Therapeutic implications of hepatitis C virus resistance to antiviral drugs

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Abstract: Treatment of chronic hepatitis C is currently based on a combination of pegylated interferon- α and ribavirin. Neither drug exerts direct selective pressure on viral functions, meaning that interferon- α /ribavirin treatment failure is not due to selection of interferon- α - or ribavirin-resistant viral variants. Several novel antiviral approaches are currently in preclinical or clinical development, and most target viral enzymes and functions, such as hepatitis C virus protease and polymerase. These new drugs all potentially select resistant viral variants both *in vitro* and *in vivo*, and resistance is therefore likely to become an important issue in clinical practice.

Keywords: hepatitis C virus, resistance, interferon- α , ribavirin, protease inhibitors, polymerase inhibitors

Introduction

Hepatitis C virus (HCV), like human immunodeficiency virus (HIV) and hepatitis B virus (HBV), is prone to develop resistance to antiviral drugs. Indeed, HCV can establish a chronic infection during which viruses are continuously generated and cleared. In addition, HCV has rapid replication dynamics: the half-life of free virions was recently estimated at 2–3 hours, and about 10^{12} particles are produced and cleared each day [Neumann *et al.* 1998]. Finally, HCV has a quasi-species distribution in a given individual, meaning that a broad spectrum of genetic variants, including potentially drug-resistant variants, are always present when antiviral therapy is initiated [Pawlotsky, 2003, 2006]. However, unlike HIV and HBV, HCV replicates in the cytoplasm of its host cells and has no known genetic form of intracellular persistence. This explains why chronic HCV infection can be eradicated by antiviral therapy. It also raises the possibility that, in future, all infected cells will be able to be cleared before resistant variants can gain a foothold.

Treatment of chronic hepatitis C is currently based on a combination of pegylated interferon (IFN)- α and ribavirin. Neither drug exerts direct selective pressure on viral functions, meaning that IFN- α /ribavirin treatment failure is not due to selection of IFN- α - or ribavirin-resistant viral

variants. Several novel antiviral approaches are currently in preclinical or clinical development [Pawlotsky *et al.* 2007] and most target viral enzymes and functions. These new drugs all potentially select resistant viral variants both *in vitro* and *in vivo*, and resistance is therefore likely to become an important issue in clinical practice.

Interferon- α and ribavirin treatment failure

Antiviral mechanisms of IFN- α and ribavirin

Interferons are natural cellular proteins with a variety of actions, including induction of an antiviral state in their target cells, cytokine secretion, recruitment of immune cells and induction of cell differentiation. Their metabolism and molecular mechanisms were recently reviewed [Chevaliez and Pawlotsky, 2009]. After subcutaneous administration, IFN- α binds specifically to high-affinity receptors that are present on the surface of most cells, including hepatocytes. IFN- α binding to its receptor triggers a cascade of intracellular reactions that activate numerous IFN-inducible genes. The products of these genes mediate the cellular actions of IFN- α . They are responsible for the antiviral effects of IFN- α , through two distinct but complementary mechanisms: (1) induction of a nonvirus-specific

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antiviral state in infected cells, resulting in direct inhibition of viral replication (and potentially also in noninfected cells, reducing the chances that they will become infected); and (2) immunomodulatory effects that enhance the host's specific antiviral immune responses and may accelerate the clearance of infected cells [Chevaliez and Pawlotsky, 2009].

Direct inhibition of HCV replication by IFN- α has been demonstrated *in vitro* in the subgenomic replicon, a synthetic replication system using HCV nonstructural proteins in HuH7 cells, as well as in the JFH1 infectious cell culture system and in primary cultures of normal human hepatocytes, the model closest to the naturally infected human liver [Kim *et al.* 2007; Lanford *et al.* 2003; Castet *et al.* 2002; Frese *et al.* 2001; Guo *et al.* 2001]. The IFN-induced proteins and enzymatic pathways involved in establishing the antiviral state are not entirely known. The numerous effectors that act in concert include the 2'-5' oligoadenylate synthetase (2'-5' OAS) system, Mx proteins, and double-strand-RNA-dependent protein kinase (PKR), as well as other, less well-characterized or unknown IFN-induced intracellular pathways [Chevaliez and Pawlotsky, 2009].

IFN- α binding to its receptors at the surface of immune cells also triggers complex and intricate effects, such as the induction of class I major histocompatibility complex antigen expression, activation of effector cells (macrophages, natural killer cells and cytotoxic T lymphocytes), and complex interactions with the cytokine cascade [Tilg, 1997; Peters, 1996]. It also stimulates the production of type 1 T-helper (Th1) cells and reduces the production of Th2 (suppressor) cells [Tilg, 1997; Peters, 1996]. Together, these properties mean that IFN- α could theoretically accelerate infected cell clearance in the presence of an adapted immune response, although this has not yet been experimentally demonstrated in HCV infection.

