were tracked via 454 sequencing over a 10 year period in two patients who failed to achieve viral clearance after SOC combination therapy supplemented with BI 201335. Frequencies of characterised resistance mutations pre- and post-therapy were also assessed. Treatment failure protease genes were engineered into sub-genomic replicons (SGRs) for phenotyping.

Methods: 454 sequencing was performed on amplicons generated from seven pre-treatment samples, in addition to one post-treatment sample, for both patients. Nested PCR, followed by barcoded adaptor ligation and bidirectional sequencing ensured complete protease gene coverage at high-depth. Genomic cDNA input for each 454 amplicon was ascertained via qPCR to avoid making erroneous claims about viral population diversity. Pre- and post-therapy protease genes were engineered into SGRs for cross-resistance testing.

Results: 454 sequence re-sampling due to low genomic copy number input was identified by qPCR. Protease amino acid population consensuses remained stable for over a decade in both patients, with a large excess of synonymous mutations observed. Post BI 201335 therapy, a quantitative reduction in sites exhibiting polymorphism occurred. In one patient, enrichment of Q80L mutations (7667/8104 reads) was observed. This mutation was present in pre-therapy samples, but below conventional sequencing detection levels (213/5512 reads). Engineering the Q80L mutation into SGRs, as well as pre- and post-treatment proteases, indicate Q80L does not confer cross-resistance to Boceprevir or Telaprevir.

Conclusions: Quantification of viral genomic input prior to 454 sequencing is essential to avoid resampling. 454 sequencing reveals a quantitative reduction in the total amount of sites exhibiting polymorphism post BI 201335 therapy, indicating a population bottleneck occurred. Enrichment of Q80L mutations occured post-treatment. Interestingly, previous studies indicate Q80L does not confer resistance to BI 201335 and our phenotyping suggest Q80L does not confer cross-resistance to Boceprevir or Telaprevir. Future therapeutic options are thus not compromised. Additionally these data suggest Q80L is not selected for during therapy, and is selectively neutral, but becomes fixed in the population due to the post-therapy bottleneck.

1190

STUDY OF ABT-267 2-DAY MONOTHERAPY FOLLOWED BY 12-WEEK COMBINATION THERAPY IN TREATMENT-NAÏVE PATIENTS WITH CHRONIC HCV GENOTYPE 1 INFECTION

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Background: ABT-267 is an HCV NS5A inhibitor currently being studied in combination with ABT-450/r (HCV protease inhibitor dosed with ritonavir, identified as a lead compound by Abbott and Enanta) and ABT-333 (non-nucleoside HCV NS5B inhibitor) +/− ribavirin (RBV) for treatment of patients with chronic HCV infection. This study assessed safety and efficacy of two doses of ABT-267 administered for 2 days as monotherapy followed by 12 weeks of combination treatment + RBV.

Methods: Treatment-naïve, non-cirrhotic patients with HCV GT1 infection received 1.5 or 25 mg ABT-267 as monotherapy for 2 days, followed by 12 weeks of 1.5 mg (Arm 1) or 25 mg (Arm 2) ABT-267 once-daily + ABT-450/r 150/100 mg once-daily + ABT-333 400 mg twice-daily + weight-based RBV 1000–1200 mg total daily dose.

Results: Twelve patients received >1 dose of study drug, of which 5 (41.7%) were male and 11 (91.7%) were white. Mean age was 49.2 years; mean BMI was 29.9 g/m^2. The majority were GT1a-infected. Two of 12 subjects discontinued prematurely: one adverse event (AE), one lost to follow up.

Mean maximal decreases from baseline during ABT-267 monotherapy were −1.6 and −3.1 $\log_{10}$ IU/mL ($p = 0.035$) in Arm 1 and 2, respectively. Additional baseline characteristics and virologic responses are in the table.

All 10 subjects who completed treatment with the pegIFN-free combination regimen of ABT-267+ABT-450/r+ABT-333+RBV achieved SVR_{12}.

Two patients reported serious adverse events (labyrinthitis and mania). Both AEs were considered unrelated to study drug by the investigator. Potentially clinically significant laboratory abnormalities were: blood bilirubin ≥2×ULN (1 patient in Arm 1, 2 in Arm 2), potassium <3 mmol/L (1 patient, Arm 2), triglycerides >5.7 mmol/L (1 patient, Arm 2).

