Structure–Activity Relationships in the Development of Allosteric Hepatitis C Virus RNA-Dependent RNA Polymerase Inhibitors: Ten Years of Research

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Abstract: Hepatitis C is a viral liver infection considered as the major cause of cirrhosis and hepatocellular carcinoma (HCC). Hepatitis C virus (HCV) possesses a single positive strand RNA genome encoding a polyprotein composed of approximatively 3000 amino acids. The polyprotein is cleaved at multiple sites by cellular and viral proteases to liberate structural and nonstructural (NS) proteins. NS5B, the RNA-dependent RNA polymerase (RdRp), which catalyzes the HCV RNA replication has emerged as an attractive target for the development of specifically targeted antiviral therapy for HCV (DAA, for direct-acting antivirals). In the last 10 years, a growing number of non-nucleoside compounds have been reported as RdRp inhibitors and few are undergoing clinical trials. Over the past 5 years, several reviews were published all describing potentially active molecules. To the best of our knowledge, only one review covers the structure–activity relationships.[1] In this review, we will discuss the reported non-nucleoside molecules acting as RdRp inhibitors according to their chemical class especially focusing on structure–activity relationship aspects among each class of compounds. Thereafter, we will attempt to address the global structural requirements needed for the design of specific inhibitors of RdRp. Med. Res. Rev., 33, No. 5, 934–984, 2013

Key words: hepatitis C; RdRp; polymerase; allosteric inhibitors; structure–activity relationship

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1. INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, including chronic hepatitis and cirrhosis that may lead to hepatocellular carcinoma (HCC). The World Health Organization (WHO) estimates that 170 million people (i.e., 2–3% of the world population) are infected with HCV worldwide, while 3–4 million are newly infected each year.

In the absence of a prophylactic vaccine, the only therapeutic option remains antiviral therapy. Chronic HCV infection is curable by treatment. Until recently, the standard of care for HCV infection consisted of a combination of pegylated interferon-alpha (IFN-α) and ribavirin administered for 24 or 48 weeks, depending on the HCV genotype. Unfortunately, this therapy is poorly tolerated, and is not successful in all patients, emphasizing the need for more effective, better tolerated treatments.

New direct-acting antiviral (DAA) drugs are at the developmental stage. Among them, the most promising DAAs target the NS3/4A serine protease that ensures polyprotein processing, that is, the maturation of viral proteins, and the HCV RNA-dependent RNA polymerase (RdRp), which catalyzes viral replication, that is, synthesis of new genomes. Two NS3/4A protease inhibitors, telaprevir, and boceprevir, were approved in Europe and the United States in 2011 for use in combination with pegylated interferon and ribavirin in both treatment-naïve and treatment-experienced patients chronically infected with HCV genotype 1. Although the use of these drugs increases clearance rates up to 70–80% in clinical trials, tolerance is poor and treatment failure is associated with the selection of viral variants that are resistant to the protease inhibitor.

The RdRp activity is carried by the HCV nonstructural 5B (RdRp) protein. It is essential for HCV replication and has no functional equivalent in mammalian cells, making it an ideal target for new anti-HCV drugs. During the last 15 years, considerable efforts were carried to unravel the structural characteristics of HCV RdRp and discover drugs that inhibit its enzymatic function.

The HCV RdRp is a tail-anchored transmembrane protein of 591 amino acids. The recombinant RdRp lacking its C-terminal 21 amino acid anchoring domain is soluble and retains RdRp activity in vitro. The crystal structure of the HCV RdRp revealed a "right hand" shape, displaying the characteristic fingers, palm, and thumb subdomains.

In addition to the catalytic site, four allosteric binding sites, including "Thumb" Pockets I and II and "Palm" Pockets I and II/III have been described as potential targets for anti-HCV drugs (Fig. 1).

During the last decade, random screening of chemical libraries and rational drug design approaches allowed the identification of potent and selective inhibitors of HCV RdRp. RdRp inhibitors include nucleoside or nucleotide analogues, that target the RdRp catalytic site, and non-nucleoside inhibitors that target one of the allosteric sites. The latter class includes families of molecules with different scaffolds. For a number of them, X-ray structures of enzyme–inhibitor complexes are available.

This review article aims at summarizing the discovery process and current knowledge on non-nucleoside allosteric inhibitors of HCV RdRp.