Ribavirin is a synthetic guanosine analog that undergoes intracellular phosphorylation. Ribavirin triphosphate is the main intracellular metabolite and is responsible for ribavirin's effects. *In vivo*, ribavirin has a significant but moderate (<0.5 log HCV RNA level reduction on average) and transient (days 2 and 3 of administration) inhibitory effect on HCV replication in about 50% of patients [Pawlotsky *et al.* 2004].

This could be related to the weak inhibitory action of ribavirin on the RNA-dependent RNA polymerase (RdRp) of *Flaviviridae*, observed *in vitro* [Lau *et al.* 2002]. However, this effect is too weak and transient *in vivo* to select viral variants resistant to ribavirin. The principal effect of ribavirin is to prevent viral breakthroughs during treatment and relapses after treatment in patients who respond to the antiviral effect of IFN- α [Bronowicki *et al.* 2006]. Ribavirin appears to accelerate the clearance of infected cells, through unknown mechanisms. Ribavirin inhibits inosine monophosphate dehydrogenase (IMPDH), thus transiently depleting intracellular GTP pools [Lau *et al.* 2002]. However, potent specific IMPDH inhibitors fail to mimic the effects of ribavirin, either alone or in combination with IFN- α [Marcellin *et al.* 2007; Hézode *et al.* 2006]. Mathematical modeling of HCV kinetics during combination therapy suggested that ribavirin makes HCV virions less infectious, thereby diminishing *de novo* hepatocyte infection in a context of efficient inhibition of virus production by IFN- α [Dixit *et al.* 2004]. Here too the underlying mechanisms are unclear. Recent studies suggest that ribavirin is an RNA mutagen, driving viral quasispecies to 'error catastrophe'; that is, loss of fitness by lethal accumulation of nucleotide mutations during replication [Contreras *et al.* 2002; Crotty *et al.* 2001, 2000]. However, no acceleration of mutagenesis has been observed during ribavirin therapy of human HCV infection [Chevaliez *et al.* 2007; Lutchman *et al.* 2007]. More recent reports suggest that ribavirin could amplify intracellular IFN- α responses, again through unknown mechanisms [Feld *et al.* 2007]. Ribavirin also has putative immunomodulatory properties, such as the ability to tilt the Th1/Th2 balance towards Th1 [Fang *et al.* 2000; Hultgren *et al.* 1998].

IFN- α -ribavirin treatment failure

IFN- α and ribavirin fail to eradicate HCV in 50–60% of patients infected with genotype 1 (and probably 4) and about 20% of patients infected with genotypes 2 and 3 [Fried *et al.* 2002; Hadziyannis *et al.* 2002; Manns *et al.* 2001]. Treatment failure in these patients is not due to IFN- α and/or ribavirin-resistant variants [Ward *et al.* 2008; Pawlotsky *et al.* 1999, 1998]. Instead, it is determined by a conjunction of several factors, including the precise drug regimen, host factors, disease characteristics, and viral factors (Figure 1).

Figure 1. Factors explaining IFN- α and ribavirin treatment failure.

Exposure to higher doses of both pegylated IFN- α and ribavirin improves the chances of HCV clearance during treatment [Fried *et al.* 2008; Lindahl *et al.* 2005]. Patient characteristics such as older age, male gender, race, overweight, active alcohol intake and intravenous drug use reduce the chances of successful treatment [Hadziyannis *et al.* 2002; Fried *et al.* 2002; Manns *et al.* 2001; McHutchison *et al.* 1998; Poynard *et al.* 1998]. Genetic factors and intrahepatic expression of various genes involved in IFN signaling prior to treatment also appear to influence responsiveness to IFN- α [Sarasin-Filipowicz *et al.* 2008; Randall *et al.* 2006; Chen *et al.* 2005]. Advanced fibrosis and cirrhosis and HIV coinfection are associated with a lower chance of a sustained virological response [Chung *et al.* 2004; Pawlotsky, 2004; Torriani *et al.* 2004; Poynard *et al.* 1998; McHutchison *et al.* 1998].

