

Hepatitis C. Development of New Drugs and Clinical Trials: Promises and Pitfalls

Summary of an AASLD Hepatitis Single Topic Conference, Chicago, IL, February 27–March 1, 2003

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Chronic hepatitis C virus (HCV) infection affects a large number of patients worldwide. While our current therapies (Table 1) are effective in approximately 50% of patients, therapy is costly, prolonged, and associated with significant side effects, and it is not suitable for certain groups of patients. For these reasons, improved treatment regimens are necessary for patients with this disease. In February 2003, the annual American Association for the Study of Liver Diseases (AASLD) Single Topic Conference devoted to viral hepatitis and to fostering development of research in this field focused on recent advances in the process of antiviral drug design and new anti-HCV drugs currently in development. The drug design process, how these agents should be evaluated in future clinical trials, the potential problems and pitfalls related to pharmacodynamics and pharmacokinetics, viral resistance, clinical trial end points and design, and drug interactions, as well as HCV targets for novel drug development and the HCV drug pipeline were discussed.

The preface and aims of the conference were to provide investigators with new knowledge and some of the necessary tools to aid in planning future rational clinical trial design in this rapidly changing field.

Preclinical Studies

New treatment for HCV requires the development of new agents with unique biological profiles that are effective against a broad range of viral genotypes. While drug or target discovery represents the beginning of the process, multiple additional studies are required before an antiviral agent becomes a clinically effective and safe drug. Using a modern systematic drug discovery and development process, the risk of failure in the clinic can be markedly reduced. In the case of small antiviral compounds, the process generally consists of the following steps (communication by R.F. Schinazi, Decatur, GA):

- The first step is the screening of already existing libraries of potential compounds or the design and synthesis of new molecules based on potential target site structure knowledge.
- It is then necessary to rapidly test the potency and toxicity of potential agents—*i.e.* the selectivity of the targets and actions.
- The stability of the chemical entity and protein binding must be evaluated under various conditions.
- The mechanisms of action of the compound must be explored with appropriate *in vitro* tools.
- Toxicology studies must be run in at least two animal species. If the toxicity profile is acceptable, then the compound joins the “hot list” of compounds to proceed.
- The metabolism of the compound must be understood, and pharmacokinetics studies must be performed in animals.
- Efficacy studies must be performed in animals.
- The ultimate preclinical steps include various studies testing drug combinations *in vitro* and *in vivo*, selection of resistant viruses, viral fitness, pyrophosphorolysis, and others.

Abbreviations: HCV, hepatitis C virus; AASLD, American Association for the Study of Liver Diseases; PCR, polymerase chain reaction; IFN, interferon; NS3, nonstructural 3; IRES, internal ribosome entry segment; NC, noncoding; RdRp, HCV RNA-dependent RNA polymerase; DC-SIGN, dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin; L-SIGN, liver/lymph node-specific intercellular adhesion molecule 3-grabbing nonintegrin; LDL, low-density lipoprotein; TGF beta, transforming growth factor beta; PDGF, platelet derived growth factor; Th, T-helper; IL, interleukin; IMPDH, inosine monophosphate dehydrogenase; NK, natural killer; ALT, alanine aminotransferase; RNAi, RNA interference; siRNA, silencing RNA; BVDV, bovine viral diarrhea virus; HIV, human immunodeficiency virus; NNI, Nonnucleoside inhibitors; HcIg, hyperimmune anti-HCV immunoglobulin; ISCOMs, immunostimulating complexes; HGF, hepatocyte growth factor; PPAR, peroxisome proliferator activated nuclear receptors; FGF, fibroblast growth factor; TIMPs, tissue inhibitors of metalloproteinases.

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Table 1. Approved, Marketed HCV Drugs

Product	Name (manufacturer)	Dosing
Interferon alfa-2a	Roferon-A (Roche)	3 MU three times/week, SC
Interferon alfa-2b	Intron A (Schering-Plough)	3 MU three times/week, SC
Interferon alfacon-1	Infergen (InterMune)	9 μ g three times/week, SC
Peginterferon alfa-2a	Pegasys (Roche)	180 μ g once/week, SC
Peginterferon alfa-2b	Peg-Intron (Schering-Plough)	1.0-1.5 μ g/kg once/week, SC
Ribavirin	Copegus (Roche)	0.8-1.4 g/day, orally*
	Rebetol (Schering-Plough)	0.8-1.4 g/day, orally*

NOTE. Ribavirin is not approved as monotherapy, but as part of a combination therapy with interferon alfa (adapted from "Retrofitting HCV drugs," BioCentury, The Bernstein Report on BioBusiness 2003;11:A3-8, with permission).

Abbreviations: MU, million units; SC, subcutaneously.

*According to HCV genotype and body weight.

Efficacy

At the present time, the relative potency of anti-HCV agents currently can be determined rapidly and reproducibly by means of enzymatic and cellular systems coupled with quantitative real-time polymerase chain reaction (PCR). Efficacy models include tissue culture and animal models in which HCV replicates efficiently and antiviral molecules can be tested for their capacity to inhibit HCV replication.

Tissue Culture Systems. Two models based on tissue culture appear to be of great interest for antiviral drug development. First, primary cultures of human hepatocytes isolated from uninfected patients, and infected *in vitro* with serum samples from HCV-infected patients represent the model closest to the physiologically infected cell.¹ They have been shown to be sensitive to infection and permissive to HCV replication.^{1,2} This system has been used to show that interferon (IFN) alfa inhibits HCV replication in a dose-dependent manner.² It provides a very good model by which to study the antiviral effects of antiviral molecules in infected hepatocytes of human origin (communication by P. Maurel, Montpellier, France). Second, RNA replicons are subgenomic or genomic HCV RNA molecules that are capable of initiating and maintaining replication in the human Huh7 hepatoma line at relatively high copy numbers.³⁻¹¹ The subgenomic or genomic replicon systems provide excellent cell-based assays to evaluate inhibitors of HCV enzymes or nucleic-acid targeting strategies and are currently widely used for this purpose. Replicon-containing Huh7 cells have also been used to produce cell-free replication complex preparations that allow testing of inhibitors uncovered in screens using purified enzymatic components, and they can also be used to determine whether "hits" in cell-based replicon screens are capable of directly inhibiting RNA replication.⁹⁻¹¹ (communication by C.M. Rice, New York, NY).