2. PALM POCKET I INHIBITORS

A. Benzothiadiazines

1. Discovery, Small Core Adjustments, and Quinolinone Substitution Pattern

The discovery of benzo-1,2,4-thiadiazines as non-nucleoside RdRp inhibitors was reported in 2001 from a high-throughput screen of the GlaxoSmithKline compound collection

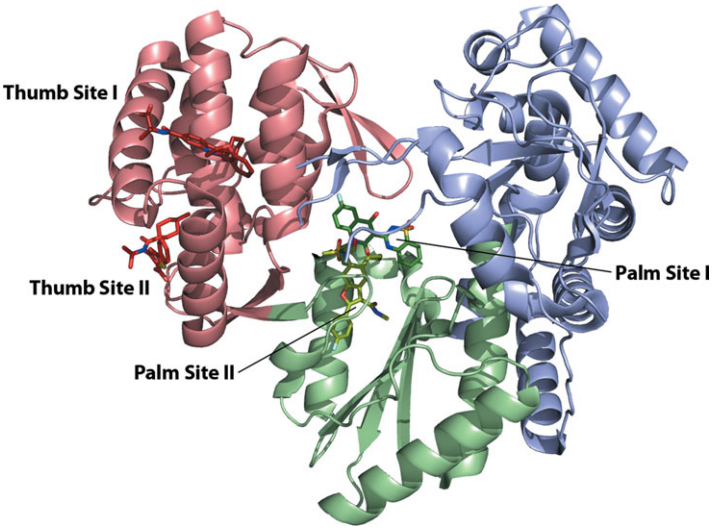


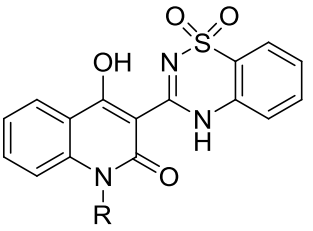
Figure 1. Structure of HCV RdRp, its three subdomains (palm, thumb, and fingers) and its four reported allosteric binding sites.

(Table I).[2] This family of compounds was shown to inhibit the initiation step of RNA synthesis, as demonstrated by the accumulation of products such as di- or trinucleotides.[3] The first identified active derivative had an IC_{50} of 80 nM against genotype 1b RdRp in an enzyme assay. Its antiviral potency highlighted the crucial role of the bulky alkyl quinolinone $N-1$ substituent (**1a**). Analogous compounds with methyl-substituted or unsubtituted $N-1$ were associated with a dramatic loss of anti-RdRp activity (500-fold increase in IC_{50}).[4] This pioneering work and resolution of the X-ray structure of **1a**–protein complex (PDB id: 2FVC) provided frameworks for further modifications on the quinolinone ring. Undertaken by GSK and Abbott Laboratories, this investigation allowed the identification of several compounds with a two-digit nanomolar IC_{50} range.

Analogs of **1** with various $N-1$ substituents revealed the high antiviral potency of 3,3-dimethylbutyl and cyclopropylethyl groups ($IC_{50} = 21$ nM for **1b** and 13 nM for **1c**, Table I). This highlighted the need of a ramified or cyclic alkyl group at this position. Indeed, simple linear alkyl chains failed to reach the same range of inhibition ($IC_{50} = 200$ nM with $R = n$-Bu and 1550 nM with $R = n$-Pr). Similarly, far less active compounds were obtained when a double bond was present or when aromatic or polar substituents were introduced in the alkyl chain (IC_{50} over 10,000 nM for compounds bearing a phenyl, ester, amino, hydroxyl, or amido group), except for some $N-1$ benzylated compounds ($IC_{50} = 82$ nM for **1d**).[5] Interestingly, the introduction of $N-1$ hydrazine-alkyl substituents led to active compounds ($IC_{50} = 60$ nM for **1e**), but none of them reached the potency of branched alkyl derivatives.[6] It clearly appears on the X-ray structure of **1a**—RdRp complex that the $N-1$ substituent binds to an adjacent little hydrophobic pocket delimited by residues Leu384, Pro197, and Tyr415 of the RdRp. This explains the negative impact of polar or too bulky $N-1$ groups. Moreover, water molecules play an important role as they allow the formation of several hydrogen bonds between the sulfonyl group and the Ser288 residue, and between the enol group and the Tyr448 residue. Aromatic edge-to-face π–π interactions are also present between the benzo ring of the benzothiadiazine moiety and residue Phe193.

