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Mini-review

Selective inhibitors of hepatitis C virus replication

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Dedicated to Prof. Erik De Clercq on the occasion of reaching the status of Emeritus-Professor at the Katholieke Universiteit Leuven in September 2006.

Abstract

Worldwide over 170 million people are chronically infected with the hepatitis C virus and hence at high risk to develop fatal liver disease. There is no vaccine available and the standard therapy [(pegylated) interferon alfa plus ribavirin] is only effective in 50–60% of patients and is associated with important side-effects. The discovery of novel antiviral strategies to selectively inhibit HCV replication has long been hindered by the lack of convenient cell culture models for the propagation of HCV. This hurdle has been overcome first with the establishment of the HCV replicon system in 1999 and, in 2005, with the development of robust HCV cell culture models. In recent years also mouse models have been elaborated that will be instrumental in assessing the in vivo efficacy of novel drugs. The viral serine protease and the viral RNA dependent RNA polymerase have shown to be excellent targets for selective anti-HCV therapy. Clinical studies with a limited number of HCV protease and polymerase inhibitors resulted in encouraging results. However, and not unexpected, preclinical evidence suggest that the virus may become rapidly resistant to such inhibitors. Combination therapy of drugs with different mode of action and resistance profiles may thus be required. Alternative strategies, such as the use of non-immunosuppressive cyclosporin A analogues with potent anti-HCV activity, may prove important, in particular since such compounds may have a resistance profile that is very different from that of protease or polymerase inhibitors.

Keywords: Hepatitis C virus; Protease inhibitors; Polymerase inhibitors; Animal model; HCV cell culture model

Contents

1. Introduction

The WHO estimated in the year 2000 that worldwide 170 million people are chronically infected with the hepatitis C virus (HCV). Following infection, in most cases, clinical symptoms are mild or even subacute. Hence many patients do not realize to be infected until there is chronic liver damage 10–30 years after the initial infection. There is no vaccine available, nor selective drugs. Current standard therapy consists of the use of (pegylated) interferon and the nucleoside analogue ribavirin. However, this therapy is only effective in 50–60% of infected individuals and is associated with serious side-effects (Fried et al., 2002; Manns et al., 2001). There is thus

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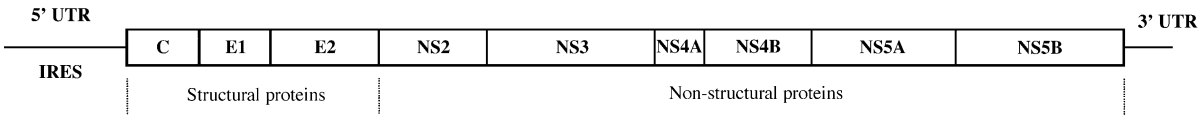

Fig. 1. Structural organization of the HCV genome.

an unmet need for potent and selective inhibitors of HCV replication.

The search for selective inhibitors of HCV replication has long been hindered by the lack of appropriate cell culture models. A major progress was made in 1999 with the generation of HCV subgenomic replicons that replicate in human hepatoma cell lines (Bartenschlager, 2002, 2005). More recently, several groups reported on infectious culture models for HCV (Wakita et al., 2005; Zhong et al., 2005; Lindenbach et al., 2005). Another limitation in anti-HCV research has been the lack of convenient animal models for antiviral drug testing. Chimpanzees can be reliable infected but represent, for obvious reasons, not the most appropriate animal model. Recently mouse models have been reported that may be instrumental in assessing the preclinical efficacy of novel drugs (Mercer et al., 2001; Meuleman et al., 2005).

The HCV genome is a 9.6 kb single-stranded RNA of positive polarity. It encodes for a large polyprotein, that, following maturation results in at least 10 proteins: the structural proteins C, E1, E2 and p7 and the non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B (Fig. 1). The viral RNA dependent RNA polymerase is encoded by NS5B. The N-terminal domain (about 180 amino acids) of the HCV NS3 protein forms, together with the co-factor NS4A, a heterodimeric serine protease that catalyses the posttranslational processing of the non-structural proteins. The C-terminal domain of NS3 encodes the viral helicase (Lindenbach and Rice, 2005). There has been a major effort in recent years to develop highly selective inhibitors of HCV replication. Most of these are inhibitors of the viral RNA dependent RNA polymerase or the viral serine protease.