Animal Models. Animal models provide a crucial link between *in vitro* experimentation and the establishment

of human clinical trials. The chimpanzee model has contributed to many advances in the HCV field,¹² but major impediments to the utilization of chimpanzees in medical research include the cost, availability, and ethical issues. In the tupaia, a tree shrew indigenous to Southeast Asia, HCV viremia is rare and, when it occurs, low and either transient or intermittent.¹³ In the "Trimera" mouse model,¹⁴ the practical window for drug administration is limited to a few days within the second week after transplantation, making this system difficult to utilize in drug screening experiments. Mercer's group recently "humanized" a murine liver through hepatocyte transplantation.¹⁵ These mice with "humanized" livers are capable of *de novo* infection with HCV, generate viral RNA levels equivalent to those of humans (10^5 to 10^6 viral RNA copies/mL), may support stable viral production for weeks or months, and can transmit the virus to other noninfected murine recipients.¹⁵ The efficacy of IFN alfa-2b, immunotherapies, antiviral gene therapy approaches, and pharmaceuticals that have shown promise in *in vitro* systems has been or is currently being tested in this model (unpublished data; communication by D.F. Mercer, Omaha, NE).

Pharmacology

The emphasis of pharmacological studies now has shifted to the early demonstration of pharmacological activity ("proof of concept") or efficacy. This shift has been aided by the development of sophisticated methods of assessment, focusing on the use of biomarkers in decision-making.¹⁶ At the preclinical stage, such models may assist initial human studies by identifying relevant exposure levels (communication by P. Glue, New York, NY).

Toxicity and Drug-Drug Interactions

There are two main types of safety risk: (1) mechanism-based toxicity, in which adverse effects are due to modulation of the same target or pathway that produces the desired therapeutic effect, and (2) compound-based

toxicity, in which adverse effects are due to secondary actions of the drug candidate and are not related to the therapeutic mechanism. Both mechanisms must be addressed prior to further development by means of *in vitro* and *in vivo* preclinical toxicity screens. In addition, many drug-drug interactions can be correlated with the inhibition and/or induction of cytochrome P450 enzyme activity. Quantitative *in vitro* prediction of clinical interactions, however, remains difficult (communication by K.R. Romines, Research Triangle Park, NC).¹⁷⁻¹⁹

Clinical Trials

After the long and arduous preclinical process, a few "survivor" compounds may reach the list of "lead candidate drugs" for clinical evaluation. Clinical evaluation then generally includes four types of trials, from phase I to phase IV. Additional issues, including viral kinetics and resistance, must be addressed at these stages.

Viral Kinetics

Using viral load monitoring and mathematical models to analyze longitudinal data yields estimates of the antiviral efficacy of therapy. Several studies have described the kinetics of HCV during IFN alfa therapy *in vivo*.²⁰⁻²⁵ Overall, these principles allow one to use viral kinetics to deduce features of the patient's response to therapy. They will prove to be particularly useful in evaluating the early efficacy of new HCV drugs, and understanding their mechanisms of action and eventual failure. They may also be important to predict the outcome of future therapy and derive appropriate clinical decision guidelines (communication by A.S. Perelson, Los Alamos, NM).²⁶

Resistance. Our current knowledge of HCV resistance to therapy includes partial understanding of the mechanisms underlying IFN alfa-based therapy failure to eradicate infection,²⁷ and a clearer view of the mechanisms underlying the resistance of other viruses (such as hepatitis B virus, human immunodeficiency virus, or herpes viruses) to specific inhibitor molecules. Viral resistance to specific antiviral drugs is characterized by the selection of minor viral populations bearing mutations conferring resistance to the specific drug. Treatment withdrawal is usually followed by restoration of the "sensitive" genotype and phenotype. Combined therapy with multiple drugs with different targets and mechanisms of action delays the emergence of resistant viruses, but multiresistant viruses may emerge after several years of treatment.²⁸ The natural variability of the HCV targets for new drugs, such as the nonstructural viral protein 3 (NS3) serine proteinase and internal ribosome entry segment (IRES),²⁹ suggests that resistant variants could be selected by future HCV inhibitors. Such events will need to be

monitored with appropriate virological tools early in the clinical development of these drugs. If viral resistance becomes a clinical issue, multiple therapy combining drugs with different targets and modes of actions will probably become the standard of care (communication by J.M. Pawlotsky, Créteil, France).

Targets for New HCV Therapies

Targets for Antiviral Inhibitors

RNA structures and viral proteins playing a critical role in the HCV lifecycle represent ideal targets for specific HCV inhibitors.

5' and 3' Noncoding Regions. The short 5' and 3' noncoding (NC) segments of the HCV genome contain structured RNA elements that are critically important for the initiation of protein translation, as well as for specific recognition of the termini of positive- and negative-strand RNAs by the HCV RNA-dependent RNA polymerase (RdRp) and subsequent initiation of RNA transcription.^{30,31} Two general approaches to the inhibition of these key steps can be envisioned. First, molecules that cleave the 5' NC segment or otherwise destabilize its higher order structure will abrogate translation of the viral polyprotein. Such drug candidates may also disrupt initiation of plus-strand RNA synthesis, since the involved RNA signals are overlapping within the genome. Similar approaches could be adopted for the 3' NC segment, potentially eliminating the first step in viral RNA synthesis. Alternatively, the growing understanding of the structures of these RNA segments at atomic level resolution³¹⁻³³ should make it possible to rationally design small molecules that bind specifically to structures within the 5' NC and 3' NC segments and that could serve as inhibitors of translation, translation initiation, and/or RdRp recognition. In both cases, the target would be a relatively large RNA structure that most likely has multiple contacts with macromolecular protein assemblies (*i.e.*, the 40S ribosome subunit or the replication complex^{31,32}). It remains to be seen whether small molecules could effectively disrupt the resulting high affinity RNA-protein interaction (communication by S.M. Lemon, Galveston, TX).

p7. Although its function is partly unknown, it has been suggested that the p7 protein could form ion channels in the endoplasmic reticulum membrane, and that this may be necessary to HCV replication. It therefore provides a potential target for antiviral intervention.

