transferred to a well of a 96-well plate and evaporated to dryness with a gentle stream of nitrogen. The samples were reconstituted with sequential aliquots of acetonitrile and 20 mM ammonium acetate. A-837093 and the internal standard were separated from each other and coprecipitated contaminants on a Clipeus 5-μm column (50 by 3 mm; Higgins Analytical, Inc.) with a 1:1 acetonitrile–20 mM ammonium acetate mobile phase at a flow rate of 0.3 ml/min. Analysis was performed on a Sciex API 2000 biomolecular mass analyzer with a turbo-ion spray interface in the negative ion mode. Detection was in the multiple reaction monitoring mode at *m/z* 516.2 to 437.0. A-837093 and internal standard peak areas were determined with Sciex TurboQuan software. The drug concentration of each sample was calculated by least-squares linear regression analysis (nonweighted) of the peak area ratio (parent/internal standard) of the spiked standards versus concentration. The method, generally evaluated over a concentration range of 0 to 6.4 μg/ml, was linear (correlation coefficient, >0.999), with mean accuracy values ranging from 96 to 107% of the theoretical values for the analysis of triplicate standards at seven separate concentrations. The limit of quantitation was estimated to be ~20 ng/ml from a 0.25-ml plasma sample.

Efficacy study with chimpanzees. The chimpanzees were housed at the Southwest National Primate Research Center at the Southwest Foundation for Biomedical Research. The animals were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* (20a), and all protocols were approved by the center's Institutional Animal Care and Use Committee. See Table 3 for the medical histories of the two chimpanzees used in this study. Neither animal had previously received any anti-HCV treatment. The design included oral dosing of the inhibitor as a suspension in aqueous solution at 30 mg per kg twice a day (BID) for 14 days, followed by a 14-day posttreatment observation. Serum samples were withdrawn at various times throughout the study period to determine the viral loads as well as the trough and the peak plasma drug levels (C_{min} and C_{max}, respectively). The C_{max} for the genotype 1a-infected animal is reported as the mean value from three samples, each taken 2 h after the a.m. dose on days 2, 3, and 4. The C_{max} for the genotype 1b-infected animal is reported as the mean value from five samples, each taken 2 h after the a.m. dose on days 2, 3, 4, 9, and 12. The C_{min} values were determined from a single sample taken from each animal 1 h before the a.m. dose on day 6. Viral load determinations were done by a TaqMan quantitative RT-PCR assay (9, 10). Plasma drug concentrations were determined by LC-MS, as described above.

Phenotype analysis of HCV NS5B genes isolated from chimpanzees. Viral RNA isolation and amplification of the HCV NS5B gene for both genotypic and phenotypic analyses were done as reported previously (24). In brief, viral RNA was isolated from serum, and the HCV NS5B gene was amplified with Superscript III reverse transcriptase (Invitrogen), followed by first-round PCR amplification with *Pfx* polymerase (Invitrogen). Adapters with restriction enzyme cleavage sites for PacI or AscI were included in the primers used for the second round of PCR. The products from the second-round PCR were first digested with PacI and AscI and were then ligated with similarly treated shuttle vector DNA, regenerating a functional subgenomic replicon. After transfection into *Escherichia coli*, the recombinant plasmid DNA was purified as a pool. The DNA pool was linearized with XbaI, and the linearized DNA was used in in vitro transcription reactions to generate RNA for transient replication analyses, as described previously (24).

Genotype analysis of HCV NS5B genes isolated from chimpanzees. Genotypic analyses of the HCV NS5B gene derived from chimpanzees at various treatment times were performed at both the population and the clonal levels. For clonal sequencing, individual colonies were isolated from *E. coli* harboring the recombinant shuttle vector plasmid generated as described above. The oligonucleotide primers used in the nested PCR described above were used to amplify cloned NS5B genes for DNA sequencing. After cloning of the HCV polymerase gene from each chimpanzee serum sample, between 20 and 40 colonies were picked and the polymerase gene from each colony was sequenced. For population analysis, the amplified product from the nested PCR was used as the sequencing template before it was cloned into the shuttle vector. Nucleotide sequences were generated by automated sequencing with a BigDye Terminator (version 3.1) cycle sequencing kit and a 3100 genetic analyzer (Applied Biosystems). The sequences were analyzed with Sequencher software (Gene Codes Corp., Ann Arbor, MI).