The next step was to maintain the alkyl chain at the $N-1$ position and to modify the quinolinone substitution pattern (Table II). It appeared from this study that substitution at

Table I. Structure and In Vitro Inhibition of Various *N*−1 Substituted Derivatives of 3-Quinolinone benzo-1,2,4-Thiadiazines Against 1b HCV RdRp



1

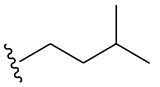
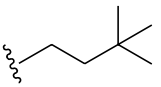
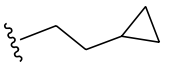
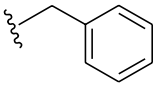
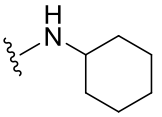
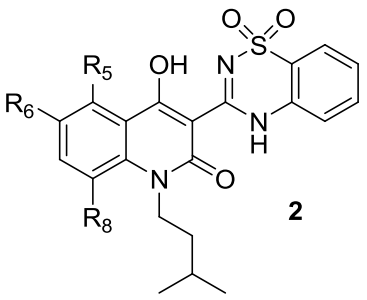
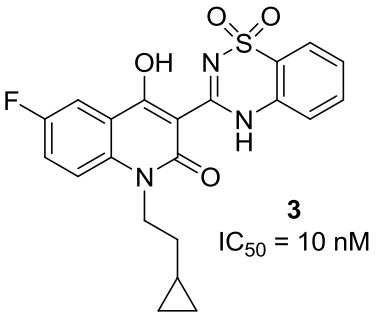
Compound	*R*	IC_{50} (nM)
1a		80
1b		21
1c		13
1d		82
1e		60

Table II. Structure and In Vitro Inhibition of Various 5-, 6-, 7-, and 8-Substituted Derivatives (**2a–e**) and the Optimized lead (**3**) Against 1b HCV RdRp



2

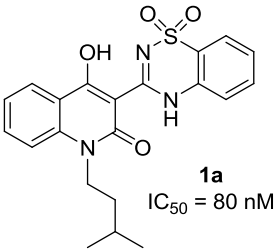


3

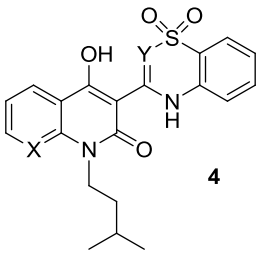
IC_{50} = 10 nM

Compound	R_5	R_6	R_8	IC_{50} (nM)
2a	H	H	F	90
2b	Cl	H	H	33
2c	H	F	H	20
2d	H	OMe	H	23
2e	H	NH-CH_2CO_2H	H	21

Table III. Modification of the Original Benzothiadiazine Core and its Effect on the Biological Activity



1a
IC_{50} = 80 nM



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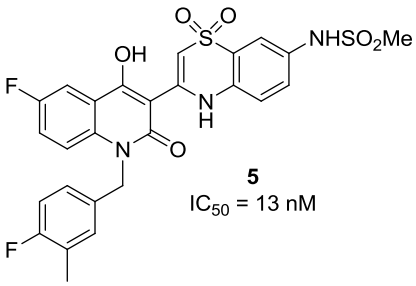


5
IC_{50} = 13 nM

Compound	*X*	*Y*	IC_{50} (nM)
4a	N	N	41
4b	CH	CH	2300
4c	N	CH	1400

position C-7 was poorly tolerated (IC_{50} = 554 nM for R_7 = F, > 10,000 nM for R_7 = Cl or NO_2), in contrast to substitution at position C-8, which gave highly potent compounds (IC_{50} = 90 nM for R_8 = F). Various substituents at positions 5 and 6 were associated with a gain of activity.

Having established that compound **2c** (IC_{50} = 20 nM) was the most active, further refinement of the *N*−1 substituent led to the identification of compound **3** that inhibited HCV RdRp with an IC_{50} of 10 nM.[5]

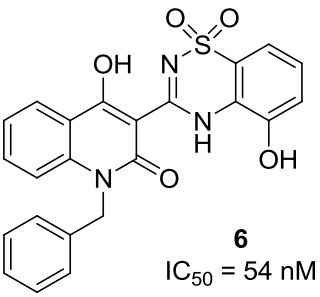
A complex of **3** with the HCV RdRp was crystallized (PDB id: 2GIQ) and X-ray structures showed a perfect superposition with **1a**, with a high conservation of close water molecules (Fig. 4A). In the binding pocket, the space around position 6 of the quinolinone core seems to be wide, while other positions are relatively close to certain protein residues. In particular, position 7 is directly in contact with residues Gly410 and Asn411, and this may explain the impact of the substitution pattern.[7]