NS3 Serine Protease. The NS3 serine protease belongs to the chymotrypsin class of proteases and is involved in HCV polyprotein maturation in which it cleaves an initially synthesized viral polyprotein into functional proteins. Three-dimensional (3-D) structures of

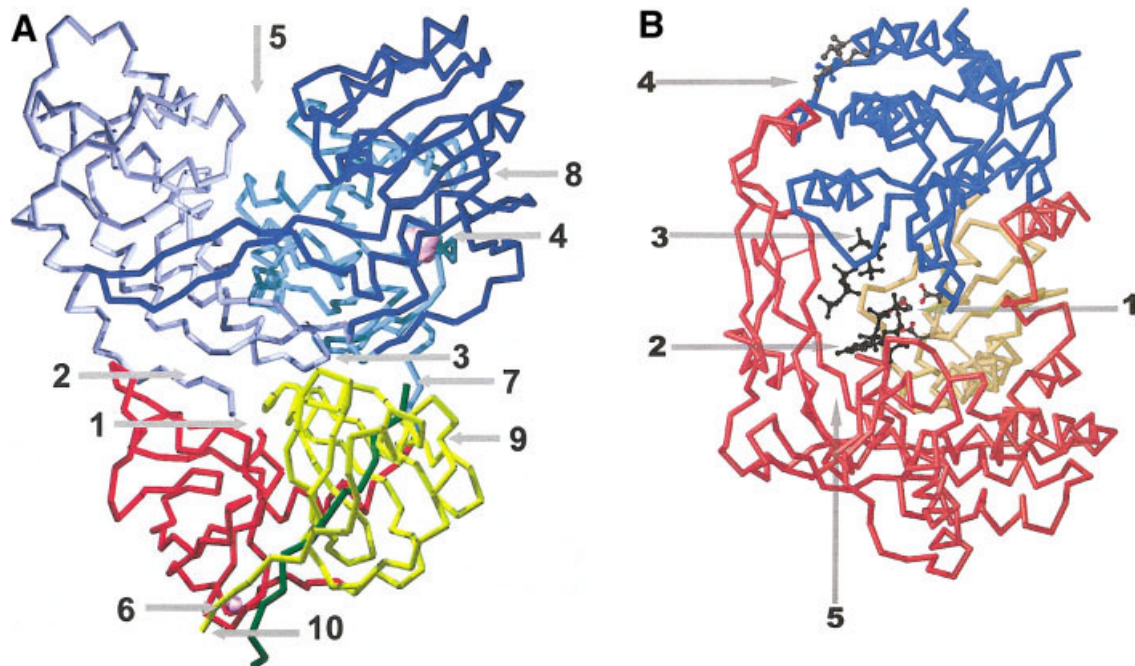


Fig. 1. (A) Inhibitor sites on the NS3/4A protease/helicase. The backbone trace of the bifunctional NS3/4A is drawn from the crystallographic coordinates (1CU1). NS3 protease subdomains are shown in **yellow** and **red**, the central portion of the NS4A cofactor is shown in **green**, and the essential Zn is indicated by a **sphere**. The three helicase subdomains are shown in shades of **blue**. The NTPase subdomain is colored cyan and one of the NTP phosphate binding locations is indicated by a **pink sphere**. The arginine-rich subdomain that is structurally-homologous to the NTPase domain is colored **dark blue**, and the third helicase subdomain is colored **light purple**. Sites for competitive inhibitors include the protease active site (1), and here the serine and histidine side chains of the catalytic triad are shown. **Arrows** (2) and (3) indicate the polyprotein substrate P and P' recognition sites, respectively, and (4) and (5) indicate the NTPase- and ssRNA-binding sites of the helicase. Locations of additional RNA-recognition sites are unknown. Molecules that prevent productive binding of essential cofactors, such as the Zn atom (6) or NS4A cofactor peptide (7), also act as inhibitors. Inhibitors of requisite interdomain motions of the helicase subdomains bind near the end of the **arrow** (8). The general region of membrane attachment of the bifunctional protease/helicase is denoted by an **arrow** (9), and interference with attachment could also prevent formation of a competent replication complex. Finally, inhibitors preventing substrate recognition by the NS2/3 protease might bind near the NS3 N-terminus (10) prior to cleavage of the polyprotein (figure prepared by P.C. Weber). (B) Inhibitor sites on the NS5B polymerase. The backbone trace of the NS5B enzyme is drawn from the crystallographic coordinates of its binary complex with UTP (1GX6). The polymerase is shown in **red**, **gold**, and **blue** for the fingers, palm, and thumb subdomain, respectively. Sites for competitive inhibitors include the polymerase active site (1), and here the side chains of the catalytic aspartic acid residues are shown. **Arrows** (2) and (3) indicate the nucleotide binding sites for polymerization and for *de novo* initiation of RNA synthesis, respectively. Bound nucleotides are indicated in **black**. An **arrow** (4) indicates the location of the allosteric surface GTP binding site, a region at the connection between fingers and thumb where several nonnucleotide inhibitors have been shown to bind. In this region, the side chains of residues that contact the allosteric GTP are indicated in **black** (Pro 495 and 499, Arg 502). Finally, an **arrow** (5) shows the template RNA binding groove (communication by F.A. Rey).

the NS3 protease with and without the requisite viral cofactor NS4A were determined by x-ray crystallography.^{34,35} Extended contacts are made between the polyprotein and NS3 protease during recognition of polyprotein processing sites. These contact regions potentially provide distinct types of inhibitor binding sites on NS3 (Fig. 1A).^{36,37} Some NS3 protease inhibitors bind at subsites utilized by the polyprotein region on the N-terminal side of the scissile bond. Many of these inhibitors achieve increased affinity for the NS3 protease through additional interactions with the active site catalytic machinery including the active site serine and oxyanion hole. Inhibitors that span the active site and thus more closely mimic the polyprotein substrate have also been synthesized (communication by P.C. Weber, Yardley, PA).

NS3 Helicase. The NS3 helicase primary function is to unwind the viral genomic RNA during replication. Studies probing the mechanism of polynucleotide unwinding have led to observations that may suggest new strategies for drug discovery.³⁸ In addition, an increasing number of 3-D helicase structure determinations, including a structure of the full-length NS3 protein having both protease and helicase domains³⁹ (Fig. 1A), has helped delineate the functionally important regions of the NS3 helicase. Structural comparisons revealed helicase domain motions that accompany the progressive unwinding of nucleic acid duplexes and that may serve as targets for specific inhibitors (Fig. 1A).⁴⁰ The NS3 helicase is characterized by the location of an NTP binding site near the interface between two structurally homologous nucleotide binding domains, the existence of a third domain

composed primarily of alpha-helices, and the occurrence of a pronounced substrate binding groove, which binds an extended polynucleotide (communication by P.C. Weber).

NS5B RNA-Dependent RNA Polymerase. The HCV RdRp (NS5B protein) is a 68 kDa protein that catalyzes RNA synthesis during replication. Several 3-D structures of active, water-soluble domains missing the hydrophobic C-terminal tail that localizes the RdRp to internal cellular membranes have been determined.⁴¹⁻⁴³ The HCV RdRp displays the classical "Finger/Palm/Thumb" motif observed in nucleic acid polymerases and a number of molecular interaction sites that can be targeted by specific inhibitors (Fig. 1B). It is, however, unique by its fully encircled active site into which nucleotides can bind in the absence of the template. The active site of the enzyme is a target for nucleoside/nucleotide analogue inhibitors. In addition, a specific GTP binding site has been identified at the surface of the enzyme, more than 30 Å away from the active site.⁴⁴ Nonnucleoside inhibitors of HCV RdRp were recently shown to bind very close to the surface GTP site, suggesting that a possible mode of action of these drugs is by inhibiting the conformational change that would be needed for RNA elongation.⁴⁵ Finally, indirect evidence suggests that some conformational changes must occur upon simultaneous binding of template and nucleotide to create the replication initiation platform. This mechanism could also serve as a target for antiviral drugs (communication by F.A. Rey, Gif-sur-Yvette, France).