RESULTS

Antiviral potency. A-837093 (Fig. 1) is a potent inhibitor of HCV NS5B polymerase. In biochemical inhibition assays with purified recombinant enzyme, A-837093 showed potencies of less than 2 nM against polymerases derived from both HCV genotypes 1a and 1b (Table 1). Similar inhibitory activities were found against genotype 1 HCV polymerases derived from patient isolates and from other standard laboratory strains, such as genotype 1b strains BK and J4 (data not shown). In subgenomic replicon replication assays, A-837093 demonstrated a 50% effective concentration (EC_{50}) of 11 nM against genotype 1a strain H77 replicon and 3 nM against genotype 1b strain N replicon, while it exhibited a 50% cytotoxic concentration of greater than 29 μM. A-837093 displayed an approx-

TABLE 1. Activity of A-837903 against recombinant polymerase enzymes and HCV subgenomic replicons

Genotype	IC_{50}[a] (nM) against recombinant enzyme	EC_{50} (nM [fold reduction]) against HCV replicon		CC_{50}[b] (μM)
		0% human serum	40% human serum	
1a strain H77	1.25	11	143 (13)	29
1b strain N	0.33	3	42 (15)	

[a] IC_{50}, 50% inhibitory concentration.
[b] CC_{50}, 50% cytotoxic concentration.

TABLE 2. Plasma concentrations of A-837093 following oral dosing of 5 mg/kg in rats, dogs, and monkeys

Animal	$t_{1/2}$ (h)[a]	C_{max} (μg/ml)[b]	T_{max} (h)[b,c]	AUC (μg · h/ml)[b]	F (%)[b,d]
Rat	7.6	0.31 ± 0.13	4.2 ± 1.8	3.3 ± 0.5	62.7 ± 8.7
Dog	10.7	1.94 ± 0.39	0.4 ± 0.1	10.5 ± 1.4	89.3 ± 11.5
Monkey	1.9	0.52 ± 0.22	2.7 ± 0.7	1.5 ± 0.5	15.2 ± 4.9

[a] Values are harmonic means.
[b] Values are the means ± standard errors of the means of three animals each.
[c] T_{max}, time to C_{max}.
[d] F, bioavailability.

imately 14-fold reduction in inhibitory activity in the presence of 40% human serum.

PK profiles in rats, dogs, monkeys, and chimpanzees. The PK properties of A-837093 were investigated in rats, dogs, and monkeys (Table 2). A-837093 displayed a good PK profile in rats and dogs, with a half-life ($t_{1/2}$) of 7.6 h and bioavailability of 62.7% in rats and a $t_{1/2}$ of 10.7 h and bioavailability of 89.3% in dogs. The areas under the concentration-time curves (AUCs) were 3.3 and 10.5 μg · h/ml in rats and dogs, respectively. The PK parameters measured in monkeys demonstrated a shorter $t_{1/2}$, a lower AUC, and a lower bioavailability than those seen in rats or dogs.

Two chimpanzees were included in this study (Table 3). Chimpanzee 1 was infected in 1991 with the HCV-1 strain of genotype 1a by inoculation with serum from an infected chimpanzee (11). Chimpanzee 2 was infected with genotype 1b HCV in 1984 from a contaminated human factor VIII blood product. A-837093 was dosed in each animal at 30 mg/kg BID for 14 days. Serum samples were withdrawn after light sedation on the days indicated in Fig. 2. To estimate the C_{min}s and C_{max}s, blood samples were taken pre- and postdosing and the concentration of A-837093 was measured by LC-MS (Table 4). The C_{min} was measured 12 h after dosing, and the C_{max} was measured 2 h after dosing. Similar C_{min}s were found for both chimpanzee 1 and chimpanzee 2 and were 0.63 and 0.74 μg/ml, respectively. The C_{max}s were 5.49 ± 1.57 μg/ml (n = 3) for chimpanzee 1 and 8.56 ± 3.68 μg/ml (n = 5) for chimpanzee 2. Taken together, these data indicate that A-837093 maintained a plasma level ranging from 9-fold (chimpanzee 1, C_{min}) to 394-fold (chimpanzee 2, C_{max}) over the plasma protein-adjusted EC_{50} (Table 1) in the chimpanzees.

