The Exploding Field of the HCV Polymerase Non-Nucleoside Inhibitors: Summary of a First Generation Compounds

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Abstract: The hepatitis C virus (HCV) RNA-dependent RNA polymerase (RdRp) is strictly essential for viral replication and it has been used as viral target for anti-HCV drug development. All small molecules which have been identified to be selective non-nucleoside inhibitors (NNI) of the HCV RdRp to date are reported.

Key Words: HCV, RNA dependent RNA polymerase (RdRp), non-nucleoside, inhibition, drug development, antiviral, NNI, small molecules.

INTRODUCTION

Hepatitis C virus (HCV) is a major human pathogen that, according to the World Health Organization, has already infected about 180 million people (around 3% of the world-wide population). Disease occurrence has a prevalence of infection around 1%, or lower, in industrialized areas (Australia, Europe and North America), while it can be as high as 5-10% in African, Latin American and Central and South-Eastern Asian countries. Overall, it has been recently estimated that 3-4 million persons are newly infected each year. HCV infection is infrequently diagnosed during acute phase, as either no or only mild symptoms are observed most of the times, and patients can remain asymptomatic for decades before developing liver cirrhosis and/or liver cancer [1]. In fact, in approximately 70% of cases, HCV escapes the immune system and establishes a persistent infection, leading to the so-called "chronic carrier status". In the long term, these chronic carriers are at risk of developing life-threatening liver diseases, including hepato-cellular carcinoma [2]. HCV is responsible for 50–75% of all liver cancer cases as well as for two thirds of all liver transplants in the developed world. As a consequence, it has been compared to a silent killer.

Unfortunately, several lines of evidence suggest that developing effective HCV vaccines will be difficult [3]. The current standard treatment for HCV-infected patients is a combination therapy of pegylated interferon-α (PEG-INFα) and ribavirin (a guanosine analogue) [4]. However, given that more than 50% of HCV-1 (the genotype predominant in Europe and US) infected patients fail to achieve a sustained virological response, the need to develop alternative and effective HCV therapeutic approaches has become mandatory and, at first, IFN modifications aimed to increase its bioavailability and pharmacokinetics, as well as new ribavirin analogues, have been investigated [5, 6]. In the last few years, given that the growth of the treatment market for HCV have been foreseen to raise from the current ~US $3 billion per year to $8 billion (and even more) by 2010 [5], many pharmaceutical companies have been pushed to intensely pursue the identification of new drugs targeted to different viral proteins. As a result, several different new HCV inhibitors have been identified and are currently under clinical trial evaluations [6, 7].

HCV is a positive(+)-strand RNA virus belonging to the *Flaviviridae* family, genus *Hepacivirus*. The HCV genome is an uncapped, linear molecule with a length of around 9600 nucleotides carrying a single long open reading frame (ORF) flanked, at both 5' and 3' ends, by short non-translated regions (NTR) [8]. The ORF encodes for a polyprotein that is proteolytically cleaved into a set of distinct products, the structural proteins being located in the aminoterminal one-third and the nonstructural replicative proteins in the remainder. Recent insights into the HCV life cycle have revealed that the minimum HCV replication complex is formed by the NTR, the viral coded proteins NS3 protease, its cofactor NS4A, the NS3 helicase, the NS4B and NS5A proteins, whose function is not completely clarified yet, and the NS5B protein, the viral RNA-dependent RNA-polymerase (RdRp) [8]. Furthermore, host cell factors may also be required for *in vivo* replication [9, 10].

Among the different steps of the HCV life cycle that can be targeted by new drugs, one of the most extensively studied has been the viral RdRp. This choice has been determined by the fact that: i) there is no cellular counterpart for such enzyme activity and it is, therefore, an highly selective viral target; ii) the RdRp good processivity makes this enzyme relatively easy to be used for inhibitors screening; iii) the experience developed in the last 20 years in the HIV field could be somehow translated into the HCV field. As a results, different classes of HCV RdRp inhibitors have been recently identified. These compounds can be classified into nucleoside inhibitors (NIs) and non-nucleoside inhibitors (NNIs). The present review will focus on the recent advances made in the development of NNIs of the HCV RdRp. To this purpose, the RdRp activity and structure will be first shortly

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described. It is worth to note that many of these compounds have entered clinical trials and that, however, for concision reasons these information are not broadly discussed but only some of them are presented.

1. HCV RNA-DEPENDENT RNA POLYMERASE

The HCV RdRp is the core enzyme of the viral replication complex and catalyzes the genomic RNA synthesis in two steps. In the first step, it synthesizes the minus(-)-strand RNA using the (+)-strand genomic RNA as template; in the second step, it replicates the synthesized (-)-strand RNA to obtain new copies of the (+)-strand genomic RNA. The enzyme can initiate *de novo* (primer independent) RNA synthesis in biochemical assays and it is assumed that *de novo* initiation actually occurs *in vivo* [8]. Initiation of RNA synthesis at the 3'-end of the RNA templates, both (+)- and (-)-strands, most probably involves specific interactions between the RdRp and structures and/or sequences of the viral RNA templates. The secondary structures of the (+)-strand RNA comprise at its 3'-NTR a polypyrimidine [poly(U/UC)] tract of variable length and a highly conserved 98 base pairs segment (X-tail) which may form three stem loops that have been extensively studied [11-13]. Both poly(U/UC) tract and X-tail are essential for RNA replication both in cell culture and *in vivo* [11-15]. Furthermore, a stem-loop structure named 5BSL3.2 has been recently identified in the 3'-terminal region of the NS5B gene which seems to be essential for the HCV RdRp recruitment at the 3'-end of the (+)-strand RNA [16, 17]. In the (-)-strand RNA it has been shown that the first 125 base pairs at the 3'-end are essential for RNA replication and that the first 341 base pairs, which may fold into six stem loops, are also involved in the RNA initiation [18, 19].

The resolution of the HCV RdRp crystal structure showed the classical polymerase right hand structural fold with palm, fingers and thumb subdomains, and no fidelity domain [20-22]. The fingers subdomain interacts with the incoming nucleoside triphosphate (NTP) and the complimentary template base, while the thumb subdomain plays a role in positioning the RNA for initiation and elongation. In the active site, the catalytic aspartates which chelate the Mg^{2+} ions have also the same geometry of the other polymerases. The HCV RdRp, however, has some structural peculiarities. A first one is the fact that the active site is fully encircled by the multiple interactions between fingers and thumb subdomains which create a tunnel through which the ssRNA template is guided to the active site [20-22]. A second one is the presence of two loops, connecting fingers and thumb subdomains, that may be the major elements responsible for its closed conformation and may also play a role in the catalytic motion of the enzyme [23]. A third one is the presence of a low affinity GTP-binding site at the interface of the fingers and thumb subdomains and residing on the protein surface about 30 Å away from the active site [24]. Interestingly, since mutations of amino acid residues in the GTP binding site impaired or ablated the HCV replication in cellular systems, while they did not affect the *in vitro* RdRp activity, it has been hypothesized that the GTP binding may be involved in the RNA synthesis possibly by modulating the HCV RdRp interaction with other viral/cellular factors [25].

2. HCV RDRP NON-NUCLEOSIDE INHIBITORS

The cloning and expression of a recombinant form of the HCV RdRp that could be used for high-throughput screening (HTS) of compound libraries have led to a rush for the identification of effective RdRp inhibitors. Noteworthy, the development of valuable compounds has been also possible by the availability of HCV RNA replicon systems which allowed the efficient propagation of genetically modified HCV in human hepatoma cell lines [26]. In this way, the HTS results obtained in biochemical assays (usually expressed as IC_{50} values) could be confirmed by the results attained in HCV RNA replicon systems (usually expressed as EC_{50} values). Consequently, in the last five years, several small molecules with different chemical structures have been identified to be HCV RdRp inhibitors, up to a recent sort of explosion of the field in which a great number of new HCV NNIs have been published. These compounds can be considered a first generation of HCV RdRp NNIs. It is worth to note that, given the broad HCV genome diversity, the evaluation of the NNIs in biochemical assays had to take into consideration the RdRp of different viral strains. The RdRp most commonly used in biochemical assays belongs to the 1b strain and the majority of the reported data refers to this enzyme. However, several derivatives have been assayed also against the RdRp of other viral strains, even though these results have not been extensively included in this review but only some cases are discussed.