HCV genotypes 1 and 4 are intrinsically more resistant than genotypes 2 and 3 to the antiviral action of IFN- α . In addition, within each genotype, different HCV strains may have very different sensitivities to IFN- α [Neumann *et al.* 2002; Pawlotsky *et al.* 2002]. In addition, clearance of infected cells in patients who respond to IFN- α often occurs later and more slowly in patients infected by HCV genotype 1 or 4 than in those infected by genotype 2 or 3 [Neumann *et al.* 2002; Pawlotsky *et al.* 2002]. The molecular mechanisms underlying this genotype- and strain-specific sensitivity to IFN- α and its influence on the final outcome of IFN- α -ribavirin therapy are unknown. It is noteworthy that treatment does not select viruses that are intrinsically

resistant to IFN- α [Pawlotsky *et al.* 1999, 1998]. In addition, because of its modest and transient antiviral effect, ribavirin does not select ribavirin-resistant viruses in patients in whom treatment fails to eradicate the infection [Ward *et al.* 2008]. A recently identified amino acid substitution thought to confer resistance to ribavirin [Young *et al.* 2003] in fact appears to be a relatively common polymorphism of the HCV RdRp with no influence on treatment outcome [Ward *et al.* 2008].

Resistance to specific HCV inhibitors

Viral resistance to specific inhibitors

Long-term administration of specific viral inhibitors frequently leads to the emergence of viral resistance; that is, the selection of viral variants bearing amino acid substitutions that alter the drug target and thereby confer reduced susceptibility to the drug's inhibitory activity. Experience with highly active antiretroviral therapy (HAART) in HIV-infected patients shows that resistance to HIV reverse transcriptase inhibitors occurs through the selection of pre-existing resistant variants and the accumulation of new amino acid substitutions that confer stepwise decreases in the level of drug susceptibility, although often at a cost of reduced replicative capacity [Clavel and Hance, 2004]. Partial resistance conferred by a substitution present in a preexisting viral population may allow the virus to replicate at a level sufficient for further mutations to accumulate. In addition, selection of so-called compensatory mutations may restore

the *in vivo* fitness of the resistant virus, allowing viral replication to return to near-pretreatment levels [Clavel and Hance, 2004]. A similar process occurs during treatment of chronic hepatitis B with HBV inhibitors such as lamivudine, telbivudine, adefovir and entecavir [Pawlotsky *et al.* 2008; Fournier and Zoulim, 2007; Shaw *et al.* 2006]. *In vitro* and *in vivo* cross-resistance has been reported between antiviral drugs targeting the same site/function. It is mediated by amino acid substitutions that confer reduced susceptibility to the different drugs.

Drug-resistant viral variants may have different kinetics in different patients, depending on the level of resistance conferred by the amino acid substitution(s) and the relative fitness of the variants *in vivo*. Resistance is usually associated with a typical escape pattern, with restoration of baseline replication levels (amino acid substitutions conferring a high level of resistance without impairing fitness in the presence of the drug). A gradual resumption of viral replication may occur if the fitness of the resistant virus is poor; a resistant viral variant may also be selected with no effect on antiviral drug efficacy if the variant naturally replicates at low levels and/or the drug retains partial antiviral efficacy [Baldick *et al.* 2008; Pallier *et al.* 2008, 2006]. The ability of resistant viruses to replicate in the presence or absence of the drug may have considerable implications for patient outcome.

HCV infection is prone to the development of resistance to specific antiviral inhibitors, for the reasons mentioned above, namely the quasispecies nature of the virus, its rapid dynamics, and the error-prone RdRp [Pawlotsky 2003; Neumann *et al.* 1998]. It has recently been estimated that all single mutants and about 10% of all potential double mutants (i.e. viruses with one and two amino acid substitutions, respectively) pre-exist in all infected patients. Thus, a group of ten or more patients will harbor all conceivable double mutants, while all potential single mutants and 0.1–1% of all potential double mutants are still produced each day during the first days of inhibitor administration [Perelson, A.S., unpublished observations].

New HCV inhibitors

Advances in virology have led to the development of novel therapeutics specifically targeting HCV [Pawlotsky *et al.* 2007]. Indeed, every step of the HCV lifecycle constitutes a potential target for

one or several classes of inhibitor molecules with one or several target sites (Table 1).

HCV entry can be inhibited by specific antibodies that neutralize infectious particles and prevent their attachment to receptor molecules, or by specific entry-blocking molecules that, theoretically, belong to one of two groups: molecules that specifically fix to HCV surface structures and neutralize the virus, and molecules that compete with infectious viral particles for receptor occupation. So far, only polyclonal and monoclonal antibodies have been tested in clinical trials. Inhibition of later steps of HCV entry, such as membrane fusion, is also theoretically possible.