Antiviral activity in HCV-infected chimpanzees. The baseline viral loads in samples taken at three time points from each animal prior to the initiation of the study were determined. The samples from the genotype 1a- and 1b-infected chimpanzees were taken 5, 17, and 40 days and 11, 21, and 35 days prior to the first A-837093 dose, respectively. Samples from the genotype 1a-infected chimpanzee had viral titers ranging from 1.7×10^6 to 2.6×10^6 genome equivalents (ge) per ml, while in the genotype 1b-infected chimpanzee, the titers ranged from 5.2×10^6 to 8.4×10^6 ge per ml.

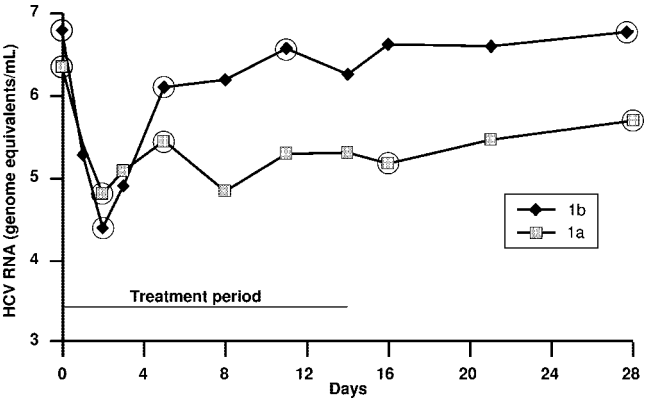

FIG. 2. HCV viral load changes during and after A-837093 treatment in the HCV genotype 1a- and genotype 1b-infected chimpanzees. Each animal was dosed with 30 mg/kg A-837093 BID for 14 days. Viral load determinations were done by a TaqMan quantitative RT-PCR assay. The samples chosen for genotypic and phenotypic analyses are circled.

A rapid and significant drop in the viral loads was observed for both chimpanzees over the first 2 days of dosing (Fig. 2). At day 2, HCV viral load reductions of 1.4 and 2.5 $\log_{10}$ copies/ml for the genotype 1a- and 1b-infected chimpanzees, respectively, were observed, providing in vivo validation of the antiviral efficacy of the benzothiadiazine polymerase inhibitor A-837093. Different viral responses were observed after 2 days of treatment in the two chimpanzees. A viral rebound to within fourfold of the baseline level was observed in the genotype 1b-infected chimpanzee by day 5. In contrast, A-837093 elicited a different viral load response in the genotype 1a-infected chimpanzee, particularly with respect to the rebound of the HCV titer. Although the initial fold drop in the HCV viral load was less in this animal, a viral load reduction of about 1.4 $\log_{10}$ copies RNA/ml was maintained in this animal throughout the treatment period, followed by a slow upward trend during the posttreatment observation period.

Genotypic analysis. Genotypic analyses were conducted to investigate the possible emergence of resistant mutants that would lead to a viral load rebound. Samples that were collected on days 2, 5, 11, and 28 from the genotype 1b-infected chimpanzee and on days 2, 5, 16, and 28 from the genotype 1a-infected chimpanzee were analyzed. Serum samples withdrawn from both animals before the start of therapy were used as the baseline controls and are indicated as day 0 samples in Fig. 2. Nucleotide sequence analyses at the population level revealed

TABLE 3. Medical history of the chimpanzees used in this study

Animal no.[a]	Age (yr)	Wt (kg)	Inoculum	No. of yr infected	HCV genotype	Baseline viral load (ge/ml)[b]
1	26	62	Chimpanzee serum	13	1a	$(1.99 \pm 0.5) \times 10^6$
2	24	61	Factor VIII	20	1b	$(6.78 \pm 1.6) \times 10^6$

[a] Both animals were males.
[b] Values are the means ± standard errors of the means of three bleed dates.