2. Current therapy for HCV

Currently the only drugs approved for the treatment of infections with HCV are (pegylated)interferon-α and ribavirin. Interferon has both direct antiviral properties and acts also as a cytokine with immunomodulatory activity (such as an increased expression of major histocompatibility complex proteins and activation of natural killer cells and macrophages). However, the combination of ribavirin with interferon-α (now most often in its pegylated form), results only in 50–60% of the patients in a sustained virological response and is, in addition, associated with important side-effects. Moreover, the effect of interferon is genotype specific, genotype 1 infections appear to be more difficult to cure than infections with other genotypes (Yoshioka et al., 1992; Fried et al., 2002; Manns et al., 2001).

Various mechanisms of action have been suggested to contribute to the (in vitro) antiviral activity of ribavirin, including inhibition of the inosine monophosphate dehydrogenase (IMP-DH) by ribavirin 5′-monophosphate, inhibition of the polymerase activity by ribavirin 5′-triphosphate or induction of a so called error-catastrophe as a result of the accumulation of a lethal number of mutations in viruses that have been cultured in the presence of ribavirin. Alternatively, in the infected host, ribavirin is also believed to exert an immunomodulatory activity, by modulating the Th1-respons (Graci and Cameron, 2006). Employing the yellow fever virus (YFV-17D), a virus that belongs, like HCV, to the family of Flaviviridae, we demonstrated recently that inhibition of IMP-DH activity by ribavirin, and hence depletion of intracellular GTP pools, is the main, if not the sole mechanism, by which ribavirin inhibits the replication of this flavivirus (Leyssen et al., 2005). Moreover, in viral populations that had received maximum antiviral pressure by ribavirin (pre-extinction populations) no evidence was obtained for a role of error-catastrophy in the antiviral effect (Leyssen et al., 2006). It remains to be studied whether or not inhibition of GTP pools is the major, or the sole mechanism by which ribavirin exerts its direct antiviral effect against HCV.

3. Cell culture models for HCV

HCV replicons are autonomously replicating (subgenomic) genetically engineered HCV RNA genomes in which the region that encodes the core to NS2, is replaced by a selectable marker. An internal ribosome entry site (IRES) mediates translation of the HCV replicase (NS3-5B). Transfection of this RNA in cells of the human hepatoma cell line Huh-7, followed by selection (for example with G418 in case the *neo*-selectable marker is used) results in cell clones in which the HCV subgenomic RNA replicates efficiently. Cell culture adaptive mutations, and a favorable host cell environment, allow replication of HCV RNAs to very high level. Many different HCV replicons have been generated, including replicons with reporter genes (such as the firefly luciferase) that allow fast and reproducible screening of large series of compounds (Bartenschlager and Pietschmann, 2005; McHutchison et al., 2006).

Very recently, efficient in vitro HCV production systems have been elaborated (Lindenbach et al., 2005; Zhong et al., 2005; Wakita et al., 2005). Crucial was the use of a genotype 2a isolate (JFH-1) from a Japanese patient with fulminant hepatitis; an isolate that proved able to replicate to very high levels in Huh-7 cells in the absence of cell culture adaptive mutations. Chimeric full-length replicons that contained JFH-1 replicase (NS3-5B) and its non-translated regions were constructed. Transfection of the appropriate cell line (Bartenschlager, 2005) with this viral RNA resulted in the production of infectious HCV particles. Virus particles generated from the authentic JFH-1 full-length genome were infectious in a chimpanzee and in a mouse model, confirming the authenticity of HCV grown in cell culture (Wakita et al., 2005; Lindenbach et al., 2006). To facilitate drug screening JFH-