Currently the most promising targets for new drug development appear to be the NS3 serine protease and RdRp (Fig. 1).

Alternative Targets

Other mechanisms of infection and disease may be suitable for future drug targets. The objectives may be to inhibit virus entry into cells by targeting the cellular receptor structures or their interactions with surface viral structures, to neutralize the virus or accelerate infected cell death by interacting with host immune responses, or to prevent liver disease progression by slowing or reversing fibrogenesis.

Virus Attachment and Entry Into Cells. The HCV genome encodes two envelope glycoproteins, E1 and E2, which are thought to be expressed as noncovalent heterodimers at the virus surface. The mechanism by which HCV enters target cells is currently unknown. The E2 glycoprotein is thought to be responsible for initiating virus attachment, and the E1 glycoprotein has been hypothesized to contain the fusion peptide responsible for the fusion of the virus and cell membranes.⁴⁶ An early interaction of envelope glycoproteins with glycosamino-

glycans has been suggested to play a role in cell recognition and tropism. Truncated soluble versions of E2 have been reported to bind specifically to human cells and were used to identify interactions with tetraspanin CD81,⁴⁷ with scavenger receptor class B type I,⁴⁸ and with dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) and liver/lymph node-specific intercellular adhesion molecule 3-grabbing nonintegrin (L-SIGN).⁴⁹ A role has also been suggested for the low-density lipoprotein (LDL) receptor.⁵⁰ The relevance of these molecules as receptors for HCV infection *in vivo* and as targets for future therapies is currently unknown and awaits the development of systems to study HCV-cell entry. One approach to overcoming the lack of a conventional cell culture system for HCV is to generate retroviral pseudotypes bearing HCV glycoproteins, which are infectious for a restricted number of human hepatoma cell lines.^{51,52} This system will allow further understanding of the early events of HCV infection in detail,^{51,52} to identify cellular molecules used by HCV, and to screen for small molecules able to block viral entry (communication by J.A. McKeating, New York, NY). This system will also be useful to assess neutralizing responses *in vitro* and to test therapies based on virus neutralization, such as specific anti-HCV immunoglobulins.

Immune Responses. The cellular immune response plays a major role in HCV infection and could represent an attractive target for therapeutic intervention. Indeed, vigorous and multispecific CD4+ and CD8+ T-cell responses during acute hepatitis C are associated with viral clearance and recovery.⁵³ Moreover, HCV-specific memory T cell responses remain detectable in the peripheral blood for decades⁵⁴ and mediate rapid viral clearance upon reexposure to the virus.^{55,56} In contrast, a lack of this response or a failure to maintain it for a sufficient time, particularly in the face of viral mutations, is associated with development of persistent infection and chronic hepatitis.⁵⁷ Once persistent hepatitis is established, the frequency of HCV-specific T cells is typically low, and their proliferative, cytokine, and cytotoxic effector functions appear to be impaired.⁵⁸ Whether successful antiviral therapy leads to transient and/or long-lasting reconstitution of cellular immune responses is controversial.⁵⁹ With regard to the future role of immunotherapy, our current knowledge of HCV-specific cellular immune responses raises the following questions: Should antiviral therapy be combined with approaches to modulate and/or induce the cellular immune response in order to induce long-lasting immunity, and does immunomodulatory therapy accelerate infected cell clearance and improve sustained virological response rates in chronic

Table 2. The HCV Pipeline*

Compound	Description	Company	Status
Interferon alfa	Oral IFN alfa	Amarillo	Phase II
Multiferon	Purified multisubtype human IFN alfa	Viragen	Phase II
Omega interferon	IFN omega	BioMedicines	Phase II
Actimmune	IFN gamma	InterMune	Phase II
Albuzeron	Fusion protein IFN alfa-albumin	Human Genome Sci.	Phase I
Peg-alfacon	Pegylated consensus IFN alfa	InterMune	Phase I
Transfersome containing IFN alfa	Transfersome containing IFN alfa	IDEA	Phase I
Viramidine	Ribavirin prodrug	Ribapharm	Phase II
Levovirin	L-isomer of ribavirin	Ribapharm/Roche	Phase I
Merimepodib (VX-497)	IMPDH inhibitor	Vertex	Phase II
ANA-245	Oral IFN-like molecule	Anadys	Phase I
Maxamine	Histamine immune modulator	Maxim	Phase II
Macrokin	Chloride matrix immune modulator	Dimethaid	Phase II
Zadaxin	Thymosin alfa immune modulator	SciClone	Phase III
ISIS-14803	Anti-IRES antisense oligonucleotide	Isis	Phase II
NM-283	Nucleosidic RdRp inhibitor	Idenix	Phase I/II
JTK-003	Nonnucleosidic RdRp inhibitor	Akros	Phase II
JTK-109	Nonnucleosidic RdRp inhibitor	Akros	Phase I
HCV-371	RdRp inhibitor	ViroPharma/Wyeth	Phase I
BILN-2061	Protease inhibitor	Boehringer-Ingelheim	Phase II
UT-231-B	Iminosugar p7 inhibitor	United Therapeutics	Phase II
Civacir	Pooled HClg	Nabi	Phase I/II
HepeX-C (XTL-002)	Anti-HCV antibody	XTL	Phase II
HCV vaccine	Structural HCV protein vaccine	Chiron/CSL	Phase I
HCV E1 vaccine	Recombinant E1 protein vaccine	Innogenetics	Phase II
HCV vaccine	Vaccine with 5 T-cell epitope peptides	Intercell	Phase II
IDN-6554 (IV and oral)	Anti-fibrotic, pancaspase inhibitor	Idun	Phase I
IP-501	Anti-fibrotic, purified phospholipid	Indevus	Phase III

*Compounds in clinical development to treat HCV infection at the date of writing, *i.e.*, May 2003 (adapted from "Retrofitting HCV drugs," BioCentury, The Bernstein Report on BioBusiness 2003;11:A3-8, with permission).

hepatitis C? (communication by B. Rehermann, Bethesda, MD).