Nucleic acid-based strategies have been developed to inhibit HCV open reading frame translation into the HCV polyprotein. Antisense DNA and RNA oligonucleotides and ribozymes have been reported to inhibit HCV gene expression *in vitro*, but their clinical development has been halted for a lack of antiviral efficacy and/or toxicity *in vivo* [McHutchison *et al.* 2006; Soler *et al.* 2004]. Small interfering RNAs (siRNA) and short hairpin RNAs (shRNA) targeting the HCV 5' noncoding region inhibit HCV translation in various models [Kanda *et al.* 2007; Korf *et al.* 2007; Prabhu *et al.* 2006]. However, because of their size and chemical composition, these molecules require parenteral administration. One limitation of sequence-based nucleic acid drug design is that it is strictly dependent on the predicted secondary structure of the target RNA sequence. The three-dimensional functional internal ribosome entry site complexed with cellular and viral proteins and ribosomal subunits offers a promising target for small-molecule inhibitors.

In the near term the most promising HCV inhibitors are highly selective potent peptidomimetic inhibitors of HCV NS3/4A protease. Several drugs have reached phase I, II or III clinical development, including telaprevir (VX-950, Vertex Pharmaceuticals, Cambridge, Massachusetts), boceprevir (SCH 503034, Schering-Plough Corporation, Kenilworth, New Jersey), TMC 435350 (Tibotec, Mechelen, Belgium), ITMN-191/R7227 (InterMune, Brisbane, California, and Roche, Basel, Switzerland), MK-7009 (Merck, Whitehouse Station, New Jersey) and BI 201335 (Boehringer-Ingelheim, Ingelheim, Germany). When administered once to three times a day, these drugs have potent antiviral

Table 1. Novel antiviral approaches in HCV infection [other approaches may be in early preclinical development without having been publicized].

Inhibition of viral entry	HCV virion neutralization Specific entry inhibitors – targeting the virus – targeting the receptor complex
Inhibition of fusion	
Inhibition of polyprotein translation	Nucleic acid-based strategies Specific small molecule inhibitors
Inhibition of polyprotein processing	NS3/4A protease inhibitors – peptidomimetic inhibitors of NS3/4A protease – inhibitors of NS3-NS4A interaction NS2 protease inhibitors
Inhibition of replication	Direct RdRp inhibitors – nucleoside/nucleotide analogs – non-nucleoside inhibitors (NNIs) NS5A inhibitors Cyclophilin inhibitors
Inhibition of late steps in the HCV lifecycle	Glycoprotein maturation inhibitors

efficacy, reducing the HCV RNA level by 3–4 log on average after 3–14 days of single-agent therapy [Sarrazin *et al.* 2007; Reesink *et al.* 2006]. Adding an NS3/4A protease inhibitor to pegylated IFN- α and ribavirin combination therapy increases the sustained virological response rate by about 20% and shortens the necessary treatment period in patients with HCV genotype 1 infection [Hézode *et al.* 2009; McHutchison *et al.* 2008; Forestier *et al.* 2007]. NS3/4A serine protease function can also be inhibited by preventing NS4A cofactor binding to the NS3 protease: this inhibits the formation of the active protease complex, thereby preventing polyprotein processing [Pottage *et al.* 2007].

The HCV replication process is particularly complex and therefore offers a large variety of targets for antiviral intervention [Pawlotsky *et al.* 2007]. Direct inhibitors of the HCV RdRp belong to two categories, namely nucleoside/nucleotide analogs and non-nucleoside inhibitors. Nucleoside/nucleotide analogs target the catalytic site of the viral enzyme. A prodrug of a cytidine analog, R7128 (Pharmasset, Princeton, New Jersey, and Roche), is in clinical development. This molecule potently inhibits viral replication and its effect is at least additive to that of IFN- α and ribavirin. Non-nucleoside inhibitors (NNIs) target allosteric sites of the RdRp. Five such sites, designated A to E, have been identified within the RdRp structure, and a number of molecules are in preclinical or early clinical development [Pawlotsky *et al.* 2007]. Site A is located in the thumb-fingertips domain of the RdRp and is a target for indoles and benzimidazoles; site B is located in the thumb domain and can be targeted by

phenylalanine derivatives, thiophenes, dihydroxypirones and pyranindoles; site C located in the palm domain is a target for benzothiadiazines, acyl-pyrrolidines, proline sulfonamides and acrylic acid derivatives; and site D can be targeted by benzofurans [Koch and Narjes, 2006]. There may also be other target sites for NNIs. At least three molecules are currently in clinical development, namely GS-9190 (Gilead Sciences, Foster City, California), which targets site E through elusive mechanisms, filibuvir or PF-00868554 (Pfizer, New York, New York), and ANA598 (Anadys Pharmaceuticals, San Diego, California).

So-called ‘NS5A inhibitors’ have been tested in various models *in vitro*. The function of NS5A in the HCV lifecycle is still unknown, the three-dimensional structure of the entire protein has not been determined, and no functional assay is available. As a result, the supposed action of these drugs on NS5A is based solely on their inhibitory effect in replicon assays and on the selection of amino acid substitutions in the NS5A protein sequence. BMS-790052 (Bristol-Myers Squibb, New York, New York) was recently shown to potently inhibit HCV replication both *in vitro* and in patients infected by HCV genotype 1.