TABLE 4. Plasma concentrations of A-837093 in chimpanzees following oral BID dosing of 30 mg/kg

Animal (genotype)	2 h after dosing (C_{max})		12 h after dosing (C_{min})	
	A-837093 concn (μg/ml)	Fold above protein-adjusted EC_{50}	A-837093 concn (μg/ml)	Fold above protein-adjusted EC_{50}
1 (1a)	5.49 ± 1.57[a]	74	0.63	9
2 (1b)	8.56 ± 3.68[b]	394	0.74	34

[a] Values are the means ± standard errors of the means of three samples.
[b] Values are the means ± standard errors of the means of five samples.

TABLE 5. Genotypes and phenotypes of virus in serum samples of HCV genotype 1b-infected chimpanzee before and after treatment with A-837093

Day	Genotype	No. of sequences/ total no. (%)	EC_{50} (nM [fold reduction])	
			A-837093	BILN 2061
0	WT[a]	26/26 (100)	0.9	1.4
2	C316Y	7/21 (33)	25 (28)	1.3 (1)
	G554D	5/21 (24)		
	C316Y + G554D	2/21 (10)		
	WT	7/21 (33)		
5	C316Y	6/15 (40)	1,200 (1,333)	1.3 (1.1)
	G554D	5/15 (33)		
	C316Y + G554D	2/15 (13)		
	WT	2/15 (13)		
11	C316Y	6/37 (16)	1,800 (2,000)	1.1 (0.8)
	G554D	24/37 (65)		
	C316Y + G554D	1/37 (3)		
	WT	6/37 (16)		
28	C316Y	1/19 (5)	350 (389)	1.3 (1)
	G554D	8/19 (42)		
	C316Y + G554D	5/19 (26)		
	WT	5/19 (26)		

[a] WT = C316 and G554.

a general picture of genetic change throughout the treatment period. Careful inspection of sequence chromatograms suggested that there was a mix of wild-type (WT) Gly and mutant Asp at residue 554 in all four of the samples taken from the genotype 1b-infected animal after treatment started, although the actual proportion of these two residues was difficult to estimate from the chromatograms. A mix of WT Cys and mutant Tyr was evident at residue 316 in samples collected on days 2, 5, and 28 from the same animal. Population sequences derived from the genotype 1a-infected animal demonstrated that residue 63 was the only position with detectable heterogeneity. His and Tyr were found at this position in every sample analyzed both pre- and posttreatment and thus represent quasispecies and are unrelated to treatment with A-837093 (data not shown).

To get a clearer picture, we analyzed sequence changes at the clonal level. Overall, 119 and 118 clones were selected at different time points from the genotype 1a- and 1b-infected chimpanzees, respectively, for sequence analysis. The sequence variation was such that for 110 different residue positions, at least one clone displayed an amino acid sequence deviation from the predose baseline consensus sequence. Although we made every effort to reduce the introduction of artifacts during sample amplification and cloning steps, there is no reliable method to distinguish whether the observed changes were naturally occurring quasispecies variants in the chimpanzees or were artificially introduced. Therefore, our analysis focused on residues previously identified as conferring resistance to this class of compounds. Previously, our group demonstrated that the benzothiadiazine class of HCV polymerase inhibitors selects resistance-associated mutations in vitro (16, 19, 20). Mutations such as C316Y, Y448H, G554D, and D559G can confer tens to hundreds fold resistance to A-837093. In the present study we observed similar mutations in the chimpanzees treated with A-837093. In the samples from the genotype 1b-infected chimpanzee, clones with the G554D mutation demonstrated a steady increase from 24% at day 2 to 33% at day 5 and reached 65% at day 11 (Table 5). On the other hand, mutants with the variant C316Y peaked on day 5 at a level of 40% and then declined to 16% on day 11. At day 28, after a 14-day posttreatment observation, mutants with G554D and C316Y constituted 42% and 5% of the clones surveyed, respectively. Clones that had mutations at both residues 316 and 554 were maintained at about 10% on both day 2 and day 5. At day 28, clones containing both G554D plus C316Y still constituted 26% of the population. Further examination indicated that the levels of WT variants (i.e., variants lacking any mutation known to be associated with resistance to A-837093) were reduced from 100% before treatment to 13% to 33% during treatment and were maintained at 26% at the end of this study.