The currently disclosed HCV RdRp NNIs can be classified into four main categories according to their binding site, Fig. (**1**): i) compounds interacting with the metal cofactor Mg^{2+} in the active site (*pyrophosphate mimic*); ii) compounds binding in a cleft at the base of the palm and thumb subdomains in the active site (*allosteric active site binders*); iii) compounds binding in an allosteric NNI site which lies in the thumb subdomain close to the GTP binding site (*allosteric thumb-1 NNI site binders*); iv) compounds binding in a second allosteric NNI site which lies in the thumb subdomain (*allosteric thumb-2 NNI site binders*).

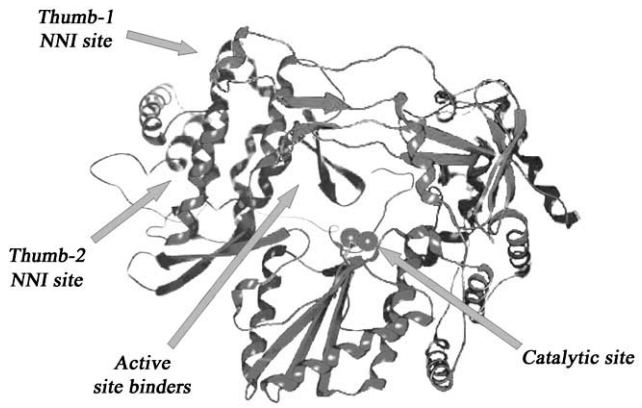


Fig. (1). Structure of HCV NS5B RdRp with known binding sites for NNI.

2.1. Pyrophosphate Mimic

Mechanistically, the HCV RdRp is thought to mediate the viral RNA synthesis through two Mg^{2+} ions, coordinated

in the palm subdomain, which serve the dual role of i) positioning/stabilizing the pyrophosphate leaving group on the incoming NTP and ii) activating the 3'-OH of the elongating RNA towards the nucleophilic attack. Phosphonophormate (PFA, foscarnet) is the prototype of the pyrophosphate analogues, targeting the DNA polymerase of herpesviruses and the reverse transcriptase (RT) of retroviruses [27, 28]. It has been proposed that PFA can act by mimicking the pyrophosphate motif of the incoming NTP substrate and inhibiting the polymerase non-competitively with respect to NTPs [27, 28]. Other compounds, with different structure, that may mimic the pyrophosphate motif and interact with the metal cofactor Mg^{2+} in the active site are the diketo acid (DKA) inhibitors of the HIV integrase and HIV Ribonuclease H [29-32]. Accordingly, when a random screening at the Istituto di Ricerche di Biologia Molocolare (IRBM), Merck Research Laboratories Rome, showed that some α-γ-DKA derivatives could inhibit the HCV RdRp, it revealed also that they act as pyrophosphate analogues [33]. In particular, a DKA derivative, compound (**1**), Fig. (**2**), inhibited in biochemical assays both HCV RdRp and HIV RT with a IC_{50} values of 5.7 and 54 μM, respectively, whereas it was inactive on both Klenow DNA polymerase and poliovirus RdRp [33]. Structure-activity relationship (SAR) studies demonstrated that the aryl group from the γ-position of compound (**1**) could be differently substituted, while the DKA moiety was essential for inhibition [33]. Among the synthesized derivatives, compound (**2**), Fig. (**2**), inhibited the HCV RdRp with an IC_{50} value of 45 nM whereas it was inactive on human DNA polymerases and HIV RT at 5 and 50 μM, respectively [33]. Given these observations, the anti-HCV activity of other DKA analogues, originally designed against the HIV integrase [30], was than predicted by a molecular modeling strategy and confirmed in biological assays with the aim of further developing the DKA scaffold for HCV inhibition [34]. More recently, mechanism of action studies have shown that compound (**2**) binds competitively to the NTP elongation pocket in the active site and inhibits both initiation and elongation steps [35].

The HTS program at IRBM also allowed the discovery of the monoethylester of meconic acid, compound (**3**), Fig. (**2**), (IC_{50} = 2.3 μM), as a lead of new selective NNIs which are mutually exclusive with compound (**1**) [36]. Since the two identified leads, compounds (**1**) and (**3**), shared an intrinsic chemical and biological instability, the 4,5-dihydroxypirimidine-6-carboxylic acid derivative, compound (**4**), Fig. (**2**), (IC_{50} = 30 μM), was designed from them to obtain compounds with more drug-like characteristics [37]. A first SAR study led to a series of 2-thiophene substituted 5,6-dihydroxypyrimidine-4-carboxylic acids that showed a 10-fold increase in potency with respect to compound (**4**) [38]. A second SAR study led to a N-methylpyrimidinone which showed an IC_{50} value of 6 μM, a good selectivity, but it was inactive in HCV replicon assays [37]. Further SAR studies led to more active derivatives, one of which is compound (**5**), Fig. (**2**), which showed an IC_{50} value of 30 nM in enzyme assays and an EC_{50} of 1.8 μM in cellular assays [39]. Biochemical studies confirmed that compound (**5**) binds to Mg^{2+} in the active site, and selection of drug-resistant HCV replicons suggested that P156 and G152 residues, within the RdRp active site, may be involved in its binding to the enzyme [39]. More recently, a new SAR study based on compound (**4**) as starting structure led to a series 5,6-dihydroxy-2-(2-thienyl)pyrimidine-4-carboxylic acids whose best derivative, however, was less active than compound (**5**) [40].

2.2. Allosteric Active Site Binders

A spherical hydrophobic pocket, comprising the amino acid residues P197, R200, L384, M414 and Y448 has been observed within the RdRp active site. This pocket is very close to the NTP binding site and can be considered an allosteric NNI binding site. Furthermore, an unique feature of the RdRp active site structure, mechanistically significant, is the

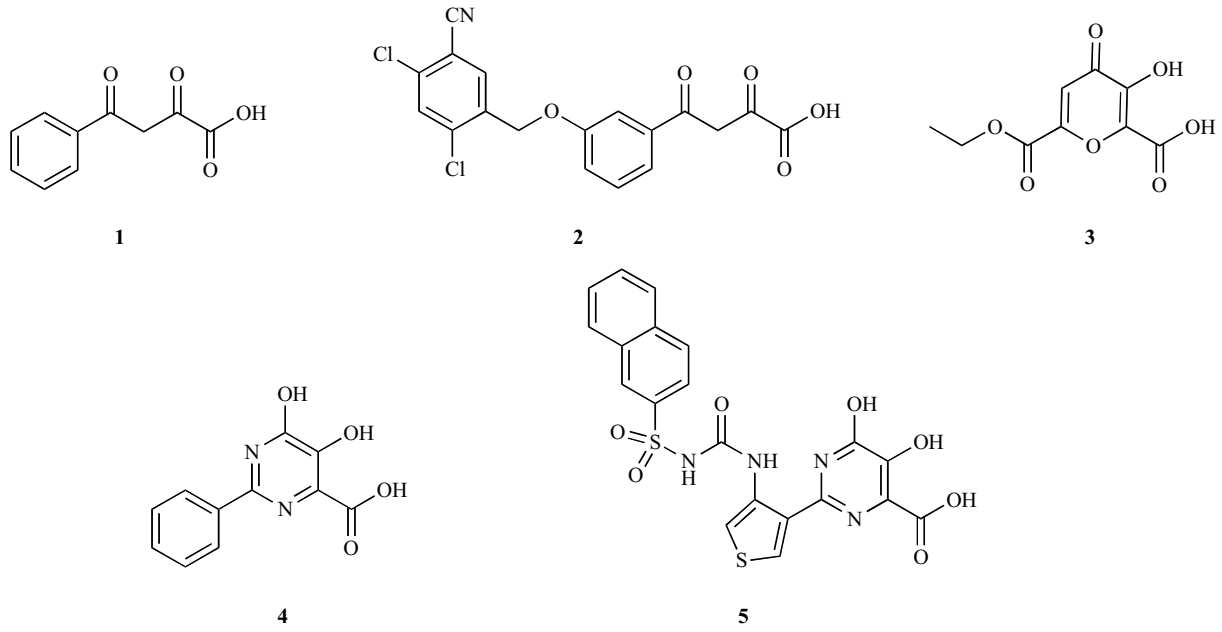


Fig. (2). Chemical structures of HCV RdRp pyrophosphate mimic inhibitors.

presence of a loop region (β-loop) which may form important interactions with the RNA template, the NTP and the ds-RNA intermediate [20-22]. This region, termed *primer-grip*, is involved in the assembly of the initiation complex and in the advancement of the RNA polymerization and can also be targeted by NNIs. The following compounds have been proposed to interact with the active site protein determinants.