Fibrosis Progression. If the virus cannot be eradicated, an alternative approach would be to slow the progression of liver disease. Hepatic fibrosis is the major histologic complication of chronic HCV infection, and it generally develops after many years, leading to cirrhosis within an estimated 10–50 years. A key pathogenic event in fibrosis progression is the transition of hepatic stellate cells from a quiescent to an "activated" state. This process is characterized by the production of increased amounts of extracellular matrix and *de novo* expression of smooth muscle alfa-actin, the latter characteristic consistent with cellular transformation to myofibroblasts.^{60–62} One of the most remarkable aspects of this response is enhanced extracellular matrix production, or fibrogenesis. Fibrogenesis after injury to the liver is characterized by significant increases in collagen (type I>III>IV) and other extracellular matrix constituents such as laminin, fibronectin, and proteoglycans (dermatan sulfate, chondroitin sulfate, heparan sulfate).^{61,63,64} This "wounding" process is complex and integrated, and it involves aspects of matrix synthesis and deposition, as well as degradation. Cross-talk between stellate cells and the extracellular matrix appears

to play a critical role in fibrogenesis, as well as a number of cytokines and small peptides, including transforming growth factor (TGF) beta, platelet derived growth factor (PDGF), endothelin, and angiotensin II. New specific, antifibrotic therapies may target at reducing or removing the primary injury, inhibiting stellate cell activation, stimulating matrix degradation, stimulating stellate cell apoptosis, or blocking the downstream effects of stellate cell activation (communication by D.C. Rockey, Durham, NC).

New HCV Drugs: The Pipeline

Various new directions are currently being explored in HCV drug development, and several specific and nonspecific anti-HCV drugs have already reached the clinical development phase (Table 2).

New Directions in IFN-Ribavirin-Based Therapy

Human IFNs have been classified according to the cell surface receptor with which they bind. Type 1 IFNs bind to the IFN alpha heterodimeric receptor IFNAR1/IFNAR2 and include the 21 nonallelic subtypes IFN alfa, IFN beta, IFN omega, and IFN tau.⁶⁵ IFN gamma binds

to a unique cell surface receptor, is classified as a type 2 IFN, and displays antiviral, antifibrotic, and immunomodulating activity that effects the T-helper (Th) 1 response.⁶⁶ Recently, three novel cytokines have been identified that share sequence identity with type 1 IFNs and bind to a novel cell surface receptor. These putative type 3 IFN molecules have been called interleukin (IL) 28A, IL-28B and IL-29. They are induced by viral infections and exhibit marked antiviral activity *in vitro*.⁶⁷

New IFNs. Challenges still exist with respect to IFN treatment optimization, including poor pharmacokinetic profiles, limited biological activity, suboptimal therapeutic indices, and limited knowledge on non-alfa, non-beta IFNs efficacy. Several groups have attempted to modify naturally occurring IFNs to improve their performance. These modifications include alteration of the primary amino acid sequences, the addition of polyethylene glycol, alterations of glycosylation patterns, and the production of fusion proteins. Consensus IFN alfa (IFN-alfacon1) is a second-generation cytokine that was engineered to contain the most frequently occurring amino acids among the nonallelic IFN alfa subtypes. IFN alfacon1 has been shown to be more effective than naturally occurring type 1 IFNs in cell culture models and equally effective in clinical trials.^{68,69} Other novel alfa IFNs have been produced by gene-shuffling the family of 20 human IFN alfa DNA encoding sequences.⁷⁰ Using this technique, a novel nonnaturally occurring type 1 IFN has been described with a 285,000-fold increase in antiviral activity when compared with IFN alfa-2b. The role of this highly active IFN in the treatment of chronic hepatitis C has yet to be elucidated.

The addition of polyethylene glycol to therapeutic IFN alfa proteins has been shown to dramatically increase plasma exposure following dosing and has led to increased response rates.^{71,72} Recently, an IFN beta molecule has been conjugated to a linear 20-kDa polyethylene glycol molecule targeted at a single site on the N-terminal amine.⁷³ This molecule is now entering clinical trials for the treatment of chronic hepatitis C. A pegylated form of IFN-alfacon1 is also being clinically evaluated. Finally, a fusion protein of IFN alfa-2 and human serum albumin, has entered clinical trials for the treatment of chronic hepatitis C.⁷⁴ This molecule displays similar *in vitro* antiviral and antiproliferative activity to unmodified IFN alfa-2, but has marked improvements in pharmacokinetic characteristics. Several new techniques for selection of novel second-generation IFNs have produced even more potent molecules, and clinical trials of these molecules are either underway or planned for the near future. Lastly, the role of additional naturally occurring IFN species, such as IFN gamma and IFN omega, is now being elucidated in

clinical trials (communication by L.M. Blatt, Brisbane, CA).

Oral IFN Inducers. Oral IFN inducers represent a class of compounds that may allow the generation of an effective immune response by induction/modulation of cytokine responses at the site of infection, or that may supplement or replace parenteral administration of IFN. The central challenge in the use of such agents as oral therapy for chronic HCV infection is the delivery of effective doses to the liver. Agents known to induce IFN alfa and other cytokines include (among many) relatively high molecular-weight agents, such as double-stranded RNA (poly I:C), and CpG oligonucleotide derivatives. In contrast to the complexities associated with oral delivery of such large molecules, low-molecular-weight molecules may offer similarly useful immune-modulating properties and a reasonable probability of oral absorption. Two advanced chemical classes include the imidazoquinolones imiquimod and resiquimod,⁷⁵ and the nucleoside analogs ANA245 and ANA971.

Imiquimod is approved for use as a topical agent in dermatology. Toxicity probably resulting from cytokine induction was reported in human studies.^{76,77} Resiquimod is currently in phase II studies for chronic viral hepatitis, and preliminary reports indicate a lack of specific detectable antiviral activity (Pockros et al., poster presented at the meeting). ANA245 is a low-molecular-weight nucleoside analog that induces multiple cytokines, including IFN alfa, and it showed immunologically mediated antiviral activity in a range of viral infection models (D.R. Averett, unpublished data, May 2003). However, its oral bioavailability is limited at high doses. Studies in humans are underway to characterize the safety and pharmacokinetics of ANA245 after intravenous administration, as a component of the development program for ANA971, a novel molecule that was shown to efficiently deliver ANA245 at levels associated with antiviral effects to the plasma of various animals (Averett et al., unpublished data) (communication by D.R. Averett, San Diego, CA).