Cyclophilins are host cell proteins that seem to play a critical role in HCV replication, probably by interacting with viral components of the replication complex. Synthetic non-immunosuppressive inhibitors of cyclophilins are being tested in patients with chronic HCV infection. DEBIO-025 (DebioPharm, Lausanne, Switzerland) is a potent inhibitor of HCV replication both

in vitro and *in vivo*. Inhibition of HCV replication is independent of the HCV genotype, and the effects of DEBIO-025 are at least additive with those of IFN- α [Flisiak *et al.* 2008; Paeshuyse *et al.* 2006]. Other cyclophilin inhibitors are in development, including NIM 811 (Novartis, Basel, Switzerland) and SCY-635 (Scynexis, Research Triangle Park, North Carolina). Inhibitors of the late steps of HCV life cycle are also in development.

Resistance to NS3/4A protease inhibitors

Peptidomimetic inhibitors of the NS3/4A protease select resistant HCV variants both *in vitro* and *in vivo*. Exposure of HCV genotype 1 replicons to low concentrations of these drugs in cell culture resulted in the selection of variants bearing amino acid substitutions that conferred various levels of resistance to the corresponding drug. Several substitutions, including those at positions 155, 156 and 168 of the protease, conferred cross-resistance to different molecules [He *et al.* 2008; Tong *et al.* 2008; Lin *et al.* 2005]. Three-dimensional modeling showed that the principal resistance substitutions are located near the NS3 protease catalytic triad. Any change at one of these positions may thus alter the affinity of the drug for the enzyme catalytic site and thereby attenuate its inhibitory activity [Welsch *et al.* 2008; Zhou *et al.* 2008, 2007]. Resistance substitutions generally reduce the catalytic efficiency of the protease [Dahl *et al.* 2007; Tong *et al.* 2006; Yi *et al.* 2006], but compensatory substitutions at other positions may restore fitness without affecting the level of resistance [Yi *et al.* 2006]. Other amino acid substitutions may reduce drug susceptibility by indirectly altering the conformation of the drug-binding site.

The level of resistance conferred *in vitro* by a given amino acid substitution may vary slightly from one drug to another. For instance, among several substitutions selected *in vitro* by both telaprevir or boceprevir, Q41R, F43S and V170A have been shown to confer slightly higher levels of resistance to boceprevir than telaprevir, whereas V36M, R155K and V36M + R155K confer slightly higher resistance to telaprevir than boceprevir, and similar levels of resistance were noted with T54A and A156V/T [Tong *et al.* 2008]. R155K is predominantly selected by ITMN-191/R7227 *in vitro*, while A156T/S confers a higher level of resistance to telaprevir than other substitutions, including R155K/Q, T54A, V170A, and V36M + R155K [He *et al.* 2008].

Replicons resistant to both boceprevir and a non-nucleoside HCV RdRp inhibitor have recently been selected by simultaneous exposure to low concentrations of the two drugs [Flint *et al.* 2008].

The liver of chimeric *scid*-Alb/uPA humanized mice contains grafted human hepatocytes that can be infected by HCV and produce relatively large amounts of the virus. Resulting HCV titers in serum are similar to those seen in humans [Mercer *et al.* 2001]. Amino acid substitutions that confer resistance to HCV protease inhibitors have been reported to be selected in HCV-infected *scid*-Alb/uPA mice treated with BILN-2061 (Boehringer-Ingelheim, Ingelheim, Germany), the first peptidomimetic NS3/4A protease inhibitor, which was dropped because of myocardial toxicity in animals [Kneteman *et al.* 2006].

In humans, most resistance data have been obtained with telaprevir. Telaprevir monotherapy selects resistant viral populations within days to weeks. The time of relapse depends strongly on the level of exposure to the drug [Kieffer *et al.* 2007; Sarrazin *et al.* 2007]. The following telaprevir resistance substitutions have been reported, by order of increasing resistance: V36A/M (3.5- to 7-fold increase in the 50% inhibitory concentration (IC₅₀) compared to wild-type sensitive virus); T54A (6- to 12-fold); R155K/T (8.5- to 11-fold); V36A/M + R155K/T (57- to 71-fold); A156V/T (74- to 410-fold); and V36A/M + A156V/T (>781-fold) (Figure 2) [Kieffer *et al.* 2007; Sarrazin *et al.* 2007]. *In vivo* fitness appeared to be the principal determinant of the kinetics of resistant variants during treatment. The most resistant, but least fit, variants were those with substitutions at position 156. They were selected early during therapy but were rapidly replaced by less resistant but fitter variants bearing substitutions at positions 155, 36 + 155 and 36 + 156 at the time of virological breakthrough [Kieffer *et al.* 2007; Sarrazin *et al.* 2007].

