Sequence and Phenotypic Analysis for Resistance Monitoring in Hepatitis C Virus Drug Development: Recommendations From the HCV DRAG

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Development of direct acting antiviral (DAA) agents for the treatment of hepatitis C virus (HCV) infection[1–3] (Supplementary Table 1) has reached the stage that human immunodeficiency virus (HIV) found itself a decade ago: rapid accumulation of information without clear consensus on the drug development pathway. The Forum for Collaborative HIV Research[4] (the Forum) assembled the HCV Drug Development Advisory Group (HCV DRAG), a group of experts from academia, industry, regulatory agencies, and community, with the goal to develop recommendations for HCV drug development. In this article we address the assessment of drug resistance through HCV sequence and drug susceptibility analysis, with the aim of streamlining HCV drug development through consensus recommendations and establishing coherent nomenclature. Table 1 lists the definitions of terms used throughout the article.

Introduction

Recent review publications have described overall concepts of HCV drug resistance.[5]

The goal of HCV therapy—sustained virologic response—requires sustained inhibition of replication and elimination of drug-susceptible and drug-resistant variants. In the absence of complete suppression, DAAs may select for pre-existing variants with decreased DAA susceptibility.[2] Rigorous HCV resistance monitoring during drug development will lay the foundation for our understanding of the clinical impact of drug resistance, and promote the development of strategies to avoid and/or overcome it.

HCV DRAG Sequence and Phenotype Analysis Recommendations

Determining Which Region to Analyze

All new drug candidates require preclinical research providing information on the genomic regions where mutations are most likely to appear, the genetic barrier to resistance, specific resistance pathways and their effect on overall drug susceptibility, and potential patterns of cross-resistance with other DAAs.

Clinical studies require sequencing and phenotypic analysis of the target viral region in variants emerging on treatment. Extra-target regions possibly involved in resistance and/or fitness should be analyzed for a subset of virologic failure samples. Inability to explain virologic failure, or suspicion of nontarget region changes, may require analysis of additional regions.

Sample Collection and Timing of Sequencing and Phenotypic Analysis

Frequent collection and long-term storage of samples allows examination of target regions according to viral load decay and rebound kinetics. Sequence and phenotypic analysis of variants present at baseline and at specific study time points provide insight into potential

Abbreviations used in this paper: **DAA, direct acting antivirals; HCV DRAG, Hepatitis C Drug Development Advisory Group; HIV, human immunodeficiency virus; RC, replicative capacity; EC_{50}, 50% inhibitory effective dose.**

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Table 1. Definition of Key Terms Used

Terms	Definition
Biochemical assays	In vitro assay used to quantify enzymatic or other biochemical activity in the absence/presence of compounds; inhibitory potency generally is represented as the 50% inhibitory concentration (K_i)
Cell-based assays	In vitro assay used to quantify viral replication and antiviral activity of compounds in cell culture HCV replication models; inhibitor potency in these systems generally is represented as the EC_{50} Cytotoxicity assays should be run in parallel (CC_{50})
Clinical resistance	The association of treatment failure with certain and specific viral genotypic or phenotypic characteristics that confer enhanced ability of the virus to replicate while on treatment (as opposed to low adherence or poor drug exposure, and so forth)
Drug susceptible/susceptibility	Preferred term for the ability of a virus to be inhibited by a drug
Genetic barrier to resistance	Ease with which a virus can escape drug pressure: at the molecular level it is represented by the number of nucleotide changes that result in resistance and it depends on viral fitness and level of resistance conferred
Quasispecies	Population of closely related but distinct viral variants
Replication capacity	Capacity of a virus or replicon to replicate (eg, reproduce and survive) in cell-based assays using an infectious virus or replicon cell-based system; most often assessed in the absence of drug 100% replication capacity is denoted as the level of replication obtained with a wild-type virus or replicon in the absence of drug
Viral fitness	Capacity of a virus to reproduce and survive in a particular environment; monitored in patients through longitudinal studies
Viral variant	Single molecular clone virus entity within a virus quasispecies
Wild-type virus	Fully susceptible predominant virus population present in treatment-naive patients at baseline, before initiating any exposure to antiviral therapy

resistance pathway(s). The two methodologies should be applied to concurrent samples as follows.

Pretreatment (baseline) samples. Pretreatment samples are analyzed to detect known or novel predominant polymorphisms and provide the comparator for mutations emerging at later time points during or after treatment. Quasispecies containing high proportions of potentially resistant variants and a high degree of sequence diversity in target gene(s) are of special interest. Phenotypic analysis should be performed on samples from patients with identified resistance mutations at baseline,[6] or who experience nonresponse.

On-treatment samples. Viremic samples are analyzed to determine specific changes associated with decreased susceptibility and virologic failure. Longitudinal intrapatient analysis during viral plateau and/or breakthrough may illuminate quasispecies evolution, which mutations most likely are associated with reduced susceptibility, and which mutations contribute to higher levels of resistance and/or improved fitness.

Phenotypic analysis should be performed on concurrent samples to assess the level of decreased susceptibility relatable to specific mutations and to the baseline sample.

Interpretation of data from samples at viral suppression needs to take into account the fact that different genotypes, subtypes, or specific regions differ in the minimum viral load required for amplification. Low template numbers negatively impact assay performance in 2 ways: (1) the relative proportion of each variant detected increases as a result of reduced amplification of co-existing minority variants, potentially yielding larger discrete changes in frequency estimates; and (2) the chances of re-sampling increases (Supplementary Figure 1).[7]

Post-treatment samples. Persistence or loss of resistant variants implies their lesser or greater fitness loss compared with wild type, respectively. The appearance or reappearance of specific variants may help distinguish between re-infection and relapse.

Relapse samples. Detection of resistant virus implies low levels of resistant variants that were not eradicated on treatment.

Technical Recommendations for Sequencing and Phenotypic Methodologies

Population and clonal analysis. Population sequencing will suffice for simple mutation patterns, but does not adequately describe linkage between different substitutions if none of them are present at levels greater than 50%. Variant frequency in mixed populations is not proportionally represented by changes in drug susceptibility, possibly owing to differing replicative capacities (RCs).[8–10]

Clonal analysis is recommended for complex mutation patterns and assessment of variant frequency (Figure 1).

Firm conclusions regarding linkage of mutations must be based on multiclonal analysis and verified by sequencing of products/clones generated from multiple independent polymerase chain reactions. The use of templates generated from single viral genomes should be considered, as has been performed for HIV.[11] Although a minimum of 20 clones should be sequenced to evaluate resistance mutations identified with population sequenc-