Ribavirin-Like Molecules. Ribavirin monotherapy is ineffective in inducing sustained viral clearance, but it significantly enhances the sustained viral clearance rate when combined with IFN. Until now, the mechanism by which ribavirin enhances IFN efficacy remains unknown. There are four proposed mechanisms of action: immune-mediated activity on the host Th1/Th2 balance; inhibition of host enzyme inosine monophosphate dehydrogenase (IMPDH) activity; weak inhibitory activity against the RdRp; and induction of RNA mutagenesis.⁷⁸ Hemolytic anemia is a frequent side effect that limits ribavirin dosing, emphasizing the need for alternative

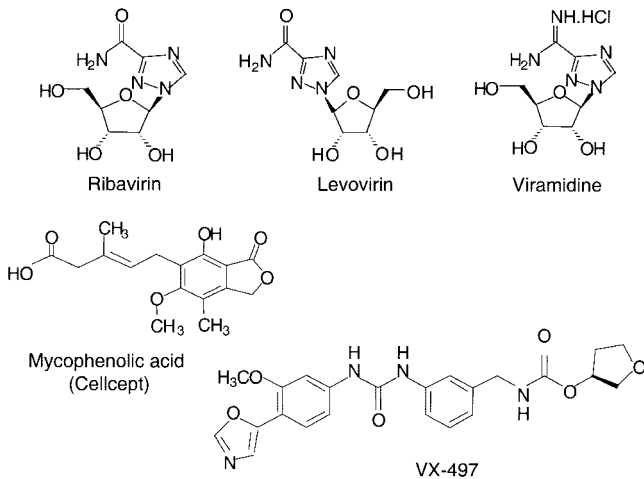


Fig. 2. Chemical structure of ribavirin, levovirin (an L-sugar analogue of ribavirin), viramidine (the amidine version of ribavirin), and two IMPDH inhibitors, mycophenolic acid and VX-497 (communication by J.Y.N. Lau).

molecules with similar mechanisms and efficacy and less toxicity.

Levovirin is the L-sugar analogue of ribavirin (Fig. 2), with similar Th1/Th2 immunomodulatory activity. However, as an L-isomer, it does not undergo metabolism to the phosphate metabolites and hence, it does not inhibit the host IMPDH nor accumulate in erythrocytes, the mechanism responsible for hemolytic anemia.⁷⁹ In preclinical studies, levovirin was well tolerated by animals and did not have mutagenic effects in conventional short-term *in vitro* and *in vivo* assays. Phase I studies in humans showed that the drug is orally absorbed and well tolerated. A proof-of-concept study in combination with pegylated IFN alfa is under way. Viramidine is the amidine version of ribavirin (Fig. 2) that can be converted by adenosine deaminase to ribavirin.⁸⁰ The first pass effect of oral dosing will lead to the delivery of viramidine to the liver. As the liver is rich in deaminases, viramidine is converted to ribavirin and its phosphorylated metabolites and may be preferentially retained in the liver, establishing an equilibrium in which more viramidine/ribavirin are concentrated in the liver compared with other tissues, including erythrocytes.⁷⁹ Rodent and chimpanzee experiments confirmed that viramidine does target the liver, and early studies suggest acceptable safety, pharmacology, and toxicology profiles. In phase I studies, viramidine showed a similar profile of adverse events similar to those of ribavirin. However, the extent of hemoglobin drop with the highest dose was lower than the hemoglobin drop with conventional combination therapy. A phase II proof-of-concept study in combination with pegylated IFN alfa is ongoing. IMPDH inhibitors, such as mycophenolic acid (Cellcept) and VX-497,⁸¹ are also currently being

studied in patients with chronic hepatitis C (Fig. 2), but preliminary results showed no/minimal direct antiviral efficacy, similar to ribavirin (communication by J.Y.N. Lau, Newport Beach, CA).

Other Immunomodulatory Drugs. Several parenterally administered immunomodulatory drugs are currently being used in combination with IFN or pegylated IFN in clinical trials. They include: (1) histamine dihydrochloride,⁸² which inhibits phagocyte-derived oxidative stress and inflammation and is currently being studied in phase II trials in combination with pegylated IFN and ribavirin, and (2) thymosin alfa-1, which promotes T-cell maturation and natural killer (NK) cells and differentiation of pluripotent stem cells. Preliminary results with this drug and IFN alfa were unclear, and thymosin alfa-1 is currently being evaluated in large phase II/III trials in combination with pegylated IFN alfa⁸³ (communication by D.R. Averett). Finally, IL-10, an anti-inflammatory drug, failed to show any beneficial histologic and antifibrotic effects in a randomized controlled trial, whereas a phase II study of IL-12, a different proinflammatory drug, suggested a lack of efficacy and significant toxicity.^{84,85}

Specific Antiviral Inhibitor Molecules

Specific antiviral molecules target functional viral components, including viral RNA structures and viral enzymes.

Nucleic Acid-Based Therapy: Antisense Oligonucleotides, Ribozymes, and siRNAs. The aim of the antisense approach is to exploit the high affinity and selectivity of nucleic acid hybridization for the development of highly specific drugs. Viral genomes contain numerous unique nucleic acid sequences not present in the human genome that have the potential to act as a virus-specific antisense target. Several groups have identified antisense oligonucleotides that inhibit the translation of HCV RNA in cell-free systems and cell culture models.^{86,87} Among them, ISIS 14803, a 20-nucleotide antisense oligodeoxynucleotide that is complementary to the IRES region surrounding the translation initiation codon has entered clinical development. ISIS 14803 decreases HCV RNA and protein levels in various *in vitro* and *in vivo* models. The HCV RNA decrease was shown to occur through RNase H cleavage of the RNA in oligonucleotide-RNA heteroduplexed regions, and potentiated the antiviral effect related to inhibition of polyprotein translation. Two clinical studies of ISIS 14803 monotherapy have been conducted.^{88,89} Reductions in plasma HCV RNA levels have been observed in 3 of 10 patients treated at 2 mg/kg (-1.3 to -2.2 log₁₀) and in 6 of 20 patients dosed twice weekly at 6 mg/kg (-1.0 to -3.8 log₁₀). Transient asymptomatic alanine aminotransferase (ALT)

flares, up to 30 times, were observed in temporal association with plasma HCV RNA reductions and in other patients with no HCV RNA reductions.^{88,89} The mechanism by which HCV RNA reductions occurred is unclear (communication by T.J. Kwok, Carlsbad, CA).

Ribozymes, another nucleic acid-based strategy, are catalytic RNA molecules that cleave specific RNA sequences. They contain a catalytic core region flanked by binding arms with specific nucleotide sequences determined by the complementary base sequence of the target RNA. Heptazyme is a synthetic, stabilized 33-mer ribozyme that is chemically modified for resistance to enzymatic and chemical degradation. This first-generation ribozyme product was developed to treat chronic hepatitis C. Preclinical results indicated that Heptazyme selectively cuts hepatitis C RNA, significantly inhibiting viral replication in cell culture.⁹⁰ Phase II results indicated that Heptazyme as monotherapy led to a reduction in serum HCV RNA levels in 10% of patients. The development of Heptazyme has been halted in favor of developing a next-generation product with an improved therapeutic index. This strategy capitalizes on recent technological advances resulting in improved stability and targeting of ribozymes to specific tissues (communication by N. Usman, Boulder, CO).