	Arm 1 (N = 6) 1.5 mg ABT-267	Arm 2 (N = 6) 25 mg ABT-267
Baseline Characteristics		
IL28B non-CC genotype, n (%)	3 (50.0)	4 (66.7)
GT1a infection, n (%)	5 (83.3)	4 (66.7)
BL HCV RNA, $\log_{10}$ IU/mL ±SD	6.4±0.5	6.1±0.5
Virologic response		
RVR (HCV RNA <LLOQ at week 4), % (n/N)	83.3 (5/6)[a]	100 (6/6)
cEVR (HCV RNA <LLOQ at week 12), % (n/N)	83.3 (5/6)[a]	83.3 (5/6)[a]
eRVR (HCV RNA <LLOQ at week 4 through week 12) , % (n/N)	83.3 (5/6)[a]	83.3 (5/6)[a]
ITT SVR_{12}, % (n/N)	83.3 (5/6)[a]	83.3 (5/6)[a]
OD SVR_{12}, % (n/N)	100 (5/5)	100 (5/5)

[a]One subject in each treatment arm prematurely discontinued study drug (one due to a serious event and one lost to follow up) and the study and were imputed as a failure.

Conclusions: ABT-267 monotherapy followed by 12 weeks of combination therapy provided high rates of SVR_{12} (Intent To Treat (ITT): 83.3%, observed data (OD) 100%). In this study, treatments were well-tolerated and virologic response was durable through 12 weeks post-treatment. Based on monotherapy response, 25 mg ABT-267 will be used in combination with ABT-450/r and ABT-333 in future studies.

1191

GS-5816, A SECOND GENERATION HCV NS5A INHIBITOR WITH POTENT ANTIVIRAL ACTIVITY, BROAD GENOTYPIC COVERAGE AND A HIGH RESISTANCE BARRIER

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Background: The polymorphic nature of the HCV NS5A gene can lead to variable antiviral responses within and between HCV genotypes. Here, we report the discovery of a second-generation NS5A inhibitor, GS-5816, that has potent in vitro antiviral activity across all HCV genotypes and retains potency against common NS5A polymorphisms and resistance mutations.

Methods: Antiviral activity was studied in 3-day HCV replicon assays using stable or transient replicons encoding the NS5A gene from different genotypes. Site-directed mutagenesis was used to introduce major polymorphs or resistance mutations. Colony reduction assays were performed in stable GT1–4 and GT6 replicon cells to assess resistance barriers.

Results: GS-5816 has potent and consistent antiviral activity against representative GT1–6 HCV strains (EC_{50}=7–59 pM; Table-1) with low cytotoxicity (CC_{50}>44 mM). Relative to nM potencies for the first-generation NS5A inhibitors, GS-5816 has potent activity against consensus GT2a and GT2b replicons encoding the dominant NS5A residue M31 (EC_{50}=17 and 10 pM respectively). Furthermore, GS-5816 has consistent potency (EC_{50} values varying <3-fold) against >50 replicons representing >90% of NS5A polymorphism in GT1–4. In colony reduction assays and in vitro resistance selections

GS-5816 has a significantly higher resistance barrier than the first-generation NS5A inhibitors; and GS-5816 has potent activity against major clinical resistance mutations (e.g. EC_{50}=130 and 121 pM against GT1a L31M and Y93C respectively, and 210 pM against GT3a A30K). GS-5816 is fully-active against mutants resistant to other classes of HCV inhibitors (e.g. Protease inhibitor resistant mutants R155K or D168Q in NS3, and nucleotide NS5B inhibitor resistant mutant S282T in NS5B), and shows additivity to moderate synergy when combined with other anti-HCV agents including sofosbuvir.
Conclusions: GS-5816 is a second-generation HCV NS5A inhibitor with potent and selective antiviral activity, broad genotypic and polymorphic coverage, and a favorable resistance profile for HCV genotypes 1 to 4 and 6. This preclinical activity profile of GS-5816 together with its preclinical pharmacokinetics supports its development as part of a regimen to treat chronic HCV infection worldwide.

Table 1. Broad Genotypic Activity of GS-5816

EC_{50} (pM)	GT1a H77	GT1b Con-1	GT2a JFH-1	GT2a J6*	GT2b MD2b*	GT3a S52	GT4a ED43	GT5a SA13	GT6a HK6a
GS-5816	13	15	9	17	10	13	9	59	7

1192
VAST MAJORITY OF DETECTED NS5A RESISTANT VARIANTS ARE NOT AMPLIFIED IN HCV PATIENTS DURING 3-DAY MONOTHERAPY WITH THE OPTIMIZED NS5A INHIBITOR PPI-668