The very early selection of resistant viral populations in patients receiving telaprevir is in keeping with the notion that these variants pre-exist in the vast majority of HCV-infected patients at the time of treatment initiation. Their rapid growth during single-agent therapy suggests that the fitness of these natural polymorphic variants is relatively high, albeit lower than that of the

Figure 2. Main amino acid substitutions conferring HCV resistance to telaprevir. The figure shows a 3D representation of the NS3 protease domain (cyan) bound to its cofactor NS4A (yellow). The three residues involved in the NS3 protease catalytic site (His57, Ser139 and Asp181) are shown in blue. The amino acid positions where substitutions have been shown to confer resistance to telaprevir are shown as balls and sticks: Val36 (yellow), Thr54 (orange), Arg155 (green), Ala156 (red) and Asp168 (pink). The second diagram is a magnified area of the residues. The third diagram shows the area in detail while the fourth attempts to convey the fact that the protein is compact and not full of empty space. Figure by Laetitia Barbotte.

wild-type sensitive virus, explaining why they are rarely detected before therapy [Bartels *et al.* 2008; Kuntzen *et al.* 2008; Lopez-Labrador *et al.* 2008]. Differences in resistance profiles between HCV subtypes 1a and 1b have been reported. For instance, amino acid substitutions at position R155 require only one nucleotide change in HCV subtype 1a isolates. Therefore,

variants with changes at this amino acid position often pre-exist and are selected by telaprevir in most patients infected with this subtype. In contrast, two nucleotide changes are required to generate an amino acid change at position R155 in subtype 1b isolates, accounting for the higher 'genetic barrier' to resistance in this subtype. Telaprevir resistance is thus less frequent in

patients infected with HCV subtype 1b, and variants bearing substitutions at position R155 are rarely selected.

HCV resistance to telaprevir is significantly less frequent when the drug is administered in combination with pegylated IFN- α or with both pegylated IFN- α and ribavirin [Hézode *et al.* 2009; McHutchison *et al.* 2008; Forestier *et al.* 2007]. The recent PROVE2 phase II trial showed that ribavirin is needed to efficiently prevent telaprevir resistance in patients receiving pegylated IFN- α and telaprevir in combination [Hézode *et al.* 2009]. This effect appeared to be due to ribavirin-induced acceleration of infected cell clearance in IFN- α -telaprevir responders, through unknown mechanisms. As a result, sustained virological responses were significantly more frequent in patients receiving the triple combination of telaprevir, pegylated IFN- α and ribavirin than in patients receiving pegylated IFN- α plus ribavirin or pegylated IFN- α plus telaprevir (without ribavirin) [Hézode *et al.* 2009]. Breakthroughs during treatment and relapses after treatment were frequent in the latter group, and were characterized by the selection of mixtures of telaprevir-resistant HCV variants with complex on-treatment and post-treatment dynamics, driven by the *in vivo* fitness of the variants. Although less frequent in patients treated with the triple combination of telaprevir, pegylated IFN- α and ribavirin, breakthroughs and relapses were also associated with the selection of telaprevir-resistant variants [Hézode *et al.* 2009]. Other amino acid changes were often observed together with primary resistance substitutions and probably conferred better fitness to the resistant HCV populations [Chevaliez S. *et al.*, unpublished observations]. Dynamic changes in viral populations generally continue after telaprevir withdrawal. Telaprevir-resistant variants sometimes acquire sufficient fitness during therapy to persist as one (or the only) dominant population. In other instances the wild-type virus takes over a few weeks or months after treatment cessation [Kieffer *et al.* 2007]. The resistant variants probably still replicate at low, undetectable levels in the latter patients. However, as HCV is incapable of episomal persistence in infected cells, resistance substitutions are not archived and it is possible that resistant variants are finally cleared as a result of competition with fitter, drug-sensitive viruses.

Few data are available on boceprevir resistance *in vivo*. Selection of boceprevir-resistant variants,

associated with virological breakthrough, has been reported to be frequent in prior nonresponders to pegylated IFN- α and ribavirin treated with the triple combination of pegylated IFN- α , ribavirin and a relatively low dose of boceprevir [Schiff *et al.* 2008]. The reported substitutions so far include those found in telaprevir resistance and V170A [Curry *et al.* 2008].