A related and new potential class of therapeutics makes use of RNA interference (RNAi), a recently discovered process whereby cells down-regulate gene expression through destruction of a specifically targeted mRNA.⁹¹ The RNAi process is mediated inside the cell by a naturally occurring protein complex that uses double-stranded RNA as a molecular guide to down-regulate genes at the posttranslational level. Rational design of double-stranded RNA permits the down-regulation of virtually any gene. In human cells, these double-stranded RNAs, known as silencing RNAs (siRNAs), show biological activity as short fragments of 20–23 residues. Stabilized siRNA compounds are currently being evaluated in the preclinical setting for their potential activity against HCV (communication by N. Usman).

p7 Inhibitors. Long-alkyl-chain iminosugar derivatives, which are known to have antiviral activity against bovine viral diarrhea virus (BVDV),⁹² were recently shown to inhibit HCV p7 ion channels,⁹³ suggesting these compounds with low toxicity profiles in animals might be used in the treatment of chronic hepatitis C. One such compound is currently in a phase II clinical trial.

HCV Protease Inhibitors. The search for inhibitors of the NS2/NS3 cleavage reaction has been hampered by the hydrophobic nature of the protein and by the autocatalytic nature of the cleavage. In contrast, a number of

peptide-based or peptidomimetic inhibitors have been developed for the NS3 serine protease and tested *in vitro*. Most of these inhibitors fall in one of three classes: (1) substrate analogues, (2) serine-trap inhibitors (or transition-state analogues), and (3) product analogues. In addition, efforts to discover nonpeptide inhibitors have also been made.⁹⁴ Optimization of an N-terminal hexapeptide cleavage product of a dodecapeptide substrate led to the discovery of BILN 2061, a small, selective, and potent inhibitor of the NS3 serine protease.⁹⁵ Inhibitor constant values of 0.30 mmol/L and 0.66 mmol/L with a noncovalent, competitive mode of inhibition were obtained for genotypes 1a and 1b, respectively. BILN 2061 was shown to retain its inhibitory efficacy in human cells and showed low nanomolar inhibition of HCV RNA replication through blockade of the NS3 protease-dependent polyprotein processing in the replicon system. BILN 2061 was administered for 48 hours in HCV-infected patients in early clinical development studies. Administration resulted in a rapid, dose-dependent HCV RNA decrease up to $-4 \log_{10}$ within 2 days at the highest doses, with a progressive return to baseline levels within a week after treatment withdrawal^{96,97} (communication by D. Lamarre, Laval, Québec, Canada). Another NS3 serine protease inhibitor, VX-950, will soon enter clinical development.

HCV NS3 Helicase Inhibitors. A few small-molecule inhibitors of the NS3 helicase with activity *in vitro* have been reported, but their inhibitory mechanisms, specificity, and potential efficacy in the clinical setting remain unclear.⁹⁴

HCV RdRp Inhibitors. Inhibitors of viral polymerases can be classified into three categories. (1) Nucleoside (substrate) analogues (cyclic or acyclic) are usually phosphorylated to their corresponding nucleoside triphosphate (nucleotide) in the cytoplasm of infected cells. The nucleotide is then typically incorporated by the viral polymerase during processive nucleic acid synthesis, leading to early termination and thus inhibition of the virus life cycle. Nucleoside inhibitors of viral polymerases are used therapeutically for human immunodeficiency virus (HIV), hepatitis B, and herpes viruses. (2) Non-nucleoside inhibitors (NNI) of clinical interest have thus far been described only for the HIV-1 reverse transcriptase. These compounds bind to an allosteric site on the enzyme surface away from the enzyme active site, possibly distorting the precise geometry of the enzyme active site so the enzymatic function is suppressed. (3) Foscarnet (phosphonoformic acid) is the prototypic and the only approved member of the pyrophosphate (product) analogue class. It is used in the treatment of infection by the herpes virus family. These agents are thought to interact

directly with the pyrophosphate-binding site of the viral polymerases. Inhibitors of the HCV RdRp that belong to each of the above classes have been identified through the use of high throughput screening and rational drug design.⁹⁸ Several of these compounds have encouraging pre-clinical profiles, including the capability to inhibit HCV RNA replication in cell culture. Preliminary *in vitro* results suggest that resistance to RdRp inhibitors may occur in the clinical setting (communication by R. de Francesco, Pomezia, Italy).

A few orally bioavailable inhibitors of the HCV RdRp are under study in early clinical trials, such as JTK-003 and JTK-109, the potential mechanisms of which remain unclear, or NM283. NM283 is an orally bioavailable pro-drug of a ribonucleoside analogue, NM107, that was discovered through its potent activity against BVDV in cell culture. NM107 triphosphate is a competitive inhibitor of purified BVDV polymerase *in vitro* ($K_i \sim 160$ nM) and acts as a chain terminator of BVDV RNA synthesis. The efficacy of NM283 against HCV genotype 1 was established in a 1-week oral dosing study in chronically infected chimpanzees. Median drops in HCV viral load of -0.83 and -1.05 \log_{10} were observed at the end of the dosing period in two animals per group in response to 8.3 and 16.6 mg/kg/day of NM283, respectively, compared with a placebo control animal. NM283 recently entered early phase clinical trials (communication by R.F. Schinazi).

Immune Therapy

Modulation of the host immune response to infection may include enhancement and broadening of the cellular immune response against HCV, inhibitory or immunostimulatory cytokine therapy, passive transfer of hyperimmune anti-HCV immunoglobulins (HClg), and application of therapeutic vaccines.