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Background: HCV NS5A inhibitor PPI-668 was assessed in a 3-Day Phase 1b monotherapy trial in HCV gt-1 patients using QD oral doses of 40, 80, 160 and 240 mg, with 8/10 patients receiving PPI-668 per cohort. Mean maximal viral load reductions of 3.54–3.75 log_{10} IU/mL were observed with doses ≥80 mg QD. A fifth cohort of gt-2/3 patients (160 mg QD) was also evaluated.
Methods: Comprehensive monitoring of HCV resistant variants was performed during dosing and post-dosing periods. HCV RNA was extracted from patient plasma samples, RT-PCR amplified and the resulting DNA subjected to population sequencing. Clonal sequencing was performed to determine if detected substitutions were genetically linked. PPI-668 susceptibility was assessed in transient transfection assays using HCV replicons encoding specific substitutions or population NS5A gene inserts from clinical samples.
Results: Known NS5A primary resistance substitutions at residues 28, 30, 31 and 93 were detected early during PPI-668 monotherapy. Among the 40 enrolled gt-1 patients, eight (one placebo) had detectable NS5A resistance substitutions at baseline. One patient (gt-1b 240 mg) was a non-responder and fully resistant at baseline, with 100% of his circulating virus encoding genetically linked R30Q+L31I+Y93H substitutions. The other six PPI-668 treated patients with baseline resistance substitutions responded well (RNA reductions of 2.82 to 3.95 log_{10} IU/mL). Resistance substitutions became detectable in all but one PPI-668 treated patient by 24–48 hr, as WT virus was rapidly eliminated. Importantly, these observed substitutions (≤30% of patient population) were not further amplified with continued monotherapy, suggesting that PPI-668 concentrations were sufficient to suppress these single-substitution HCV variants. Susceptibility (EC_{90}) of replicons derived from PPI-668 treated patient samples were generally at or below C_{min} levels, confirming the advantageous PK profile of PPI-668 and its ability to cover single-substitution resistant variants. No significant differences were observed in the overall resistance patterns across the four PPI-668 treated gt-1 cohorts.
Conclusions: NS5A resistance variants frequently pre-exist among HCV patients, emphasizing the need for combination therapy and optimized NS5A inhibitors such as PPI-668 that achieve plasma/liver levels high enough to suppress single substitution HCV variants. Further studies of PPI-668 in combination with other DAAs are warranted.

1193
RESISTANCE ANALYSES USING DEEP AND POPULATION SEQUENCING AFTER 3 DAY MONOTHERAPY WITH GS-9669, A NOVEL NON-NUCLEOSIDE NS5B INHIBITOR IN GENOTYPE 1 HCV PATIENTS

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Background: GS-9669, a novel NS5B non-nucleoside inhibitor (NNI, site II), displayed potent antiviral activity in HCV genotype (GT) 1 subjects during a multiple ascending dose clinical trial at 50 mg, 500 mg QD and 50 mg, 100 mg, 500 mg BID for 3 days. This study characterizes the virologic resistance observed in this phase I trial.
Methods: The full-length NS5B gene was amplified and population sequenced for all patients (GT1a, n = 50; GT1b, n = 20) at baseline (BL), Day 4 (or earlier timepoint if viral load was <1000 IU/mL) and Day 17. Illumina deep sequencing analysis was performed for GT1a (n = 7) and GT1b (n = 7) patients dosed with 500 mg QD at multiple timepoints through Day 17 (n = 90). NS5B from 46 patients was cloned into an NS5B shuttle vector and phenotypic analyses were performed.
Results: Population sequencing showed that from the on-treatment (Day 1 or 2) or end of treatment (Day 4 or 5) samples, resistance-associated mutations (RAMs) at NS5B positions A486, R422 and L419 were detected in the majority of patients who received 500 mg QD and ≥50 mg BID, but only in 1/8 patients receiving 50 mg QD. Substitutions at position M423 were observed only in GT1a patients receiving low doses (50 and 100 mg BID). Deep sequencing detected RAMs as early as 24hr post dosing in 5/8 patients receiving 500 mg QD. At end of treatment, 100% of tested samples had at least 4 RAMs by deep sequencing compared to 75% by population sequencing. On Day 17, mutations were still detected in 92% of patients (compare to 44% by population sequencing). New mutations in GT1b isolates (M423T, V494A/I I482N/T) were observed by deep sequencing but not by population sequencing. Phenotypic analysis demonstrated that viral isolates with multiple RAMs had reduced susceptibility to GS-9669 and VX-222, but wild-type susceptibility to other classes of HCV inhibitors including sofosbuvir (NI), GS-9451 (PI), GS-5885 (NS5A) and ribavirin.
Conclusions: Similar to other NNIs, RAMs were detected shortly after suppression of the wild-type virus. The lack of cross-resistance between GS-9669 resistant-mutants and sofosbuvir, GS-5885, GS-9451 and ribavirin, makes GS-9669 a candidate for use in combination with these inhibitors.