HTS of the GlaxoSmithKline compound collection allowed the identification of several benzo-1,2,4-thiadiazine derivatives [41]. Among them, the *N*-isopentyl derivative, compound (**6**), Fig. (**3**), has been the first deeply characterized NNI: i) it inhibited in biochemical assays the HCV RdRp with an IC_{50} value of 80 nM, while it was not active against the Bovine Viral Diarrhea Virus (BVDV) RdRp and the human DNA polymerases; ii) it did not compete with the HCV RdRp substrates (both template and NTPs); iii) it showed in cellular assay an EC_{50} value of 0.5 μM [41]. Further studies demonstrated that compound (**6**) may form a ternary complex with the enzyme and the RNA template and that it interferes with the RNA synthesis initiation (both *de novo* and primer-dependent) rather than with the elongation process, possibly inducing a rapid dissociation of the enzyme from the RNA template [35, 42]. Selection of HCV RNA replicons resistant to compound (**6**) showed that a single mutation M414T may reduce its RdRp potency of inhibition by >100 folds [43]. Further drug resistance studies confirmed the importance for drug binding of the M414 residue, which lies in the α-helical region of the thumb subdomain with its side chain directed into the active site, and indicated a role for drug binding also for residues C451, which is in the β-hairpin, G558, which is close to the C-terminal end of the recombinant enzyme, and H95 [44]. Noteworthy, the H95 residue is in the fingers subdomain, distant from the potential NNI binding site, and it has been hypothesized that the H95R mutation may influence the binding of the ligand indirectly, possibly interacting with the bound template [44]. Recently, co-crystal structure of the HCV RdRp-compound (**6**) complex confirmed that it fit into a site at the interface between palm and thumb subdomains and interact, in particular, with residues F193, M414 and Y448 [45]. SAR studies on the N-1 alkyl group and on the C5-C8 positions of compound (**6**) brought to compound (**7**), Fig. (**3**), which showed IC_{50} and EC_{50} values of 10 and 38 nM, respectively, and favorable pharmacokinetic properties in rats, dogs and monkeys [45].

Based also on the above observations, the Abbott Laboratories synthesized a series of quinolone and naphthyridione benzothiadiazines in which the α-carbon of the N-1 side chain was replaced with either an oxygen or a nitrogen [46]. In particular, they found that the nitrogen-for-carbon replacement at the N-1 position of 4-hydroxy-quinolin-3-yl benzothiadiazines yielded potent inhibitors of which compound (**8**), Fig. (**3**), is a good representative showing both IC_{50} and EC_{50} values of 0.1 μM [46]. Further substitution of compound (**8**) with an ethancarbamyl group led to compound (**9**) (A-782759), Fig. (**3**), which showed an EC_{50} value of 70 nM, no cellular toxicity up to 63 μM and a good pharmacokinetic profile in animals [47]. Selection of HCV replicons with decreased susceptibility to compound (**9**) revealed mutations in the amino acid residues N411S, M414T, Y448H and H95Q [47]. The N411 residue clusters with the M414 and Y448 residues in the α-helical region of the thumb subdomain, close to the interface between palm and thumb subdomains [44, 47]. The Abbott Laboratories further synthesized several benzothiadiazine analogues among which the ones with a sulfonamide group were very potent [48]. Compound (**10**), Fig. (**3**), a representative of them, showed IC_{50} and EC_{50} values of 6 nM and 3 nM, respectively, but it was also strongly bound by plasma proteins [48]. Compound (**10**) was than differently substituted to obtain a variety of sul-

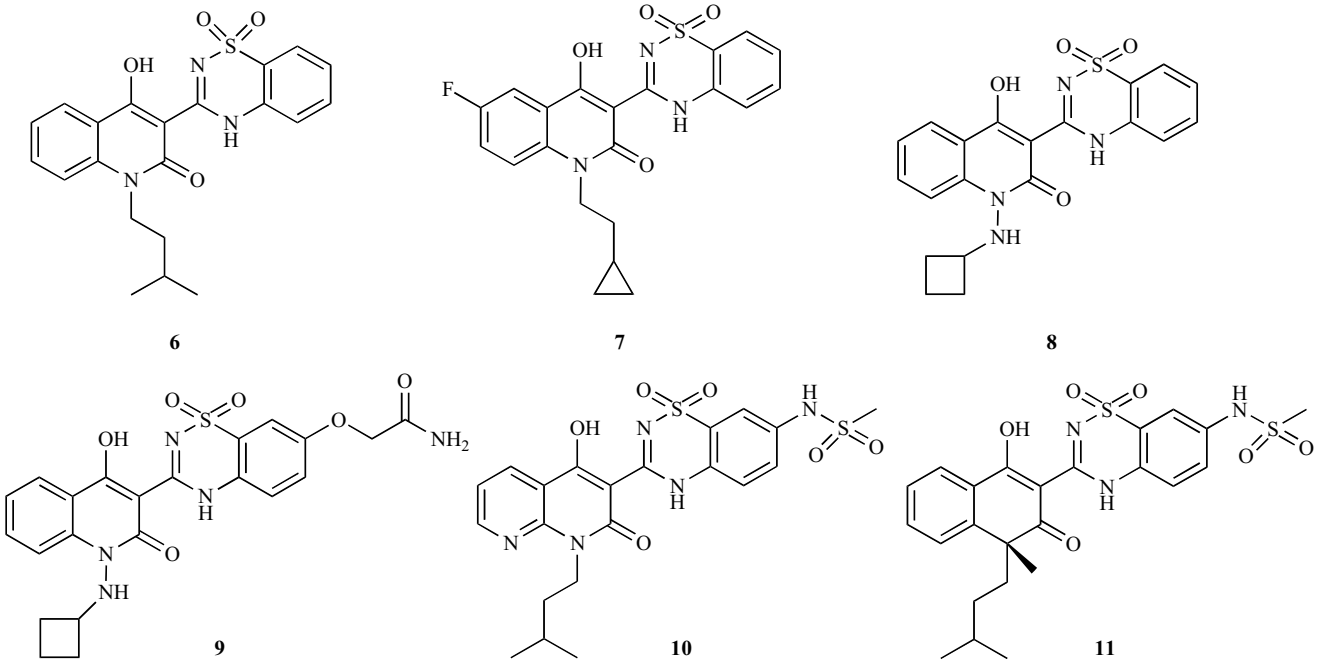
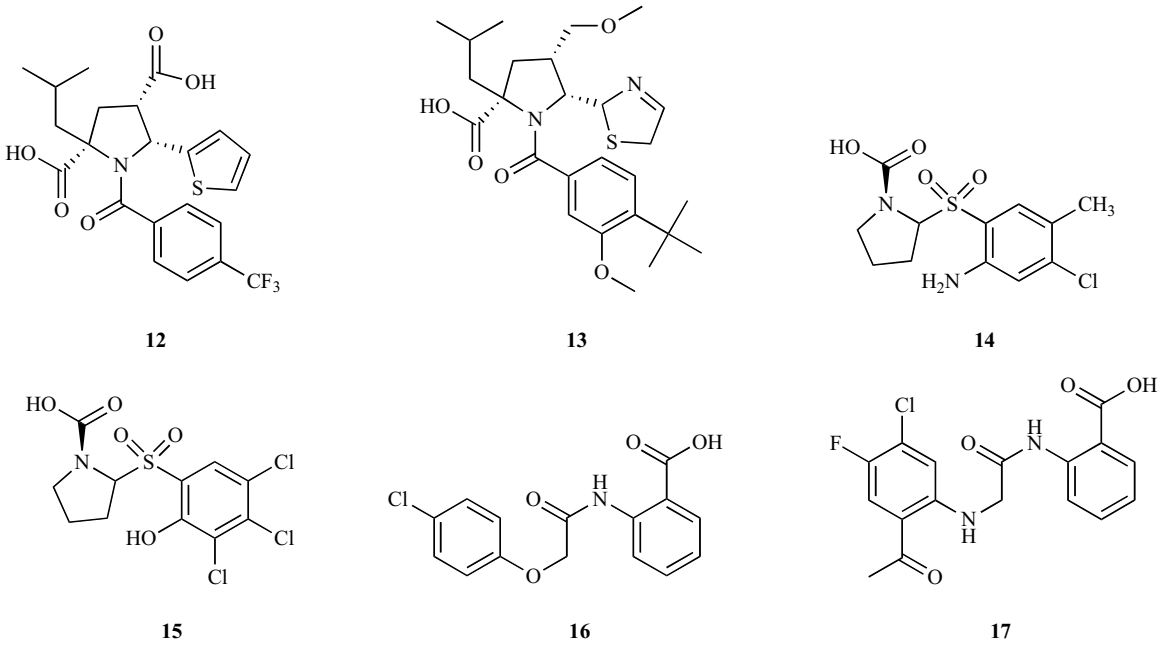