A novel class of nonpeptidomimetic inhibitors of HCV NS3/4A protease has been described. These agents appear to inhibit NS4A cofactor binding to NS3, a step required to activate the protease function. The lead molecule, ACH-806 or GS-9132 (Achillion, New Haven, Connecticut, and Gilead Sciences), has been reported to reduce HCV replication by approximately 1.5 log on average. Its development has been halted because of nephrotoxicity, but other molecules of the same class are under study. *In vitro* experiments based on the replicon system have identified two single mutations (C16S and A39V) at the N-terminus of NS3. Both are involved in interactions with the central hydrophobic region of NS4A and confer resistance to ACH-806 but no cross-resistance to peptidomimetic NS3/4A inhibitors such as telaprevir and boceprevir [Yang *et al.* 2008].

Resistance to nucleoside RdRp inhibitors

Nucleoside analog inhibitors of RdRp select resistant variants in the replicon system. 2'-methyl nucleosides and 4' azido-cytidine have been reported to select substitutions at positions S282 and S96/N142, respectively [Ali *et al.* 2008; Le Pogam *et al.* 2006]. These RdRp substitutions result in a moderate loss of antiviral activity but in a large reduction in replicative capacity [McCown *et al.* 2008; Dutartre *et al.* 2006]. This contrasts with substitutions selected by NS3/4A protease and non-nucleoside RdRp inhibitors, that confer higher levels of resistance and do not profoundly affect replicative capacity. Therefore, expansion of viral populations bearing substitutions that confer resistance to nucleoside RdRp analogs is expected to occur more rarely and less rapidly during treatment.

The single S282T substitution within the active site of the RdRp leads to a loss of sensitivity to 2'-methyl nucleosides in both the replicon model and cell-free RdRp enzyme assays. Resistance results from a combination of reduced affinity of the mutant RdRp for the drug and from an increased ability to extend the incorporated

nucleoside analog [Migliaccio *et al.* 2003]. Administration of MK-0608 (Merck), a 2'-methyl nucleoside, to HCV-infected chimpanzees selected S282T/R/I substitutions that were transiently present at the time of post-treatment relapse but were rapidly replaced by wild-type S282 variants (Olsen, D. *et al.*, unpublished data). In a phase IIb trial, nonresponders to IFN- α and ribavirin were treated with valopicitabine (NM 283, Idenix Pharmaceuticals, Cambridge, Massachusetts), another 2'-methyl nucleoside, the clinical development of which has been halted because of gastrointestinal toxicity. A modest reduction in HCV RNA was observed in patients receiving valopicitabine monotherapy for 48 weeks. Several patients who relapsed after 16–20 weeks harbored HCV variants bearing the S282T substitution. Late relapse could be due to the only modest effect of valopicitabine on wild-type virus replication, leaving little space for resistant viruses to replicate. The S282T substitution has been reported to confer a 3- to 6-fold loss of sensitivity *in vitro* to PSI-6130, the active derivative of R7128. R7128 monotherapy at various doses for a few weeks did not select resistance-associated substitutions. Longer administration in combination with pegylated IFN- α and ribavirin is under study.

R1626 (Roche) is a 4' azido cytidine which selects the S96T and N142T substitutions *in vitro* [Ali *et al.* 2008]. It has been administered alone at increasing doses for 14 days, and in combination with pegylated IFN- α and ribavirin for up to 4 weeks. No *in vivo* selection of resistance substitutions has been reported so far [Pockros *et al.* 2008; McCown *et al.* 2008], but development of this drug has been halted because of severe lymphopenias.

Resistance to non-nucleoside RdRp inhibitors

A number of non-nucleoside RdRp inhibitor families have been identified. Several compounds are in preclinical or early clinical development. These molecules essentially target five allosteric sites, designated A to E, at the surface of the RdRp. In the replicon system *in vitro*, they have been shown to select numerous resistance-associated substitutions at positions generally close to their target sites [Flint *et al.* 2008; Howe *et al.* 2008, 2006; Le Pogam *et al.* 2008, 2006; Lu *et al.* 2007a, 2007b; Mo *et al.* 2005; Tomei *et al.* 2004, 2003; Migliaccio *et al.* 2003; Nguyen *et al.* 2003]. In the chimpanzee model, A-837093

(Abbott, Abbott Park, Illinois), a benzothiadiazine targeting non-nucleoside site C, has been shown to select the following resistance-associated substitutions during 28 days of administration: G554D/S, D559G/N, and Y448C/H in a genotype 1a-infected animal; C316Y and G554D in a genotype 1b-infected animal [Chen *et al.* 2007].