1 variants were generated that express the firefly luciferase gene (Wakita et al., 2005). The infectious virus system should also simplify the selection for drug resistance. Selection of replicons that are resistant to antiviral drugs has been rather cumbersome. Indeed, selection must be performed in the presence of a selecting agent (such as G418 in case of *neo* replicons) and there is always a risk that the host cell is (in part) responsible for the resistance (for example by an increased efflux of the drug). In contrast to replicons, the infectious HCV systems also allow to explore the entire life cycle of the virus (from attachment and penetration to release of novel infectious particles). It may thus be expected that this novel culture system will result in the identification of compounds that inhibit novel, yet unexplored targets.

4. Animal models

The chimpanzee (*Pan troglodytes*), whose genome has a 98.77% sequence homology with the human genome, played a crucial role in the discovery of HCV. In 1978, 3 years after the disease was first observed in man, it was found that chimpanzees suffer from non-A, non-B (NANB) hepatitis when infected with samples of patients with NANB hepatitis (Alter et al., 1978). Disease progression of HCV in chimpanzees closely resembles that in humans, although the liver disease is milder in these animals than in man (Muchmore et al., 1988). Although the chimpanzee is still the best available animal to study antiviral therapy against HCV, the use of this endangered species as a laboratory animal is for obvious reasons not evident. There have been some reports on the infection of the Tupaia with HCV. These small animals, reproduce easily in captivity, adapt well to the environment in an animal facility and are relatively inexpensive and easy to handle. The fact that only 20–25% of the animals develop a viremia following infection, limits the use of this model for drug efficacy studies (Xie et al., 1998).

The "trimera mouse" has been proposed as a possible model for HCV infections. In this model, normal or BNX (beige/nude/X-linked immunodeficient) mice receive a total body irradiation after which they are rescued with bone marrow from SCID mice and subsequently transplanted under the liver capsula with human liver fragments infected ex vivo with HCV. Viremia levels, although variable, lasted for about 1 month (Ilan et al., 2002). Convincing evidence that this model is useful for the evaluation of antiviral drugs is still awaited. Perhaps the most promising animal model is the uPA transgenic mouse model. In these transgenic mice, overexpression of the murine urokinase-type plasminogen activator (uPA) in the liver, results in extensive liver toxicity ultimately leading to hepatic insufficiency (Sandgren et al., 1991). Backcrossing of these transgenic mice onto a genetically immunodeficient mouse strain, resulted for example in uPA+/+ SCID/Beige or uPA+/+ SCID mice; that can be transplanted with human primary hepatocytes. The xenotransplantation of human liver cells results in a repopulation of the destroyed liver tissue. Intraperitoneal infection of the animals with human serum that contains HCV, resulted in successfully transplanted animals in long-term HCV infection (in some cases up to 21 weeks). These models allow the assessment of the in vivo efficacy of anti-HCV drugs (Mercer et al., 2001; Lindenbach et al., 2006; Leroux-Roels, personal communication).

5. Selective inhibition of viral enzymes

Most antiviral drugs that are being used in the clinical setting today are inhibitors of the viral polymerase (for example the polymerase inhibitors of herpesviruses, of the human immunodeficiency virus and of the hepatitis B virus) and protease inhibitors in case of HIV (De Clercq, 2004). The design of antiviral drugs that inhibit the function of the HCV proteases and polymerases thus appears to be logic.