Fig. (3). Chemical structures of HCV RdRp allosteric active site binders: benzothiadiazines.



12 **13** **14**

15 **16** **17**

Fig. (4). Chemical structures of HCV RdRp allosteric active site binders: pyrrolidine, proline sulfonamide and anthranilic acid derivatives.

famide carbamates which, however, did not display an improvement in potency with respect to compound (**10**) [49]. The Abbott Laboratories additionally modified compound (**10**) synthesizing a set of unsymmetrical 1-hydroxy-4,4-dialkyl-3-oxo-3,4-dihydronaphthalene analogues, observing that the saturated dialkyl derivatives were the most potent among the tested compounds, but they did not improve the potency of the starting derivative [50]. A further optimization of compound (**10**) led to the identification of compound (**11**) (A-837093), Fig. (**3**), which showed in cellular assays an EC_{50} value of 6 nM and it had, in animal pharmacokinetic studies, an excellent metabolic profile achieving high plasma and liver concentrations [51]. When HCV replicons were selected in the presence of compound (**11**), the mutations S368A, Y448H, G554D, Y555C and D559G were observed, all located within or close to the known benzothiadiazine binding site [45, 51]. Interestingly, the M414T mutation that was selected by other benzothiadiazine analogues was not selected by compound (**11**) and, in fact, a mutated M414T replicon showed only a two-fold decrease in susceptibility to compound (**11**) [51]. It also worth to note that these mutations strongly reduced the replication capacity in cell culture of HCV replicons [51]. Recently, compound (**11**) has been evaluated in the chimpanzee model and found active [52].

The GlaxoSmithKline HTS program, performed on their compound collection, also allowed the identification of a series of pyrrolidine derivatives which initially had a modest anti-RdRp activity [53]. SAR studies led to the synthesis of compound (**12**), Fig. (**4**), which inhibited the HCV RdRp in biochemical assays with an IC_{50} value of 0.3 μM, but it was inactive in cellular assays [53]. The resolution of the X-ray structure of compound (**12**) in complex with the HCV RdRp showed that acyl pyrrolidine derivatives bind in a pocket of the palm region close to the active site [54] and guided further SAR studies performed to increase their cellular activity and pharmacokinetic properties [54, 55]. As a result, the replacement of the 4-CF_3 group by a bulkier 4-*tert*-butyl in the benzamide and the conversion of a carboxylic acid in an methyl ether led to compound (**13**), Fig. (**4**), which showed both IC_{50} and EC_{50} values of 0.4 μM and displayed a good pharmacokinetic profile in rats [54]. Further details of the molecular interaction of acyl pyrrolidines and the HCV RdRp have recently been reported [56]. Noteworthy, the GlaxoSmithKline acyl pyrrolidine analogue GSK625433 has been recently reported to have good pharmacokinetic profile in pre-clinical animal species[1] and it is currently under phase I clinical evaluation.

A discovery program made by Wyeth Research have recently identified two new series of active site binders. The first one is represented by the lead proline sulfonamide derivative compound (**14**), Fig. (**4**), which, in biochemical assays, inhibited the HCV RdRp with an IC_{50} value of 3.1 μM, it did not inhibit human, calf and HIV-1 DNA polymerases, and it had a K_D value of 1.6 μM [57]. Determination of the crystal structure of the HCV RdRp-compound (**14**) complex revealed that compound (**14**) binds approximately 10 Å from the motif D catalytic aspartic acids in the active site interacting, most significantly, with the β-loop containing the Y448 residue, and also with residues M414, Y415, Q446 and G449 [57]. SAR studies focused to determine the importance of both aryl group substituents and proline ring itself led to compound (**15**), Fig. (**4**), which showed an IC_{50} value of 80 nM [57]. The second series of inhibitors is represented by an anthranilic acid derivative, compound (**16**), Fig. (**4**), which showed an IC_{50} value of 1.6 μM [58]. SAR studies performed to assess the effect of i) the substitution of the methylene linker; ii) the introduction of different substituents in

[1]Amphlett, E.E.; Bright, H.; Chambers, L.; Cheasty, A.; Fenwick, R.; Haigh, D.; Hartley, D.; Howes, P.; Jarvest, R.; Mirzai, F.; Nerozzi, F.; Parry, N.; Slater, M.; Smith, S.; Thommes, P.; Wilkinson, C.; Williams, E. *J Hepatol* **2007**, *46*, Suppl 1: S225.

the phenoxy ring of compound (**16**); iii) the introduction of different groups in an aniline structure, led to compound (**17**), Fig. (**4**), which inhibited the HCV RdRp activity in biochemical assays with an IC_{50} of 17 nM and the HCV replicon multiplication with an EC_{50} of 2.0 μM [58]. The resolution of the X-ray crystal structure of the HCV RdRp and both compounds (**16**) and (**17**) suggested that they bind in the same site where the proline sulfonamides bind, and interact with residues R386, Y415, Y448 and, in the case of compound (**17**) also with a pocket close to residue F193 [58].

In the effort of developing new HCV RdRp NNIs, Pfizer identified the (*2Z*)-2-(benzoylamino)-3-(5-phenyl-2-furyl) acrylic acid, compound (**18**), Fig. (**5**), which showed a moderate activity in biochemical assays with an IC_{50} value of 6.7 μM [59]. A first SAR study combined with structure based drug design led to the synthesis of compound (**19**), Fig. (**5**), which showed an IC_{50} value of 0.1 μM, but had an EC_{50} value in HCV replicon assays of 28 μM [59]. X-ray crystal analysis revealed that compound (**19**) binds in the primer-grip site within the active site, interacting mainly with residues Y415, Y448 and S556, and also with a deep pocket formed by residues P197, L384, M414, Y415 and Y448 [59]. Compound (**19**) inhibited both *de novo* initiation and elongation reactions and exhibited reasonable pharmacokinetic properties [59]. Further SAR studies, performed in the effort to increase the HCV replicon inhibitory activity, revealed that the introduction of substituents in the aryl ring of compound (**19**) did not increase its potency, while modification of its carboxylic acid led to compound (**20**), Fig. (**5**), which showed IC_{50} and EC_{50} values of 2.7 and 8 μM, respectively. Further characterization of compound (**20**) showed that it binds at the primer-grip site and that it also has reasonable pharmacokinetic properties [60].

