ANA598 displays potent *in vitro* antiviral activity against diverse clinical isolates of genotype 1 HCV in a transient replicon shuttle vector system

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ABSTRACT*

BACKGROUND: ANA598 is a novel HCV non-nucleoside polymerase inhibitor that is in clinical development for the treatment of chronic genotype 1 hepatitis C virus infection. Because of the high error and replication rate of the HCV NS5B polymerase, extensive sequence variation exists within and between infected individuals. To assess the variability of antiviral activity among HCV patient isolates and predict possible range of responses in a clinical setting, a transient replicon shuttle vector system was developed. This enabled the rapid determination of the *in vitro* potency of ANA598 against a diverse set of clinical isolates from sera of patients with HCV infection. This shuttle vector system also enables the characterization of drug sensitivities from patient sera before and during treatment with direct antivirals.

METHODS: The genotype 1b transient replicon system was modified to contain unique restriction sites at the 5' and 3' ends of the NS5B gene sequence to allow insertion of NS5B PCR amplicons from patient sera. The wild type NS5B gene sequence was replaced with a defective copy to prevent background contamination during cloning and, to boost the replication signal, a synthetic Polio virus IRES was introduced before the Luciferase gene. HCV RNA was isolated from patient sera and reverse transcribed. Nested PCR was used to amplify the NS5B gene to contain the unique restriction sites and cloned into the shuttle vector system. Several hundred clones from each patient sample were pooled and evaluated in the shuttle vector system to determine the EC_{50} of ANA598. The pooled clones were also population sequenced to compare the genotype and phenotype of each sample.

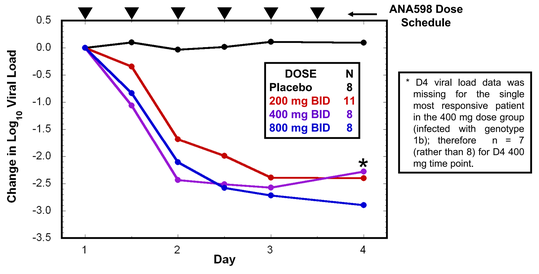
RESULTS: HCV genotype 1a and 1b patient samples from the US, Puerto Rico and the Netherlands were evaluated to determine the variability of *in vitro* antiviral activity. Nanomolar antiviral potency was observed for the clinical isolates tested with a mean EC_{50} for genotype 1b of 2.8 nM (n=10, 0.13 – 14.5 nM) and 29.2 nM for genotype 1a (n=9, 8 – 52 nM). Two genotype 1b samples contained an Y415F polymorphism and demonstrated reduced activity for ANA598 against these isolates (14.5 nM and 7.1 nM). F415 is the conserved amino acid for genotype 1a NS5B and may contribute to the difference in ANA598 activity between genotypes 1a and 1b.

CONCLUSION: All clinical isolates were fully sensitive to inhibition by ANA598. This is consistent with the robust clinical response observed upon administration of ANA598 as a single agent in HCV patients infected with genotype 1 virus *[EASL 2009: abstract LB 1055]*.

*Updated abstract

BACKGROUND

- ANA598 is a non-nucleoside polymerase inhibitor in clinical development for the treatment of chronic genotype 1 hepatitis C virus infection
- ANA598 binds to NS5B 1b with subnanomolar potency (K_D ~0.7nM) and dissociates slowly from the enzyme with a $t_{1/2}$ ~2 hours
- ANA598 exhibits nanomolar potency in assays with stable HCV replicons: EC_{50} (1b) ~3nM and EC_{50} (1a) ~50nM
- ANA598 exhibits no appreciable cellular cytotoxicity in the Huh-7 cell line as indicated by an XTT CC_{50} >100μM and cellular GAPDH EC_{50} >100μM
- The NOAEL for ANA598 in 26W rat and 39W cynomolgus monkey toxicology studies is 1000 mg/kg/day, the highest dose tested
- Low potential is observed for P450-mediated drug-drug interactions
- In treatment naive HCV genotype 1 patients, ANA598 resulted in rapid and sustained reductions in HCV RNA with median reductions at end of treatment (EOT, Day 4) exceeding 2 log_{10} at all dose levels. At 200 mg BID, the median viral load reduction was 2.4 log_{10} (range 0.4 to 3.4 log_{10}), at 400 mg BID, 2.3 log_{10} (range 1.6 to 3.5 log_{10}), and at 800 mg BID, 2.9 log_{10} (range 2.2 to 3.4 log_{10}). *[EASL 2009: abstract LB 1055]*



HCV NS5B SHUTTLE VECTOR CONSTRUCTION

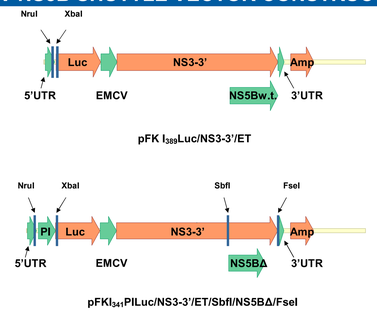


Figure 1. The transient replicon vector pFKI$_{389}$Luc/NS3-3'/ET containing the firefly luciferase reporter gene and the cell culture adaptive mutations (E1202G, T1280I, K1846T) was obtained from Dr. Bartenschlager[1]. This vector was modified by site directed mutagenesis to contain a unique SbfI and FseI site at the 5' and 3' ends of the NS5B gene sequence. A synthetic DNA fragment containing the Polio Virus IRES element was inserted between the NruI and XbaI sites to increase the luciferase signal[2] and a defective NS5B gene sequence replaced the wild type sequence to prevent any background replication signal.