5.1. The NS3-4A protease as target

The amino-terminal domain of the HCV NS3 protein forms together with the co-factor NS4A a heterodimeric protease, it cleaves the viral polyprotein into single functional proteins. Although the NS3-4A protease is, akin to for example trypsin, a serine protease, the substrate specificity is very different from that of host cell serine proteases (Lindenbach and Rice, 2005; Chen and Tan, 2005). The substrate-binding region can be considered as rather flat or shallow which may make it more difficult to design selective inhibitors (De Francesco and Migliaccio, 2005). Despite this, several groups succeeded to design highly selective inhibitors of this enzyme and hence of the replication of the virus. The macrocyclic peptidomimetic BILN-2061 (Boehringer Ingelheim) (Fig. 2) a non-covalent inhibitor of NS3-4A protease inhibitor, was the first HCV protease inhibitor to enter clinical trials. The compound resulted, within hours after administration, in a rapid decline of HCV RNA levels. Activity was most pronounced in HCV genotype-1 infected patients (Lamarre et al., 2003). The inhibition was transient as viral (RNA) levels returned to base-line upon cessation of therapy. However, the hope is that longer treatment with protease inhibitors, either alone or in combination with other compounds, may result in (markedly) higher rates of sustained response than with the current standard therapy. As could be anticipated, selective pressure in the replicon system with BILN-2061 resulted

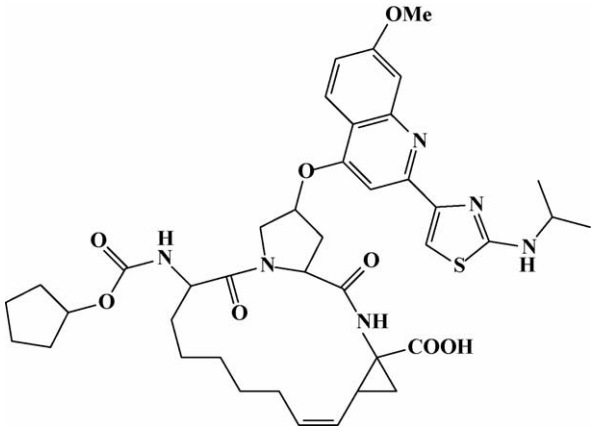

Fig. 2. BILN 2061.

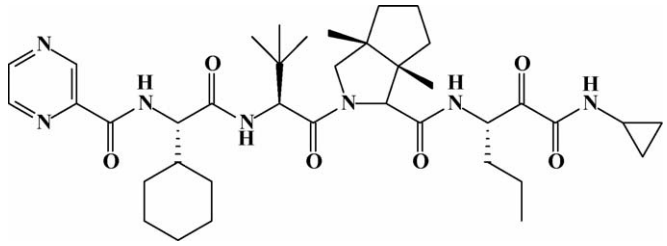

Fig. 3. VX-950.

in drug-resistant replicons, a single mutation (either arginine 155, alanine 156 or aspartate 168) proved sufficient to confer this resistance. In viral isolates of genotype 3 aspartate 168 is replaced by a glutamine, this could possibly explain the reduced activity of BILN-2061 in genotype-3 infected patients. However, BILN-2061 proved cardiotoxic and the development of the compound was halted (Lamarre et al., 2003; Reiser et al., 2005; Hinrichsen et al., 2004).

VX-950 is yet another peptidomimetic inhibitor of the viral protease VX-950 (Fig. 3) that was discovered using a structure-based drug design approach. VX-950 has a different mode of binding to the viral protease than BILN 2061. The latter is a non-covalent, reversible serine protease inhibitor, whereas VX-950 forms a covalent but reversible complex with the HCV NS3-4A serine protease in a slow process (slow-on). This allows formation of the covalent bond between the HCV protease and VX-950. Once formed, the covalent complex dissociates slowly back to the free enzyme and inhibitor with a half-life of about 1 h. This may provide a sustained inhibitory antiviral effect (Perni et al., 2006). Also in contrast to BILN-2061, VX-950 retains good activity against genotype 2 HCV proteases (Perni et al., 2006). The major in vitro resistance mutation against VX-950 is a substitution of Ala 156 to Ser. Replicons carrying this mutation remained sensitive to BILN 2061 (Lin et al., 2005). Reciprocally, the dominant in vitro resistance mutations against BILN 2061, substitutions of Asp 168 with Ala (D168A) or Val (D168V), remained fully sensitive to VX-950 (Lin et al., 2004, 2005). In a 14-day phase 1b trial of VX-950 in genotype 1 HCV-infected patients, a 4.4-$\log_{10}$ median reduction in the plasma viral load was observed in a group of patients dosed with 750 mg of VX-950 every 8 h. In some patients the virus became undetectable at day 14 of dosing (Reesink et al., 2005).