HTS of a small-molecule compound library performed by Amgen Inc. identified a benzydilene derivative, compound (**21**), Fig. (**5**), as selective HCV RdRp inhibitor with an IC_{50} value of 1.5 μM and no activity on both BVDV RdRp and HIV-1 RT [61, 62]. SAR studies designed to understand the role of the arysulfonyl and the 3-amino groups, and to assess the effect of different substituents in the structure of compound (**21**) led to derivatives with nanomolar potency of inhibition, one of which is compound (**22**), Fig. (**5**), (IC_{50} = 200 nM) [61]. X-ray analysis revealed that compound (**22**) inhibits the HCV RdRp by covalently binding the highly conserved C366 residue, located at the junction between palm and thumb subdomains in a hydrophobic pocket only 8 Å from the GDD motif of the RdRp active site, and by interacting with residues F193, M414, Y415 and Y448 [61, 62].

HTS made by Valeant Pharmaceuticals International identified the isothiazol derivative compound (**23**), Fig. (**5**), that showed IC_{50} and EC_{50} values of 5.9 and 2.0 μM, respectively, as lead for further development [63]. X-ray analysis of the complex HCV RdRp-compound (**23**) proved that, similarly to compound (**22**), compound (**23**) forms a covalent bond with the C366 residue of the primer-grip, it binds to the N316, M414 and Y415 residues in the active site, and it makes also contacts with the Y448 of the β-loop of the thumb subdomain [63]. SAR studies on compound (**23**) brought to the synthesis of compound (**24**), Fig. (**5**), which showed IC_{50} and EC_{50} values of 200 nM and 100 nM, respectively, and a CC_{50} value of 81 μM [63].

2.3. Allosteric thumb-1 NNI Site Binders

An hydrophobic cleft in the thumb subdomain, near the C-terminus of the HCV RdRp and at a distance of 10-15 Å from the low affinity GTP-binding site, has been identified

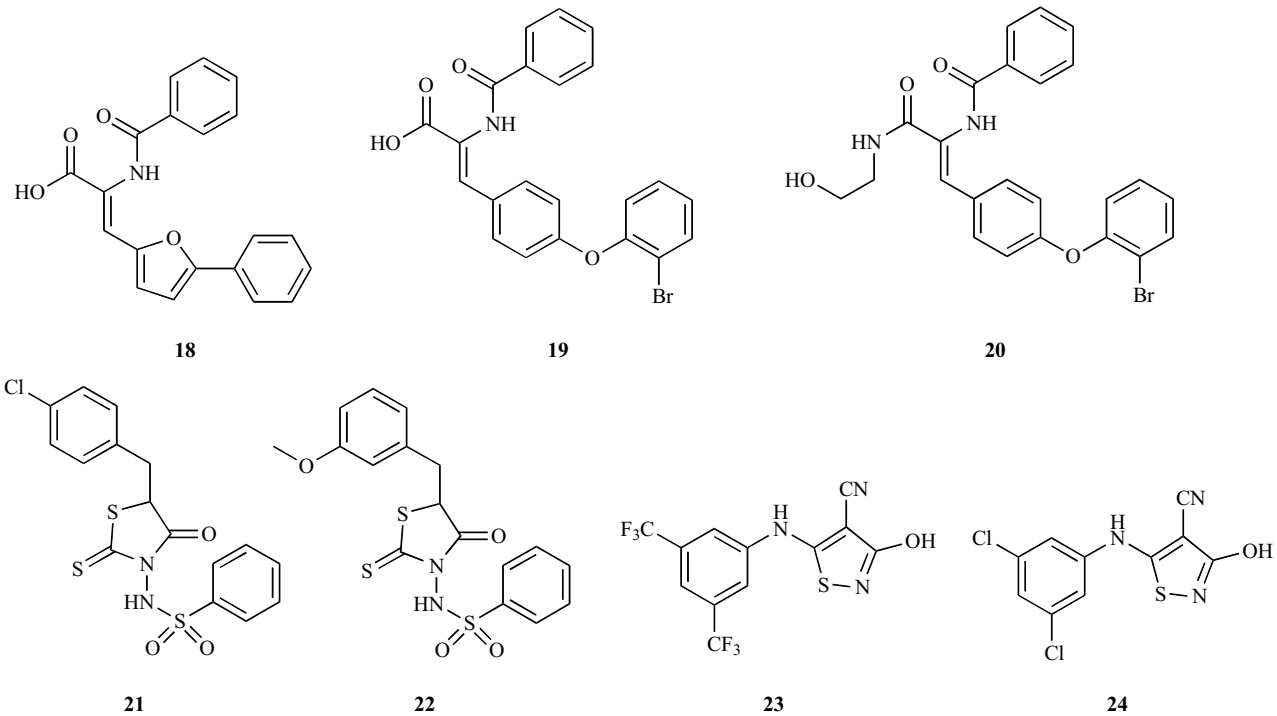


Fig. (5). Chemical structures of HCV RdRp allosteric active site binders: benzoylamino-phenyl-furyl acrylic acid, benzydilene and isothiazol derivatives.

as an allosteric drug binding site (thumb-1 NNI binding site). The following compounds have been proposed to bind to this enzyme site.

Boehringer Ingelheim has identified, from a combinatorial library, a series of benzimidazole derivatives exemplified by compound (**25**), Fig. (**6**), which showed an IC_{50} value in biochemical assays of 12 μM [64]. A first SAR study performed on compound (**25**) led to eliminate the side amide substituent and, since the further removal of the N-1 substituent was not tolerated, to insert different groups in the C-2 position of compound (**25**). Among the synthesized derivatives, compound (**26**), Fig. (**6**), was one of the most active in biochemical assays (IC_{50} = 1.6 μM), however, it had no effect in the HCV replicon system [64]. A second SAR study performed on compound (**26**) brought to a tryptophan derivative, compound (**27**), Fig. (**6**), which showed an IC_{50} value of 50 nM, but it was still devoid of cellular activity [65]. Kinetic studies performed on different benzimidazole analogues, comprised compound (**27**), to investigate their mechanism of action proved that: i) they inhibit the HCV RdRp catalyzed initiation reaction, while they have no effect on the RNA elongation; ii) they are non-competitive with respect to NTP incorporation; iii) they inhibit the productive binding of the RNA template-primer [66, 67]. Further development of compound (**27**), by replacement of the ionisable acidic functions with different neutral entities, reduced the potency of inhibition in enzyme assay, but improved the antiviral activity in the HCV replicon assay [68]. The best de-