The first non-nucleoside analog administered to HCV-infected patients was HCV-796, a benzofuran targeting site D (Viropharma, Exton, Pennsylvania, and Wyeth, Madison, New Jersey). In patients infected with HCV genotype 1, this drug reduced HCV replication by approximately 1.5 log at doses between 500 and 1500 mg bid. However, most patients experienced a relapse after 3–5 days of administration, and a C316Y substitution was found in the majority of cases, the same as that selected in the replicon system by this class of drugs [Flint *et al.* 2008; Howe *et al.* 2008]. Development of this drug has been halted, owing to ALT elevation during long-term administration in combination with pegylated IFN- α and ribavirin. Other non-nucleoside inhibitors of HCV RdRp belonging to various classes are currently in development.

Resistance to other inhibitors of HCV replication

HCV replication can also be inhibited by targeting components of the replication complex. NS5A inhibitors are currently being developed. Although their precise mode of action is not known, they have been shown to select resistance substitutions in the NS5A sequence in the replicon system.

Cyclophilin inhibitors are nonimmunomodulatory analogs of cyclosporine A. Several molecules have been shown to potently inhibit HCV replication, suggesting that cyclophilins, which are host cell proteins, are an important functional component of the replication complex. DEBIO-025 has reached clinical development and has been shown to possess potent antiviral activity independent of the HCV genotype. Interestingly, *in vitro* studies have shown that cyclosporine A and other cyclophilin inhibitors, including DEBIO-025, select variants bearing substitutions in the NS5A and NS5B protein sequences [Robida *et al.* 2007; Fernandes *et al.* 2007]. These findings suggest that the role of cyclophilins in HCV replication involves interaction with NS5A, an important regulator of the NS5B RdRp.

Prevention of HCV resistance to specific inhibitors

Twenty years of experience in the treatment of HIV infection have shown that viral resistance to specific inhibitors can be prevented if potent drugs without cross-resistance are used in combination and if exposure is optimized. This ensures a high genetic barrier to resistance; that is, a low likelihood: (1) that pre-existing variants bear the many substitutions needed to escape the simultaneous antiviral effects of the different drugs, and (2) that such variants, if present, are fit enough to replicate at high levels [Richman, 2000]. Several *in vitro* studies have shown that multiresistant variants are unlikely to be selected if two or more inhibitors are used together [Coelmont *et al.* 2008; Mathy *et al.* 2008; Koev *et al.* 2007]. However, the more complex quasispecies populations and high replication levels encountered in humans mean that at least three different inhibitors without cross-resistance will probably be needed to efficiently inhibit HCV replication until the virus is cleared. Trials of combination therapy with specific anti-HCV inhibitors will start soon, and will show how many different inhibitors must be used together.

In the meantime, specific inhibitors are being used in combination with pegylated IFN- α , the only commercial antiviral drug active against HCV. High relapse rates have been noted with a combination of pegylated IFN- α and telaprevir, owing to selection of telaprevir-resistant variants both during and after therapy [Hézode *et al.* 2009]. Adding ribavirin markedly reduces but does not abolish the risk of resistance by accelerating the clearance of infected cells in IFN responders [Hézode *et al.* 2009]. Within a couple of years a triple combination of pegylated IFN- α , ribavirin and an HCV inhibitor is likely to become the standard treatment for chronic hepatitis C, both in treatment-naïve patients and in non responders to previous pegylated IFN- α -ribavirin therapy. Adherence to the prescribed regimen will be a major determinant of success.

Conclusion

Overcoming toxicity and resistance is the main challenge in HCV drug development. All candidate HCV inhibitors can select resistant variants. These variants are present in large numbers before treatment, and most are sufficiently fit to emerge early during therapy, thus filling the 'replication space' left by inhibition of the

wild-type virus and often persisting after treatment withdrawal. Although HCV resistance substitutions are not genetically archived (HCV is incapable of genomic integration or episomal persistence), fit resistant populations may persist for years and replicate at detectable or undetectable levels after exposure to a specific HCV inhibitor. Therefore, drug development must take into account the risk that transiently inadequate exposure to drugs may select resistance substitutions and thus disqualify patients from receiving subsequent curative treatment with the same drug(s) or class of drugs. Clinical trials of specific HCV inhibitor combinations lacking cross-resistance are eagerly awaited. Indeed, in a recent study, HCV was eradicated from one out of three chimpanzees treated for a few weeks with a protease plus a nucleoside polymerase inhibitor (Olsen D. *et al.*, unpublished data). The possible place of ribavirin in IFN- α -free drug regimens will need to be determined.

Conflict of interest statement

The author has received research grants from Gilead Sciences, Roche, Schering-Plough and Vertex. He serves as an Advisor to Abbott, AstraZeneca, Biotica, Boehringer-Ingelheim, Bristol-Myers Squibb, DebioPharm, GenMab, Gilead Sciences, Glaxo-SmithKline, Jansen-Cilag, Madaus, Merck, Novartis, Ono Pharma, Roche, Schering-Plough, Tibotec and Vertex.

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