The protease inhibitor SCH 503034 (Shering Plough) (Fig. 4) is an orally active compound. In particular mutation A156T resulted in a >100-fold resistance to the compound. Replicons containing this mutation proved less fit than the wild type replicons (Yi et al., 2006; Tong et al., 2006a,b). The reduced fitness of the resistant variant may suggest that development of high-level resistance in the clinical setting may be slow. As will be likely the case with several other compounds, combination therapy with IFN-α should also greatly reduce the potential emergence of resistance. In an ongoing phase II study of genotype-I infected patients (non interferon responders), 400 mg of SCH 503034 thrice daily as single therapy resulted in a mean HCV RNA reduction of 2 $\log_{10}$ (Zeuzem et al., 2005). Recent studies suggest that NS3-mediated cleavage of host factors may abrogate cellular response to IFN-α. Blockage of NS3 protease activity is therefore expected to inhibit HCV replication by both direct suppression of viral protein production as well as by restoring host responsiveness to IFN-α (Gale and Foy, 2005; Malcolm et al., 2006).

5.2. The NS5B RNA dependent RNA polymerase as a target

The HCV polymerase can be viewed as a right hand, where the palm domain contains the active site of the enzyme and where the fingers and the thumb are responsible for the interaction with the RNA. The fingertips are two loops that extend from the fingerdomain and that make contact with the thumbdomain (in the so called closed conformation). This results in the formation of a tunnel in which the RNA, template as well as the nascent RNA, are contained. In the 'open' conformation, the contact between the finger and the thumbdomain is disrupted (Bressanelli et al., 1999; De Francesco and Migliaccio, 2005).

5.2.1. Nucleoside inhibitors

NM283 (Valopicitabine, Idenix/Novartis) is the oral prodrug of the nucleoside analog 2′-*C* methylcytidine (Fig. 5). Intracellular phosphorylation of the compounds (as well as of 2′-*C* methyl purine analogues) to their 5′ triphosphate results in the formation of the active inhibitor of the viral polymerase (Olsen et al., 2004; Carroll et al., 2003). The compounds are non-obligate chain terminators (Migliaccio et al., 2003). RNA elongation may represent the most probable target of 2′-modified nucleotide analogues, because it is more permissive to inhibition than initiation (Dutartre et al., 2005). Replicons resistant to 2′-*C* methylcytidine contain the S282T mutation in the viral polymerase and show a reduced fitness; mutant viruses are less fit than wild-type virus (Migliaccio et al., 2003; Ludmerer et al., 2005). Valopicitabine results in a dose-dependent 1.2-$\log_{10}$ decline in HCV RNA in

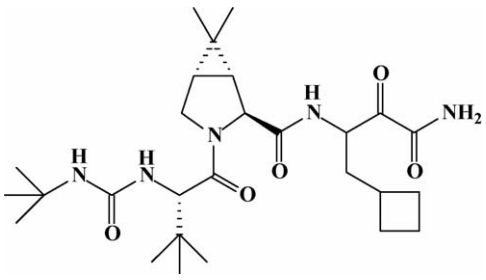

Fig. 4. SCH 503034.

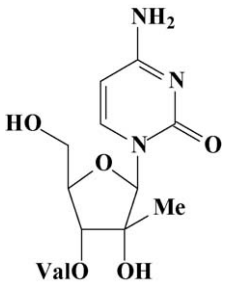

Fig. 5. NM283.