Modification of the original core structure led to various effects. The replacement of the quinolinone moiety by the analogous naphthyridone indicated that these two rings have similar influence on the activity (Table III). The *N*−1 benzyl quinolinone and naphthyridone derivatives showed similar inhibition potencies (IC_{50} = 82 and 83 nM, respectively). The *N*−1 3-methylbutyl naphthyridone was even more active than the quinolinone analog (IC_{50} = 80 nM for **1a**, 41 nM for **4a**).[8] Removal of a nitrogen atom from the benzothiadiazine ring led to much less active derivatives. Benzothiazine analogs of **1a** and **4a** were indeed 30 times less active (IC_{50} = 2300 nM for **4b**, 1400 nM for **4c**).[9] However, there were some exceptions. One of them was compound **5**, a potent inhibitor of HCV RdRp (IC_{50} = 13 nM, Table III).[10] Modifying the sulfonyl, the enol, the amide carbonyl, or the carbon at position 3 of the quinolinone led to inactive compounds with IC_{50} > 10,000 nM.[5] A number of further modifications of the benzothiadiazine rings led to alternative cores, such as benzoisothiazoles or thienothiadiazines among others. These new moieties often showed less interesting biological activities.[11,15]

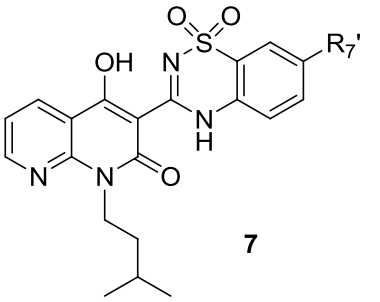
2. Benzothiadiazine Core Substitutions and Optimizations

With the previous results in hand, Abbott Laboratories initiated a study on 3-naphthyridone substituted benzothiadiazines in order to explore the influence of the benzothiadiazine core substitution on compound activity. Screening of libraries of analogs led to the conclusion that positions 5′ and 7′ were more suited for modification. Compounds **6** and **7a** were representative

Table IV. Structure and In Vitro Inhibition of 5′ and 7′ Substituted Derivatives of Benzothiadiazine Against 1b HCV RdRp



6
IC_{50} = 54 nM



7

Compound	R_7'	IC_{50} (nM)
7a	OH	42
7b	OCH_2CONH_2	16
7c	$NHSO_2Me$	6

(IC_{50} = 54 and 42 nM respectively, Table IV). Further focus on the 7′-substitution revealed the key role of this position in RdRp inhibition. Indeed, a large number of compounds with a sulfonamide or an amidoalkoxy group at position 7′ exhibited very potent inhibition (IC_{50} = 3–16 nM vs. 41 nM without the substitution). Compounds **7b** and **7c** reached IC_{50} of 16 and 6 nM, respectively. The series also showed increased potency against genotype 1a RdRp (IC_{50} = 2–46 nM vs. 810 nM without substitution).[8] This work shed light on the importance of the presence of a sulfonamide or an amidoalkoxy group at position 7′. The crucial influence of these substituents can be clearly seen in the X-ray structures of complexes of RdRp with a sulfonamido-substituted compound and **7b** (PDB id: 3G86 and 3HHK, Fig. 4A). Indeed, these two well-sized groups fill an additional polar pocket, forming hydrogen bonds with residue Asn291 for amidoalkoxyl, with additional residues Asp318 and Ser556 for sulfonamide. It can be highlighted that the two substituents exhibit a good superimposition and subsequently have similar effects on the activity (Table IV).

Further studies focused on combined benzothiadiazine and quinolinone core optimizations led to a number of molecules with one-digit nanomolar IC_{50} values (Fig. 2). For instance, compounds **8** (A-782759), **9**, **10**, and **11** are representative of this class.[12–15] In particular, compound **9** showed an impressive potency with very efficient inhibition of HCV RdRp (IC_{50} = 0.4 nM).[13]

3. Effects of Quinolinone Heterocyclic Core Modifications: Pyridinones, Pyridazinones, Pyrrolo-Pyridazinones, Cyclopentapyridines, Quinolizinones

The removal of the A ring of former benzothiadiazines was extensively investigated, leading to single six-membered ring derivatives. The development of pyridinone- or pyridazinone-based inhibitors **12** and **13** provided interesting results. These new moieties often came with a benzo ring mimic, hydrophobic 5- or 6-substituent. Among these derivatives, 5-substituted pyridinone compounds failed to reach the same inhibitory activities as quinolinone equivalents (IC_{50} = 23 nM for the most active 5-(2-furyl)-**12**), but they were of interest for cellular replication inhibition (Table V).[16] Reduced 5- or 6-ethyl or isopropyl analogs were even more active (IC_{50} < 10 nM for **14**), with a critical sensitivity to bulkier substituents.[17] Regarding the pyridazinone moiety, the influence of substituents at position 5 was extensively studied, and the thienyl substitution was identified as the most beneficial (IC_{50} < 10 nM for **13a** and **13b**).[18–20] Some compounds