CONSTRUCTION OF CHIMERIC REPLICONS

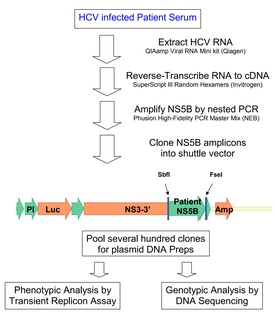


Figure 2. HCV infected sera were acquired from several sources. US samples were purchased from ProMedDx Norton, MA. Samples from Puerto Rico were obtained from the Fundación de Investigación de Diego. Samples from the Netherlands were from the Anadys ANA773-601B study *[AASLD 2009: abstract 1560]*.

PHENOTYPIC ANALYSIS

Table 1. Characterization of clinical isolates using ANA598

Cell ID	Clinical Isolate #	Genotype	Geography	EC_{50} (nM)	Comment
1462	con1	1b		0.9 ±0.3	pFKI$_{389}$Luc/NS3-3'/ET
1572	1228844	1b	US	0.5	
1573	1519358	1b	US	0.4	
1574	659049	1b	US	14.5	Y415F
1575	660021	1b	US	7.1	415Y/F mixed
1576	660043	1b	US	0.13	SbfI site in NS5B
1577	663152	1b	US	1.5	
1578	891249	1b	US	0.15	
1592	FD	1b	Puerto Rico	1.1	
1593	FA	1b	Puerto Rico	0.9	
1596	08015073 206	1b	Netherlands	1.3	
1533		1a		35 ±2	1a clone
1571	1432949	1a	US	15	
1583	LS	1a	Puerto Rico	41 ±18	
1585	JA	1a	Puerto Rico	14 ±10	
1587	1194386	1a	US	8	
1588	1194392	1a	US	16	
1589	1228824	1a	US	19 ±11	
1590	1422384	1a	US	48 ±22	
1605	08015096 207	1a	Netherlands	52 ±17	
1606	09000411 210	1a	Netherlands	50 ±10	

Transcripts of HCV replicons were generated using the Megascript T7 kit. Exponentially growing Huh7- Lunet cells were transfected with 4μg of replicon RNA with a Gene Pulser MXcell. Transfected cells were plated into 96-well plates with 7,500 cells per well. Compounds at various concentrations were added to the cells after 2 hours and were cultured for 4 days. The cells were lysed with the Bright-Glo Reagent and luciferase activity was measured with a Victor 2 luminometer. The EC_{50} values were determined using a standard four-parameter dose response equation. 18 genotype 1b isolates were originally examined. 5 failed to amplify and 3 contained an internal ScaI restriction site which is used to linearize the vector for transcription. 20 genotype 1a isolates were originally examined. 4 failed to amplify, 4 had a signal to noise ratio too low to measure and 3 had an internal ScaI restriction site. An HDV ribozyme has replaced the ScaI site in the current shuttle vector to address the prevalence of ScaI in the NS5B gene sequence (Data not shown). The potency of ANA598, in an independent transient 1a and 1b replicon system using a distinct panel of clinical isolates, was 1.1 – 14.7 nM (n = 10) and 0.6 – 2.6 nM (n = 8), respectively.

GENOTYPIC ANALYSIS

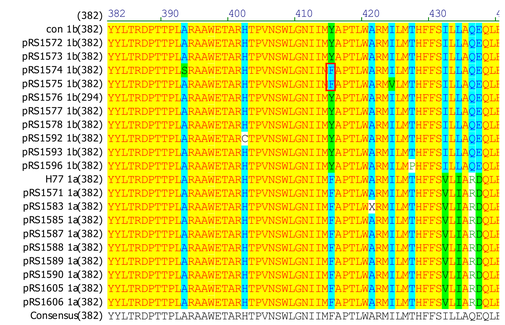


Figure 3. NS5B nucleotide population sequence data was determined by automated dye sequencing and assembled using LaserGene 8.0 SeqMan software. The protein sequence was translated and aligned using Clustal W with Vector NTI 11 along with the NS5B 1b "con1" sequence and NS5B 1a H77 as genotypic references. The red box indicates two genotype 1b samples that contain a Phenylalanine (Phe, F) substitution at position 415 instead of the more common Tyrosine (Tyr, Y).