Hyperimmune Anti-HCV Immunoglobulins. Experimental studies with chimpanzees provided compelling evidence that the neutralization of epitopes located in the hypervariable region 1 of the HCV envelope gene prevented infection of susceptible animals.^{99,100} It has been hypothesized that multiple infusions of HClg containing neutralizing antiviral antibodies may modify virus replication and the clinical course of the infection. Therapeutic HClg could also be particularly valuable in preventing recurrent hepatitis C in HCV-infected liver transplant recipients. HClg has been prepared from virus-inactivated, HCV RNA-negative, 5% IgG obtained from 460 anti-HCV-positive plasma donors. In three chronically experimentally infected chimpanzees, passive HClg transfer decreased the HCV RNA load in two chimpanzees and decreased ALT levels in all three animals once the

level of passively transferred anti-HCV E2 reached a plateau. Both markers returned to baseline when HClg infusions were stopped.¹⁰¹ In three other chimpanzees acutely infected with HCV, multiple infusions of HClg significantly shortened the length of HCV viremia and prevented acute hepatitis in comparison with control animals treated with immunoglobulin preparations without anti-HCV. HCV RNA disappeared from serum after a significantly shortened period of viremia in two animals, but it recurred in one of the two, when the level of anti-HCV E2 declined to negative values. The third animal had a fluctuating pattern of serum HCV RNA levels closely related to the changing level of anti-HCV E2 resulting from HClg infusions. Recurrent HCV viremia was followed by enzymatic and histopathologic evidence of either acute or chronic hepatitis.^{101,102} The mechanisms by which HClg affected the rate of HCV replication require further study. A clinical trial is in progress in infected patients to investigate the safety and pharmacokinetics of HClg in liver transplant recipients (communication by K. Krawczynski, Atlanta, GA).

Therapeutic Vaccines. Recent data on immune responses in the chronic phase of infection suggest that a therapeutic vaccine capable of stimulating functional CD4+ and CD8+ T-cell responses in the chronic carrier may be of benefit.^{58,103} To this end, various HCV recombinant polypeptide and plasmid DNA vaccines have been tested in nonhuman primates and shown to be capable of priming broad, functional CD4+ and CD8+ T-cell responses. Figure 3 shows various possible approaches to therapeutic vaccination. A recombinant E1/E2 vaccine was shown to prime the induction of viral neutralizing antibodies and CD4+ T-cell responses, and it prevented infection or evolution toward chronicity in a significant number of chimpanzees tested relative to control animals. Another vaccine formulation, based on yeast-derived fusion polyprotein comprising HCV genotype 1 NS3-NS4-NS5-core sequences combined with an immunostimulating complexes (ISCOMs) adjuvant, was capable of priming broad CD4+ and CD8+ T-cell responses in chimpanzees. It is now intended to test optimal formulations in chronic hepatitis C patients for their ability to increase the long-term response to pegylated IFN plus ribavirin (communication by M. Houghton, Emeryville, CA).

Other experiments have shown that cellular immune responses to the E1 envelope protein are almost absent in patients with chronic active hepatitis C, while long-term responders to IFN alfa therapy have, on average, higher levels of E1 antibodies.^{104, 105} A clinical-grade HCV E1 protein produced and purified from mammalian cells is in use for phase I and II clinical trials of therapeutic vacci-

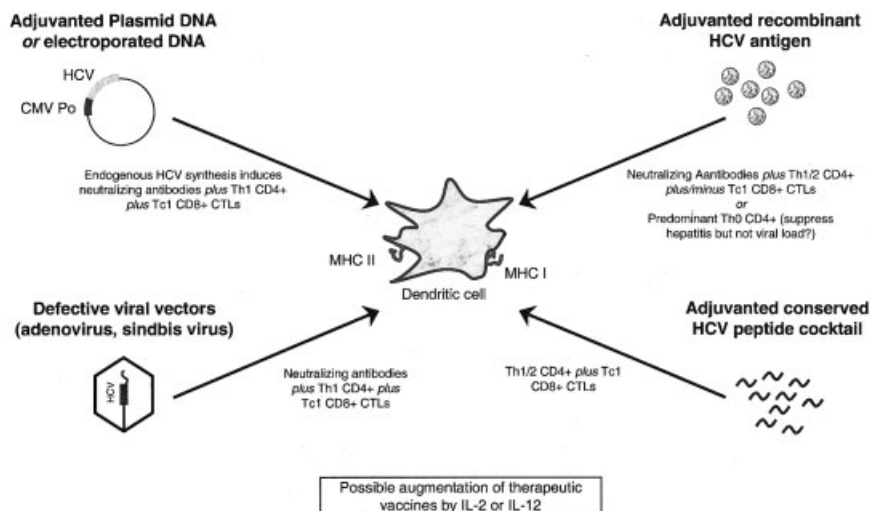


Fig. 3. Approaches to therapeutic vaccination (communication by M. Houghton).

nation. In a healthy volunteer study, the E1/alum vaccine successfully stimulated both humoral and cellular immune responses. Signs and symptoms of inflammation at the site of injection were present in a minority of the volunteers and were the only reported adverse events.¹⁰⁶ In a subsequent phase IIa study, conversion from a negative to a strong E1-specific Th response was observed in the vast majority of patients. The levels of anti-E1 antibodies increased on average 3- to 4-fold after the second course of E1 injections. The proportion of patients showing a significant T-cell response to E1 increased from 9% at baseline to 91% at study end. The T-cell responses included a strong Th1 component with high IFN gamma levels. The E1-treated patients showed a significant decline of ALT as compared with baseline, and liver fibrosis had improved by one point or more in 38% of the patients (both correlated with the increase in E1 antibody levels). HCV E2 antigen levels in the liver declined, while serum HCV RNA levels remained unchanged. Further patient follow-up and maintenance therapeutic vaccinations are being conducted to examine the long-term disease-modifying effects, and additional placebo-controlled studies are ongoing (communication by G. Maertens, Gent, Belgium).

Antifibrotic Approaches

The most effective way to eliminate hepatic fibrosis is to clear the primary cause of liver disease. Therefore, curative antiviral therapy remains the best "antifibrotic" approach. However, when this goal cannot be achieved, several antifibrotic approaches are theoretically possible, for which various agents are currently being evaluated (communication by S.L. Friedman, New York, NY):

- Reduce inflammation or the host response to avoid stimulating stellate cell activation

- Directly down-regulate stellate cell activation
- Neutralize proliferative, fibrogenic, contractile and/or proinflammatory responses of stellate cells
- Stimulate apoptosis of stellate cells
- Increase the degradation of scar matrix.

Conclusion

The clinical research environment and the rapid tempo related to new drug development for patients with hepatitis C is exciting. While many opportunities exist to improve the outcome for our patients with newer and more targeted therapies, the process will be arduous, academically stimulating, associated with many promises and pitfalls, but eventually rewarding. As outlined in this conference, and as investigators and practitioners, we must consider and incorporate numerous preclinical, clinical, and regulatory issues into the conduct of our clinical trials related to this disease. The aim will be to achieve patient-centered, high-quality clinical research with meaningful results that we can convey to our patients without reservation. A large number of HCV functions and disease features can now be targeted for drug development. As a result, many new HCV drugs are currently in the pipeline, with several of them having already reached the clinical phase of development. It is clear that only a few of these candidates will finally be approved for clinical use in the treatment of HCV infection. It is, however, encouraging to foresee multiple therapies with complementary targets that could lead to cure of infection in a greater proportion of patients.

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