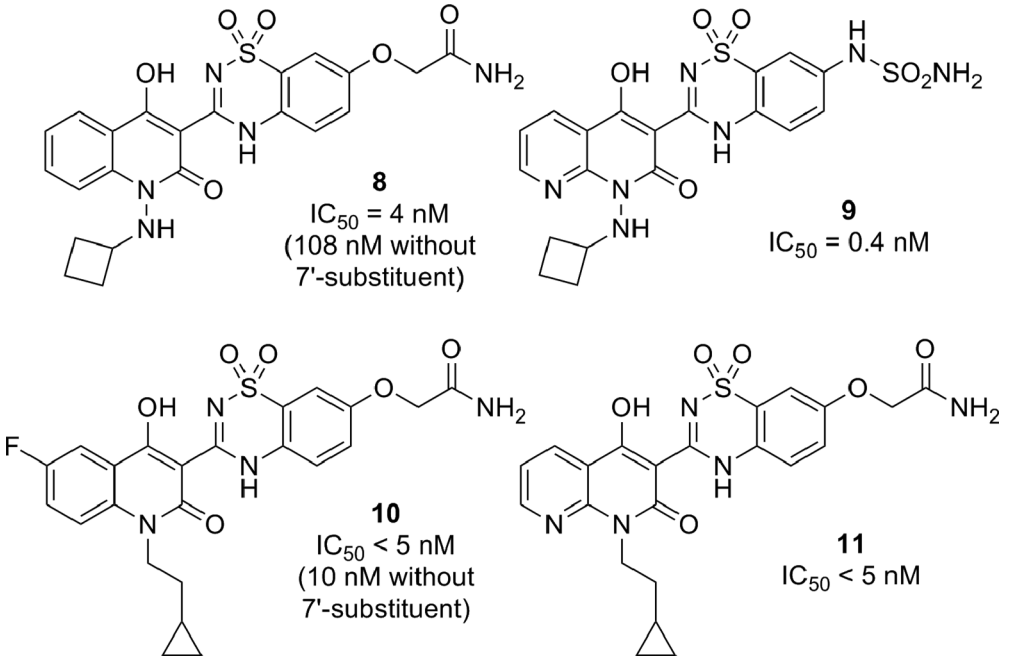
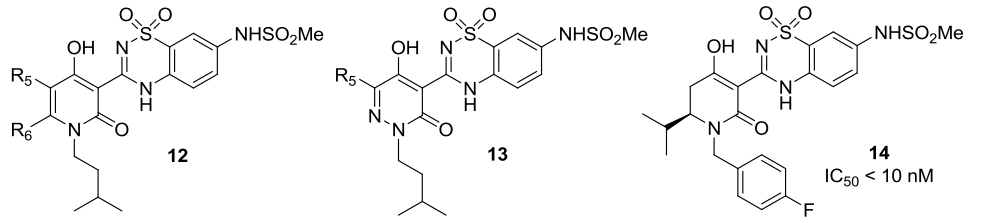


Figure 2. Structure and inhibition of 7′-substituted benzothiadiazine against 1b HCV RdRp.

Table V. Structure and In Vitro Inhibition of Various 5- and 6-Heterocyclic or Aliphatic Substituted Derivatives of Monocyclic Analogs of Original Benzothiadiazines Against 1b HCV RdRp