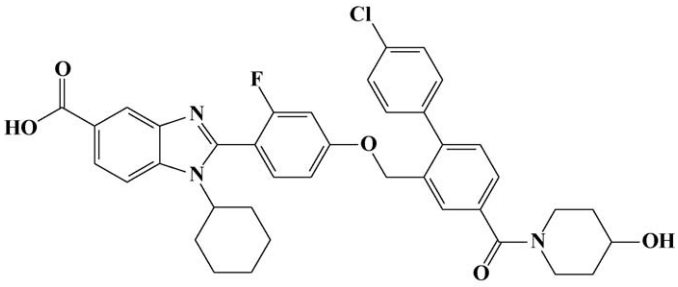

Fig. 6. Benzimidazole derivative.

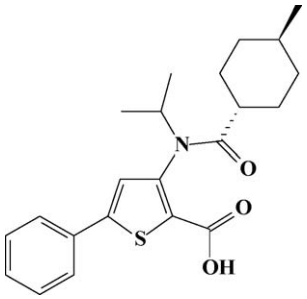

Fig. 7. Thiophene derivative.

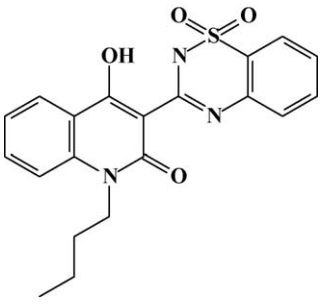

Fig. 8. Thiadiazine derivative.

HCV genotype-1 infected patients receiving 800 mg daily for 2 weeks (Afdhal et al., 2004). The combination of valopicitabine with interferon resulted in a reduction of viral load of more than 4 $\log_{10}$, and viral RNA was undetectable in a number of patients (O'Brien et al., 2005). Phase III studies with valopicitabine are planned in 2006.

5.2.2. Non-nucleoside inhibitors

A number of structurally unrelated series of non-nucleoside HCV polymerase inhibitors have been reported; these include, but are not limited to, benzothiadiazines, benzimidazoles/diamides, disubstituted phenylalaline/thiophene amides and substituted pyranones (Dhanak et al., 2002; Love et al., 2003; Sarisky, 2004; Tomei et al., 2003; Wang et al., 2003). The first NNI of HCV that entered clinical trials were JTK-109 and JTK-003 (Japan Tobacco). These benzimidazole derivatives (Fig. 6) act as allosteric inhibitors and block the polymerase before elongation (Tomei et al., 2003). Resistance of HCV replicons to this class of compounds maps to P495 (Tomei et al., 2003). Co-crystalization studies of the HCV polymerase with a related analogue suggests that these compounds bind on the surface of the thumbdomain, at a position that is in the "closed" enzyme conformation occupied by one of the fingertips. The binding of the compound to this cavity forces the enzyme in the "open" configuration (Di Marco et al., 2005).

In November 2005, ViroPharma/Wyeth announced preliminary results from phase 1b proof of concept studies with HCV-796. The patient cohort with the highest exposure to HCV-796 achieved a peak mean HCV viral load reduction of 1.4 $\log_{10}$ on day four of a 14-day dosing period. HCV-796 was found to be generally well tolerated, with favorable pharmacokinetics and no dose-limiting toxicities (www.vivopharma.com; link tested 27-3-2006).

Apart from the compounds that are known to have entered clinical trials, several other NNI of HCV are in (advanced) preclinical development. Thiophene derivatives (Fig. 7) are reversible allosteric inhibitors of the enzyme (Chan et al., 2004a,b). Replicons resistant to the compounds contain mutations at position 419 and 423 at the base of the thumb; which is different from the position where the JT-compounds bind. Binding of the thiophene analogues to this allosteric site also results in a conformation change of the enzyme that may explain the antiviral activity (Chan et al., 2004a,b; Wang et al., 2003).

Several benzothiadiazines (Fig. 8) were reported that target the viral polymerase. Although this class of compounds induce a variety of mutations in the enzyme, only methionine 414 is believed to be part of the inhibitor-binding site. This residue is located in the inner surface of the thumbdomain, close to the catalytic site of the enzyme. The allosteric binding site of the benzothiadiazines is thus again different from that of the thiophene and the benzimidazole derivatives (Tomei et al., 2004).