The situation with the genotype 1a-infected animal was somewhat different. During the treatment and recovery period, the presence of variants at residues G554 and D559 was observed (Table 6). However, unlike the variants with single mutations detected in the genotype 1b-infected chimpanzee, mutants with two variants at each location were found in samples from this animal. Asp and Ser changes at residue 554 and Gly and Asn at residue 559 were selected. In addition, variants of Cys or His were found at residue 448, replacing the WT Tyr, on day 28. However, in contrast to what was observed in the genotype 1b-infected chimpanzee, the proportion of clones harboring mutations at these locations remained low throughout the treatment period, reaching a peak of 58% at day 16 and declining to 30% by day 28.

Phenotypic analysis. Drug susceptibility studies with pools of shuttle vector clones derived from treated chimpanzee samples were carried out. BILN 2061, an HCV NS3 protease inhibitor whose EC_{50} value is independent of the sequence of the NS5B gene, was used as a control compound (17). A-837093 demonstrated an EC_{50} value of 0.9 nM against a sample derived from the genotype 1b-infected animal before dosing, which is in the same inhibitory range seen for this compound against standard laboratory replicon strains. EC_{50} values for A-837093 against each pooled sample are listed in Table 5. On day 2, an EC_{50} of 25 nM was measured, indicated greater than 25-fold drug resistance. The day 5 and day 11 samples exhibited more than 1,000-fold drug resistance. At the end of the study on day 28, which was 2 weeks postdosing, the sample still exhibited an EC_{50} of 350 nM, more than 380-fold

TABLE 6. Genotypes and phenotypes of virus in serum samples of HCV genotype 1a-infected chimpanzee before and after treatment with A-837093

Day	Genotype	No. of sequences/ total no. (%)	EC_{50} (nM [fold reduction])	
			A-837093[a]	BILN 2061
0	WT[b]	27/27 (100)	3.5	3.1
2	G554S	2/20 (10)	7.7 (2.2)	2.7 (0.9)
	WT	18/20 (90)		
5	G554D	2/19 (11)	4.5 (1.3)	3.1 (1)
	D559G	2/19 (11)		
	D559N	1/19 (5)		
	WT	14/19 (74)		
16	G554D	6/33 (18)	13.8 (3.9)	2.8 (0.9)
	G554S	2/33 (6)		
	D559G	8/33 (24)		
	D559N	2/33 (6)		
	G554D + D559G	1/33 (3)		
	WT	14/33 (42)		
28	Y448C	1/20 (5)	8.2 (2.3)	3.3 (1.1)
	Y448H	3/20 (15)		
	G554D	2/20 (10)		
	WT	14/20 (70)		

[a] Inhibition curves for A-837093 were biphasic, indicating a mixed population of sensitive and highly resistant variants. EC_{50} values are those for the predominant, sensitive population.
[b] WT = Y448, G554, and D559.

above the WT level. In this genotype 1b-infected chimpanzee, the reduction of inhibitor potency coincided with the presence of C316Y and G554D mutations, thus accounting for the drug resistance phenotype observed.