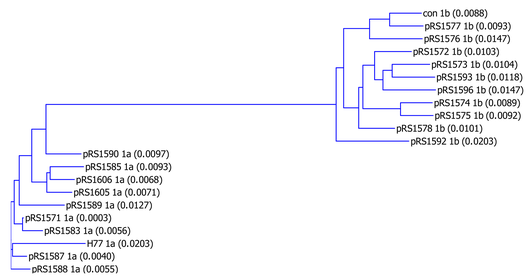


Figure 4. Phylogenetic guide tree calculated using the Neighbor Joining method from within Vector NTI 11, with the ignore gaps and Kimura's correction turned on, confirming the genotype assignment of the clinical samples.

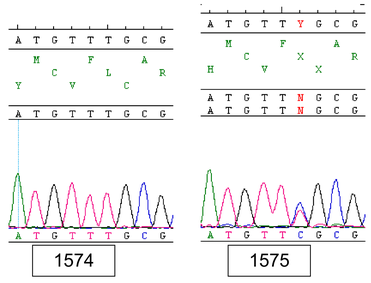


Figure 5. Chromatograms from Clinical isolate population sequencing showing the mixed Tyr and Phe codons at position 415 in sample 1575 which may help explain the intermediate phenotype observed for this sample.

ANA598 ACTIVITY AGAINST GENOTYPE 1a AND 1b REPLICONS CONTAINING TYR AND PHE AT NS5B POSITION 415

Table 2. Comparison of NS5B 415 Tyr and Phe in genotype 1a and 1b replicons

Mutation	Genotype	EC_{50} (nM)	Replication Capacity (%)
wt	1b	0.9 ±0.3	100
Y415F	1b	26 ±10	43 ±12
wt	1a	41 ±6	100
F415Y	1a	2.0 ±0.4	101 ±11

Site directed mutagenesis was used to introduce Phe at position 415 in the NS5B 1b sequence and Tyr at position 415 in the NS5B 1a sequence. The samples were assessed in the transient replicon assay. The frequency for Y415 in genotype 1a and F415 in genotype 1b in The Los Alamos HCV Sequence Database is 1.2% and 1.3% respectively[3].

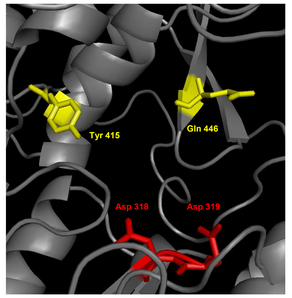


Figure 6. Cartoon ribbon drawing of the NS5B 1b "bk" palm binding site. In yellow are two residues that are different between genotype 1a and 1b near the active site catalytic Asp residues in red. Glutamine (Gln, Q) in genotype 1b is replaced by Glutamic Acid (Glu, E) in genotype 1a 99.4% in the Los Alamos HCV Sequence Database. Although position 316 (not highlighted) is an Asparagine (Asn, N) in the "bk" structure and Cysteine (Cys, C) in 99.4% of 1a and 59% of 1b sequences, that natural variation has little to no effect on ANA598 potency.

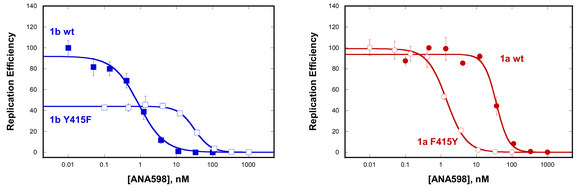


Figure 7. Dose response curves for ANA598 in the NS5B 1a and 1b 415Y/F transient replicon assay.

CONCLUSIONS

- ANA598 is a highly potent non-nucleoside inhibitor of HCV genotypes 1b and 1a NS5B polymerases from a diverse set of clinical isolates with a mean EC_{50} for genotype 1b of 2.8 nM and 29.2 nM for genotype 1a.
- The difference in activity observed for ANA598 against genotypes 1a and 1b can be attributed in part by the variation at position 415 in the NS5B gene sequence.
- The population sequence analysis confirmed the lack of pre-existing variants, known to reduce the potency of ANA598, in any of the clinical isolates amplified *[AASLD 2008: abstract 1908]*.
- Clinical isolate sensitivity to ANA598 is consistent with the robust response observed upon administration of ANA598 as a single agent to HCV patients infected with genotype 1.

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2. Friebe P, et al., J. of Virology, 2001: 75:12047-12057
3. Kuiken C, et al., Bioinformatics, 2005; 21(3):379-384

DISCLOSURES

Richard E. Showalter – Employee of: Anadys Pharmaceuticals, Inc., Stock Holder of: Anadys Pharmaceuticals, Inc.; Peggy Thompson – Employee of: Anadys Pharmaceuticals, Inc., Stock Holder of:Anadys Pharmaceuticals, Inc.; Kevin R. Steffy – Former Employee of: Anadys Pharmaceuticals, Inc., Stock Holder of: Anadys Pharmaceuticals, Inc.; James R. Appleman – Employee of: Anadys Pharmaceuticals, Inc., Stock Holder of: Anadys Pharmaceuticals, Inc.