Obviously, various NNI of the HCV polymerase target different sites of the enzyme and thus exhibit a different resistance profile. When combined in a carefully planned way, various NNI of HCV may thus constitute a potent therapeutic option while offering the potential to prevent or delay the emergence of resistant virus.

In addition to "classical" non-nucleoside analogues inhibitors (NNI) several pyrophosphate mimics, including dihydropyrimidine carboxylic acids and diketoacids derivatives have been reported as inhibitors of HCV polymerase through an interaction with the catalytic metal ions in the active site of the enzyme (Summa et al., 2004; Summa, 2005). We will here not further elaborate on these classes of molecules.

5.2.2.1. Cyclosporin A and analogues, virus host cell interactions as antiviral targets. Recently CsA was shown to exhibit anti-HCV activity in vitro (Nakagawa et al., 2004; Watashi et al., 2003). Cyclosporin A (CsA) (Fig. 9) is an 11 amino acid (AA) cyclic peptide from which seven AA are *N*-methylated and three AA are uncommon, i.e. (4*R*)-4-[(*E*)-2-butenyl-4-methyl-L-threonine] (Bmt) in position 1, (L)-alpha-amino-butyric acid in position 2 and (D)-alanine (D-Ala) in position 8 (Fig. 9). As early as 1988, Teraoka et al. (1988) reported that CsA had a beneficial effect on Non-A, Non-B hepatitis in two chimpanzees chronically infected with HCV. After treatment with CsA was terminated, the disease recurred in both chimpanzees (Teraoka et al., 1988). Following these early reports, three clinical tri-

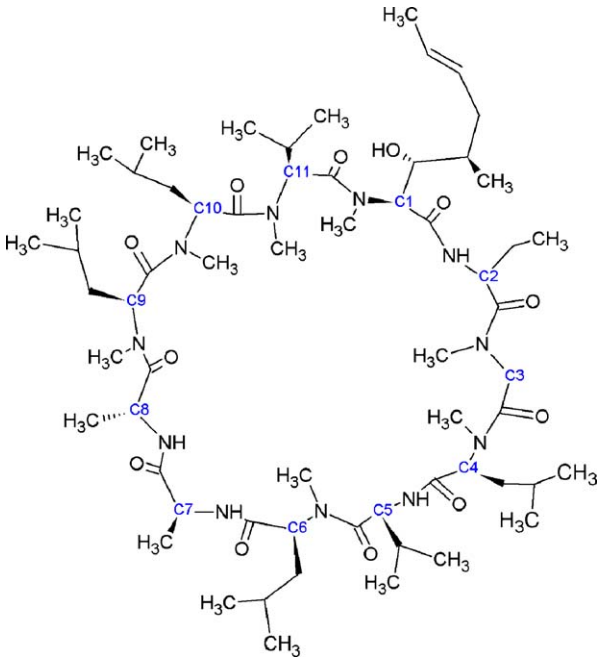

Fig. 9. Cyclosporine A.

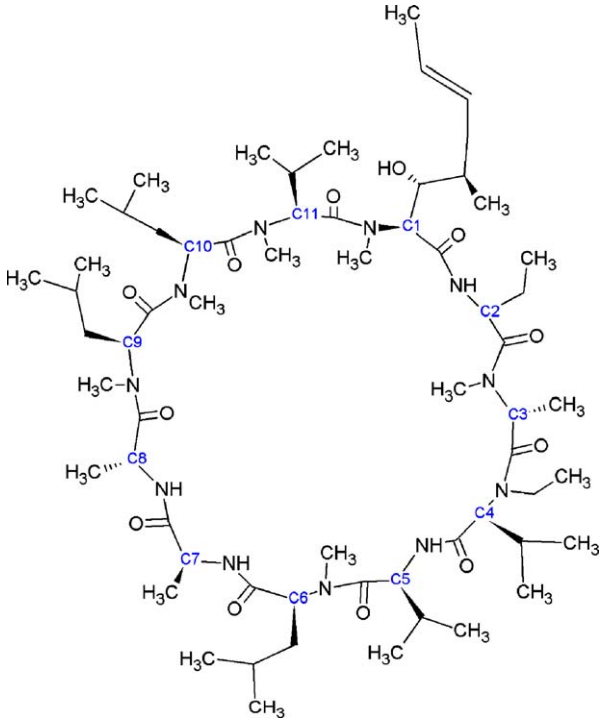

Fig. 10. Debio-025.