A different pattern was observed when we examined the inhibitory potency of A-837093 against samples derived from the genotype 1a-infected animal. EC_{50} values of 3.5 to 13.8 nM for A-837093 were measured, showing no more than a fourfold change after the initiation of treatment relative to that for the pretreatment sample, despite the high degree of resistance that the Y448H, G554D, and D559G mutations are known to confer when they are present in HCV subgenomic replicon clones (16). However, examination of the inhibition curves for some of these samples (Fig. 3) illustrates the difficulty in determining the phenotype of pooled samples containing resistant mutants in the presence of a significant proportion of WT clones, as was the case for samples from this animal. The EC_{50} values calculated for each of these samples are similar, as indicated by the point at which the dashed line at 50% inhibition first intersects the curves. However, it is clear from looking at the day 5 and the day 16 curves that they have a biphasic nature, making interpretation of drug susceptibility problematic. We have previously observed that when the phenotype of pooled samples containing both WT replicons and those with resistance-associated mutations is determined, if the mutations confer a reduction in replication fitness, very little shift in drug susceptibility relative to that of the WT is observed unless the resistant mutants constitute a significant majority of the samples in the pool (18).

DISCUSSION

In this study we report on the antiviral efficacy of the polymerase inhibitor A-837093 in reducing the viral load in both HCV genotype 1a- and 1b- infected chimpanzees. After an initial drop within 2 days of treatment initiation, the viral RNA load rebounded quickly in the genotype 1b-infected chimpanzee, but only a partial rebound was observed in the genotype 1a-infected animal. Analysis of serum samples from both animals identified the previously characterized in vitro resistance-associated mutations C316Y, Y448H, G554D, and D559G, which were likely responsible for the rebound (16, 19, 20).

Somewhat different responses toward A-837093 were displayed by the two chimpanzees used in this study. The levels of viral load reduction were not identical, despite similar drug levels in the sera of both animals. Although it is not clear why different responses were observed in the two animals, A-837093 was about fourfold less potent against a genotype 1a laboratory strain subgenomic replicon than against a genotype 1b laboratory strain (Table 1). A similar difference in potency was observed between the day 0 samples from the genotype 1a- and 1b-infected chimpanzees used in this study (Tables 5 and 6). In addition to the difference in the fraction of resistance-associated mutations observed in samples taken from the two animals, which may be related to the strength of selective pressure, different types of resistance-associated mutations were also observed. Single-residue substitutions known to confer high-level resistance to A-837093, such as C316Y and G554D, accounted for the majority of the changes observed in samples from the genotype 1b-infected animal, whereas mixtures at key residues, such as Y448H/C, G554D/S, and D559G/N, were seen in samples from the genotype 1a-infected animal. Some of the additional variants that were detected in the chimpanzee samples, such as Y448C, G554S, and D559N, were not found during the in vitro selection studies with subgenomic replicons. It is possible that these variants might confer lower levels of resistance, and the small number of each variant found in the genotype 1a-infected animal may have been the result of incomplete selection due to the lower potency of A-837093 in this chimpanzee compared to that in the

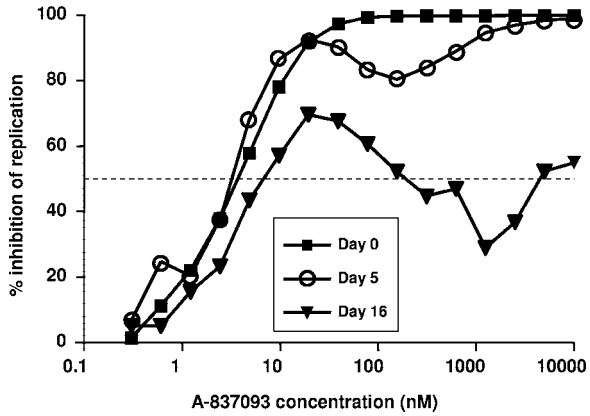

FIG. 3. Curves for inhibition by A-837093 of HCV subgenomic replicon containing NS5B cloned from the genotype 1a-infected chimpanzee samples before treatment and on days 5 and 16 after the initiation of treatment with A-837093. The dashed line indicates 50% inhibition level.

genotype 1b-infected animal. Alternatively, these mutations may exhibit a larger fitness cost and may thus be less likely to be selected during drug treatment in vitro due to their lower prevalence.