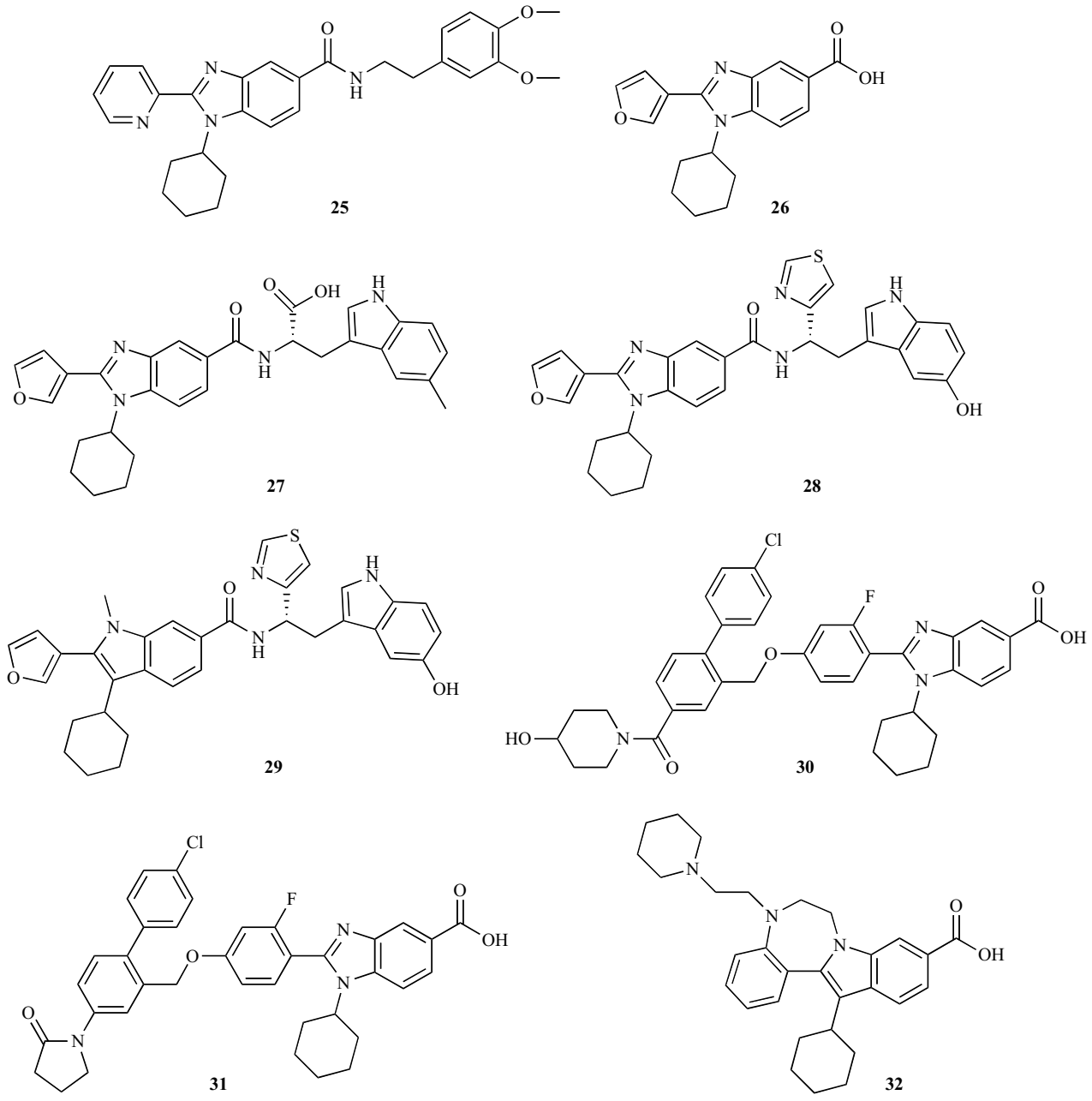


Fig. (6). Chemical structures of HCV RdRp allosteric thumb-1 NNI site binders: benzimidazoles.

rivative of this series was compound (**28**), Fig. (**6**), which showed IC_{50} and EC_{50} values of 0.3 and 1.7 μM, respectively [68]. Drug-resistance studies demonstrated that HCV replicons resistant to benzimidazoles were mutated at residues P495, P496 and V499 [67, 69]. Noteworthy, the P495 residue makes contact with the regulatory GTP, being a key residue in the GTP binding site. More recently, modifications of the imidazole core were attempted with the aim to improve the cellular penetration. In particular, replacement of the polar benzimidazole core by lipophilic indoles improved the anti-HCV activity in cell-based assays [70]. The most potent inhibitor of this series was compound (**29**), Fig. (**6**), which showed IC_{50} and EC_{50} values of 100 and 50 nM, respectively [70].

Benzimidazole derivatives were also developed by Japan Tobacco and a representative analogue, compound (**30**), Fig. (**6**), which showed IC_{50} and EC_{50} values of 0.28 and 0.35 μM, respectively, was extensively studied [69]. More recently, a SAR study was published in which some 2-[(4-diarylmethoxy)phenyl]-benzimidazoles were reported to inhibit the HCV RdRp in both enzyme and cellular assays [71]. These derivatives were further modified to obtain compounds bearing different substituents in the biphenyl ring [72]. Among them is compound (**31**) (JTK-109), Fig. (**6**), that showed IC_{50} value of 17 nM, EC_{50} value of 0.32 μM, favorable pharmacokinetic and safety profiles, and which, therefore, had been considered a promising clinical candidate [72]. Subsequently, compound (**31**) has been modified in a SAR study that brought to a new series of tetracyclic compounds comprising compound (**32**), Fig. (**6**), that showed IC_{50} and EC_{50} values of 9 and 35 nM, respectively [73].

Starting from the previously characterized benzimidazoles [69], the IRBM developed a new series of indole-*N*-acetamide derivatives whose first lead was compound (**33**), Fig. (**7**), that inhibited the HCV RdRp activity in enzyme assays with an IC_{50} value of 11 nM, it was effective in the HCV replicon system with an EC_{50} value of 300 nM and it showed a good pharmacokinetic profile [74]. Compound (**33**) was than optimized through the resolution of the X-ray crystal structure of the HCV RdRp-compound (**33**) complex, that revealed that it binds in the thumb subdomain occupying a liphophilic site and interacting with residues P495 and R503, and a subsequent SAR study that led to a series of new derivatives with nanomolar activity in both enzyme and cellular assays [75]. Further structural data confirmed that these indole-based inhibitors bind, indeed, in a space close to the GTP binding site but which is clearly distinct from it, even though the two sites share some amino acid residues, in particular P495 [69, 76]. A compound similar to the ones reported [75], compound (**34**), Fig. (**7**), (IC_{50} = 6 nM, EC_{50} = 150 nM), was stopped in its development due to the formation of glucuronide conjugates of its carboxylic acid as major circulating metabolites [77]. Therefore, in the effort to circumvent this problem, new series of indoles in which C6 carboxylic acid of compound (**34**) was replaced have been synthesized and recently disclosed [77]. One of these derivatives is compound (**35**), Fig. (**7**), (IC_{50} = 4 nM, EC_{50} = 120 nM) [77].

It is worth to note that, in the effort of developing the benzimidazole core, the IRBM synthesized also a series of thieno[3,2-*b*]pyrrole derivatives, some of which were active in biochemical assays at nanomolar concentrations and in cellular assays at micromolar concentrations [78].

Another series of indole analogues, pyrano[3,4]indoles, were identified by Wyeth Research and Viropharma through HTS of the Wyeth proprietary chemical library. The hit, compound (**36**), Fig. (**7**), was found to be a selective inhibitor of the HCV RdRp with an IC_{50} value of 3.0 μM [79]. A preliminary SAR study led to compound (**37**) (HCV-371), Fig. (**7**), which inhibited the RdRp from different HCV genotypes with IC_{50} values in the 0.3-1.4 μM range and showed anti-replicon activity in cells with an EC_{50} value of 4.8 μM [79, 80]. When compound (**37**) was characterized through X-ray and drug-resistance studies it was shown to bind to the pocket on the surface of the thumb subdomain close to the GTP binding site and to interact, specifically, with residues L419 and M423 [81]. The analogues disclosed in another published SAR study did not significantly improve the potency of inhibition showed by compound (**37**) [82].

A series of pteridine derivatives identified through HTS by Valeant Pharmaceuticals International had also some resemblance to the benzimidazoles described by Boehringer Ingelheim [83]. The hit, compound (**38**), Fig. (**7**), was moderately active against the HCV RdRp (IC_{50} = 15 μM) [83]. A SAR study was performed to improve its potency of inhibition, but the disclosed analogues of this series showed only a moderate activity in cellular assays [83]. More recently, Valeant Pharmaceuticals International reported the identification of other derivatives that also bear some similarity to benzimidazole analogues, the quinaxolines, whose most active reported derivative, compound (**39**), Fig. (**7**), inhibited the HCV RdRp in enzyme assays with an IC_{50} value of 0.6 μM [84].