als were initiated in which the effect of CsA (in combination with IFN) was studied in patients with chronic hepatitis C. The results of these trials showed some on-treatment virological response in a small pilot study and a higher sustained virological response rate for IFN-CsA combination therapy as compared to IFN monotherapy (Cotler et al., 2003; Inoue and Yoshiba, 2005). Of note, blood CsA concentrations were related to the virological response (Inoue and Yoshiba, 2005). However, the immunosuppressive properties of CsA would be undesirable if the compound would be used as an antiviral for the treatment of a chronic viral infection. We recently reported on a novel synthetic non-immunosuppressive cyclosporin analogue, i.e. Debio-025 (Fig. 10) that is 10-fold more potent as an inhibitor of HCV replication than CsA, both in subgenomic HCV replicon-containing hepatoma cells, as well as in productively HCV infected cells (Paeshuyse et al., 2006). Debio-025 proved in vitro about equipotent to the HCV protease inhibitor VX-950 and the HCV polymerase inhibitor 2′-*C* methylcytidine. The compound proved also particularly efficient in curing replicon containing cells from their replicon (i.e. no replicon remained after treatment). Since Debio-025 is also a potent inhibitor of HIV-1 replication, and has an excellent safety profile, it may be an attractive drug candidate for the treatment of HCV infections, particularly including in HIV-1/HCV coinfected patients (Paeshuyse et al., 2006).

Since CsA and Debio-025 are effective in the subgenomic replicon system (that does not encode structural HCV proteins) their mode of action against HCV cannot be linked to an effect on structural proteins, and is thus almost per definition associated with the HCV replicase. The most likely mechanism by which CsA and Debio-025 inhibit HCV replication is *via* inhibition of the PPIase (peptidyl-propyl *cis*/*trans* isomerase) activity of cyclophilins. The anti-HCV activity of CsA was shown not to involve the calcineurin/NFAT-mediated pathway that the compound shares with FK506, an immunosuppressive drug that lacks anti-HCV activity (Nakagawa et al., 2005). Watashi and coworkers reported recently that cyclophilin B (Cyp B) acts as a functional regulator of the HCV RNA-dependent RNA polymerase (RdRp) and that the anti-HCV activity of CsA is mediated by disturbing this interaction (Watashi et al., 2005). This is in line with the observations of Nakagawa and coworkers who observed a reduction in HCV replication following the knock-down of cyclophilin A, B or C (Nakagawa, 2005 185/id). It will be important to further understand the precise role of cyclopylins in HCV replication and the modalities by which compounds such as Debio-025 interfere with their function. In particular, a drug such as Debio-025, will likely have a resistance profile that is very different from that of polymerase or protease inhibitors, and may therefore be an important option for the treatment of HCV infections (Rice and You, 2005).

6. Conclusion

There has, in recent years, been a remarkable progress in the development of potent and selective inhibitors of HCV replication. Several well tolerated drugs are currently in clinical development; encouraging results have been obtained with some of these. Combination therapy, with drugs that have a different mode of action, will most likely be necessary to achieve a rapid and potent antiviral response, and to suppress, delay or even avoid the development of drug resistance. Although the viral NS3 protease and the viral polymerase have so far received most attention, alternative targets in the viral replication cycle, such as the one targeted by cyclosporine A and related non-

immunosuppressive analogues, should be further explored. The recent elaboration of robust cell culture systems for HCV replication, will hopefully provide a boost to the discovery of drugs with yet other molecular targets.

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