High levels of phenotypic resistance were observed in sample pools derived from the genotype 1b-infected animal, whereas sample pools derived from the genotype 1a-infected chimpanzee showed less than a fourfold increase in the EC_{50} value. Closer inspection of inhibitor-titration curves generated with samples taken from the genotype 1a-infected chimpanzee revealed that the curves had a biphasic nature, suggesting the presence of drug-resistant variants in the presence of a significant percentage of the WT, as previously described for pooled samples derived from HCV-infected individuals (18). This is consistent with the findings of the clonal analysis, which demonstrated that WT sequences were maintained in this animal at a level of at least 40% throughout the study period.

A structural model of the NS5B polymerase indicates that the residues involved in the binding of benzothiadiazines are largely conserved between subtypes 1a and 1b (data not shown). It is therefore expected that the same type of resistance-associated mutations might be found in samples from these two animals, since 24 of 26 residues that are located within 5 Å of the inhibitor binding site are identical. However, G554D was the only major resistance-associated mutation common to both subtypes. In contrast to C316Y, which was identified in genotype 1b samples, Y448H and D559G were found in genotype 1a samples. As most of the reported in vitro resistance selection studies use replicons from a single subtype (genotype 1b strain Con1), there are insufficient data to evaluate if the selection of subtype-specific treatment-related resistance-associated mutations is a common theme for HCV resistance, regardless of the class of inhibitor. In the human immunodeficiency virus (HIV) field, most of the treatment-related resistance-associated mutations were shared across subtype boundaries in patients undergoing highly active antiretroviral therapy. With the availability of more sequence information, in recent years some subtype-specific treatment-related resistance-associated mutations were observed in both HIV protease and reverse transcriptase (5, 6).

The detection of a higher frequency of Y448H/C variants over time in the samples from the genotype 1a-infected chimpanzee and the presence of clones containing C316Y and G554D in the genotype 1b-infected chimpanzee 14 days after treatment ended are notable. In contrast to the experience with HIV treatments, in which WT viruses generally repopulate following treatment cessation, the presence of drug resistance-associated variants Y448H, C316Y, and G554D 2 weeks after the termination of therapy implies that the fitness levels of these resistance-associated mutation-containing strains are similar to those of the WT strains in these two animals. It is also noteworthy that the mutant with the C316Y and G554D double mutation was present at a higher proportion in the sample taken from the genotype 1b-infected chimpanzee at day 28 than in samples taken at earlier time points. Reliable methods for determining the fitness of various resistant mutants in HCV from clinical samples need to be developed before the significance of the increase in prevalence of this mutant with double mutations can be evaluated. The potential for these resistance-associated mutations to persist in the absence of drug selection pressure should be investigated further. In vitro studies with recombinant genotype 1b strain N replicon clones containing Y448H or G554D showed that each possessed less than 30% of the replication capacity compared to that of the WT replicon (16). The presence of an adaptive mutation(s) in NS5B or in other genomic regions outside of NS5B, which may improve the fitness of resistant viruses, should be considered.

Finally, it should be acknowledged that since the current study included only one animal each infected with genotype 1a and 1b viruses, individual differences in both viral and host factors that might be involved in drug metabolism and the immune response should be taken into account when the results presented here are interpreted.

In summary, A-837093 is a potent and specific inhibitor of HCV polymerase. Its antiviral efficacy was demonstrated by using the chimpanzee model in the current study. The ability to select drug resistance-associated mutations by this inhibitor is in line with that in studies with other classes of inhibitors against both HCV polymerase and protease (17, 22, 23, 27). Several studies have demonstrated that combination therapy with different classes of inhibitors is able to overcome the selection of drug resistance-associated mutations against individual agents and eradicate HCV in replicon systems (7, 19, 22). To verify the possible selection of subtype-specific treatment-related mutations as well as the presence of persistent mutations, careful follow-up for both genotypic and phenotypic changes after the termination of treatment should be carried out. The data presented here clearly demonstrate that the chimpanzee model is a valuable tool for the development of treatments for HCV infection.

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