Very recently, a new series of benzimidazole-coumarin conjugates have been reported to inhibit the replicon activity in cell-based assays in the low micromolar range [85]. Noteworthy, the further SAR elaboration of these derivatives led to the imidazopyridine derivative GS9190[2] developed by Gilead whose phase I clinical trial has been recently reported[3]. However, a safety issue linked to abnormality in the heart's rhythm emerged and a lower dosage is currently under evaluation.

2.4. Allosteric thumb-2 NNI Site Binders

A second hydrophobic pocket has been located in the thumb subdomain, near the second last helix in the C-terminal region, around 35 Å away from the active site (thumb-2 NNI binding site). The two allosteric sites (thumb-1 and thumb-2 NNI binding sites) are 14 Å apart and may be functionally linked. The following compounds have been reported to bind to the thumb-2 NNI binding site.

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[2]Vliegen, I.; Paeshuyse, J.; Marbery, E.; Peng, B.; Shih, I.; Lehman, L.S.; Dutartre, H.; Selisko, B.; Canard, B.; Bondy, S.; Tse, W.; Reiser, H.; De Clercq, E.; Lee, W.A.; Puerstinger, G.; Zhong, W.; Neyts, J. AASLD, Nov 2-6, 2007, Boston, MA

[3] Bavisotto, L,; Wang, C,; Jacobson, I,; Marcellin, P,; Zeuzem, S,; Lawitz, S,; Lunde, N.M,; Sereni, P,; O'Brien, C,; Oldach, D,; Rhodes, G; and the GS-9190 Study Team. AASLD, Nov 2-6, 2007, Boston, MA

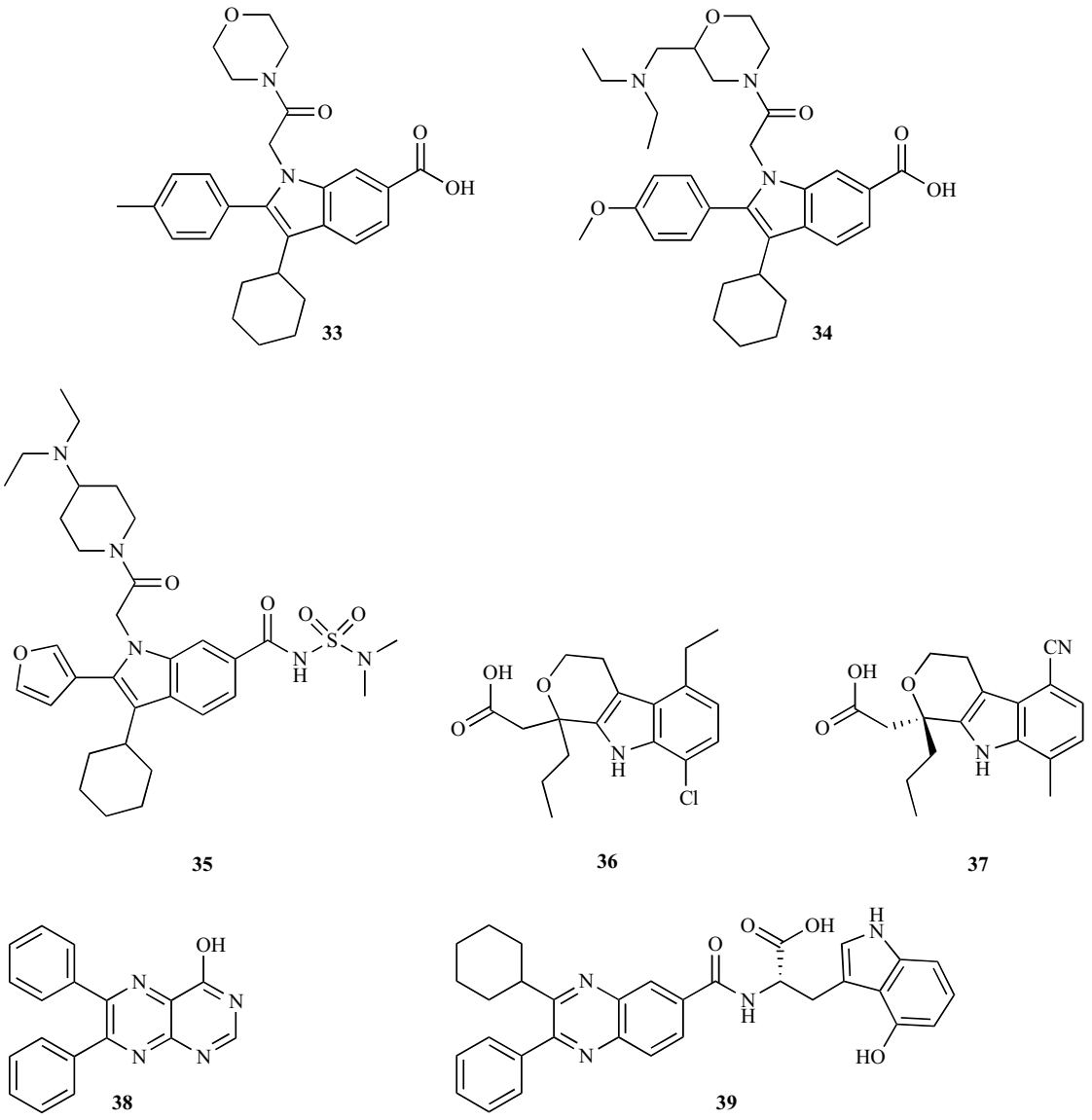

Fig. (7). Chemical structures of HCV RdRp allosteric thumb-1 NNI site binders: indole and pteridine derivatives.

A series of *N*,*N*-disubstituted phenylalanines were discovered by Shire BioChem through HTS. Their lead, compound (**40**), Fig. (**8**), inhibited the HCV RdRp activity with an IC_{50} value of 5.7 μM [86]. SAR studies were performed to improve its potency [86, 87]. The resolution of the crystal structure of the complex of the HCV RdRp with a phenylalanine derivative, compound (**41**), Fig. (**8**), (IC_{50} = 2.2 μM), demonstrated that compound (**41**) binding site consists of two hydrophobic pockets, the primary pocket is formed by amino acid residues L419, R422, M423, L474, H475, Y477 and W528, while the secondary pocket involves residues S476 and Y477 [88].

HTS performed by Shire BioChem allowed also the identification of series of thiophene-2-carboxylic acids with moderate activity on the HCV RdRp in biochemical assays, compound (**42**), Fig. (**8**), (IC_{50} value = 14 μM) [89]. SAR studies led to the synthesis of compound (**43**), Fig. (**8**), which showed IC_{50} and EC_{50} values of 1 and 5 μM, respectively [89, 90]. Further SAR study on this class of compounds were also performed by Shire BioChem together with ViroChem [91]. It is worth to note that, very recently, ViroChem Pharma reported the anti-viral activities of derivative VCH-759 that, in a double blind clinical trial, was well tolerated and showed 2 log viral load reduction[4] and it is now in phase IIa, and derivative VCH-916 which in a phase I study was safe and well tolerated[5].

In another study, a thiophene-2-carboxylic derivative was reported by Roche to bind in the thumb-2 NNI site, mainly taking contacts with amino acid residues S476 and Y477, and to select for drug resistant HCV replicon variants mutated at residues L419, M423 and I482 [92].

[4]Cooper, C.; Lawitz, E.J.; Ghali, P.; Rodriguez-Torres, M.; Anderson, F.H.; Lee, S.S.; Proulx, L. AASLD, Nov 2-6, 2007, Boston, MA.

[5]Proulx, L.; Bourgault, B.; Chauret, N.; Larouche, R.; Tanguay, M.; Thibert, R. EASL, Apr 23-27, 2008, Milan, Italy.

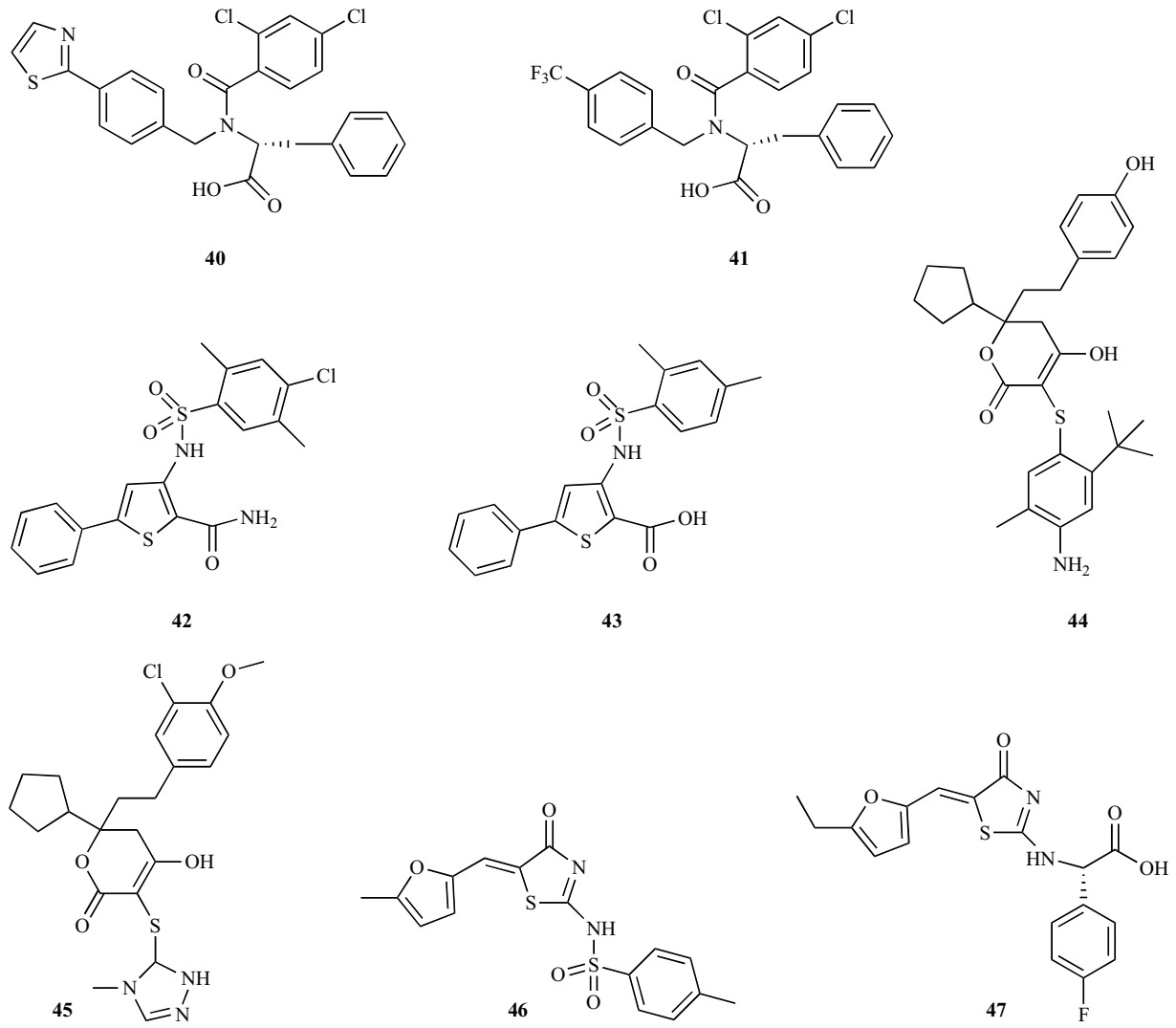

Fig. (8). Chemical structures of HCV RdRp allosteric thumb-2 NNI site binders: phenylalanine, thiophene-2-carboxylic acid, dihydropyranone and thiazolone sulfonamide derivatives.

Also HTS performed by ViroPharma Incorporated led to the identification of tetrahydrobenzothiophene derivatives that inhibited the HCV RdRp in biochemical assays at submicromolar concentrations [93]. Furthermore, a virtual screening program made by Achillon Pharmaceuticals, aimed to dock a library of available lead-like compounds in the thumb subdomain of the HCV RdRp, allowed the identification of new dialkyl substituted thiophene derivatives which were active in the HCV replicon assay with EC_{50} values in the 0.9-2.0 μM range [94].

Dihydropyranones were identified by Pfizer in an HTS of proprietary compounds. One of these derivatives, compound (**44**), Fig. (**8**), inhibited the HCV RdRp activity with an IC_{50} value of 0.93 μM [95]. Compound (**44**) was shown to interact with amino acid residues S476, Y477, L497, R501 and other residues of the thumb-2 NNI site [95]. A first structure-based optimization of compound (**44**) was recently disclosed [96]. The SAR study led to the synthesis of compound (**45**), Fig. (**8**), which showed an IC_{50} value in biochemical assays of 38 nM, however, similarly to all the so far published dihydropyranone derivatives, it was devoid of antiviral activity in cellular assays [96]. Noteworthy, Pfizer recently reported[6] that the NNI PF-00868554, which binds to the thumb site, showed an IC_{50} and EC_{50} values of 9.6 and 59 nM, respectively. This compound is currently under phase I evaluation.

Aminothiazole derivatives were originally reported by NeoGenesis Pharmaceuticals and Schering-Plough to inhibit the HCV RdRp activity in the low micromolar range [97]. More recently, thiazolone sulfunamide derivatives were disclosed by Valeant Pharmaceuticals [98, 99]. Among them, compound (**46**), Fig. (**8**), inhibited the enzyme activity with an IC_{50} value of 11 μM and it was shown to interact mainly with amino acid residues S476, Y477 and R501 [98, 99]. Compound (**46**) was used as lead to obtain new analogues through a structure-based drug design approach that led to the synthesis of compound (**47**), Fig. (**8**), (IC_{50} = 3.0 μM) [99, 100]. Compound (**47**) was than further modified to obtain derivatives, comprising thiazolone-acylsulfonamides,

[6]Shi, S.; Herlihy, K.; Irvine, R.; Binford, S.; Lewis, C.; Nonomiya, J.; Patick, A. AASLD, Nov 2-6, 2007, Boston, MA

that inhibited the HCV RdRp in biochemical assays with IC_{50} values in the 5-20 μM range [100, 101].

2.5. Other NNIs

A few other series of compounds have been identified as HCV RdRp inhibitors in biochemical assays which, however, are devoid of activity in HCV replicon assays and whose binding to the protein has not been clarified yet. Among them are: i) [(naphthalene-1-carbonyl)-amino]-acetic acid derivatives, whose most potent published compound showed an IC_{50} value of 0.12 μM, have been identified by Wyeth [102]; ii) 5-cyano-6-aryl-2-thiouracil derivatives, whose most potent derivative showed an IC_{50} value of 3.8 μM, have been disclosed by Valeant Pharmaceuticals International [103].

Finally, it is worth to note that in the last years several clinical trials have been performed on NNIs and several candidates which seemed to be promising in preclinical studies were halted due to toxicity and/or insufficient efficacy. As an example, the promising Wyeth/ViroChem Pharma NNI candidate HCV-796 has been halted in its development due to safety issue that emerged in a Phase II trial. Interestingly, however, these studies have also indicated that the NNIs resistance mutations observed *in vivo* correlated well with the ones observed *in vitro*. Therefore, the lack of cross-resistance among the NNIs coming from the described four NNI classes, due to the fact that each NNI class binds to a specific enzyme pocket, gives the opportunity to optimistically consider the possibility (and opportunity) to attempt a combination therapy which may include the simultaneous use of NNIs belonging to the diverse NNI classes. In conclusion, despite the fact many NNI have been already identified, the HCV treatment will not improve significantly until more effective, less toxic, more affordable NNIs will be available, particularly for patients who are unlikely to respond to, or unable to tolerate, the current standard of care.

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