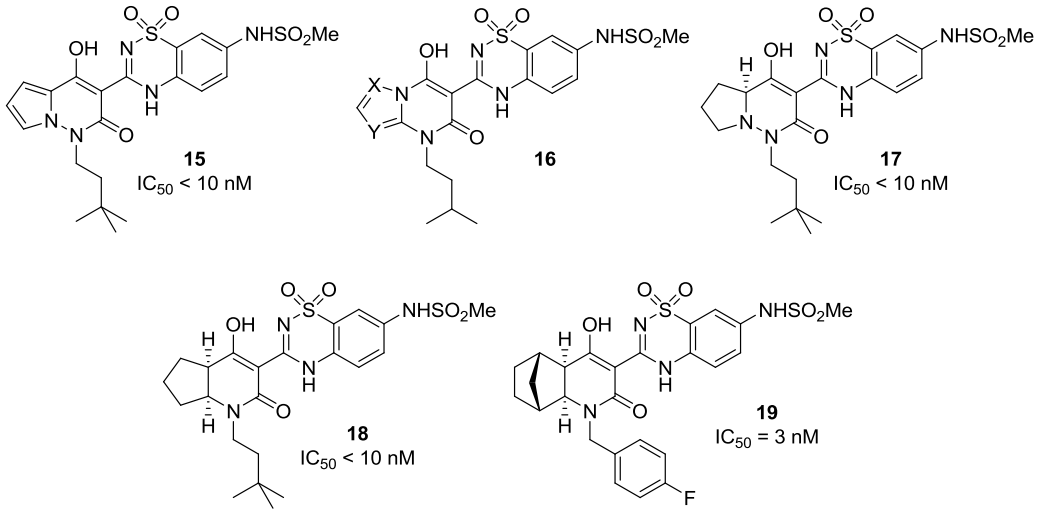


Compound	R_5	IC_{50} (nM)
13a	2-thienyl	<10
13b	3-thienyl	<10
13c	1-pyrrolidinyl	14

bearing an additional *N*-methyl group on the sulfonamine substituent and some benzothiazine-based compounds designed with a 5-thienyl substituent were associated with an important activity loss.[21,22] 5-amino substituted derivatives were also far less active, except for a couple of compounds. Among them, **13c** showed an interesting inhibitory value (IC_{50} = 14 nM).[23]

Based on previous results on the pyridazinone core, the pyrrolo[1,2-*b*]pyridazinone moiety was assessed by Anadys Pharmaceuticals. Interesting compounds emerged from this study, such as **15** which showed good inhibition (IC_{50} < 10 nM).[24,25] Varying the nitrogen atom position in the pyrrolopyridazinone subclass led to three compounds, **16a**, **16b**, and **16c**, with IC_{50} below 5 nM (Table VI).[26] Reduction of the pyrrole core into pyrrolidine afforded **17**, a structural analogue of compound **15** sharing the same potency (IC_{50} < 10 nM).[27] Removal of the pyrrolidine nitrogen led to a new chiral subclass of molecules bearing a fused cyclopentanyl

Table VI. Structure and Inhibition of Alternative Heterocyclic Compounds Against 1b HCV RdRp



Compound	*X*	*Y*	IC_{50} (nM)
16a	H	N	<5
16b	N	N	<5
16c	N	H	<5

moiety, showing high potency (compounds **17** and **18**).[28] The six-membered fused ring analogs were also evaluated, but frequently led to less active compounds.

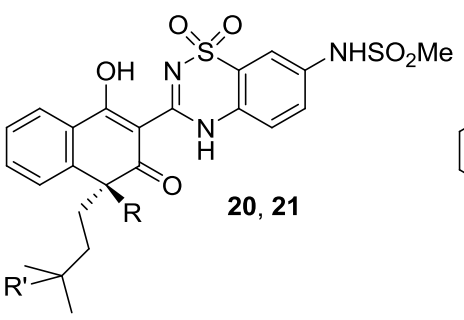
Anadys Pharmaceuticals focused on partially saturated quinolinone cores with a methylene or an ethylene bridge on ring A inhibitors. Compound **19** is a very potent inhibitor against replicon-harboring cells (EC_{50} = 3 nM).[29–33] Considering the good activities of both very hydrophobic and more polar alternative cores to quinolinone, it appeared that the sterical effect is predominant, as confirmed by available X-ray structures (complexes containing **13a**, **16a**, and **17** with RdRp (PDB id: 3CDE, 3H98, and 3CVK, respectively)). The X-ray structures showed the absence of hydrogen bonds and polar interactions in this part of the pocket. The thiophene moiety of **13a** seems to behave like the previous quinolinone 6-substituents, as it strongly interacts with amino acids at the edge of the cavity (Fig. 4B).

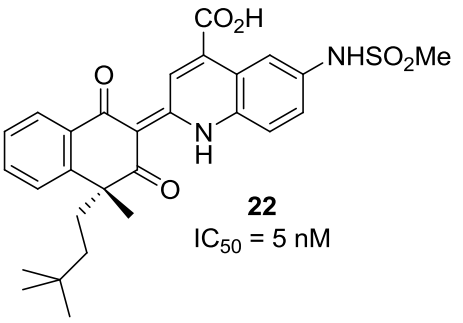
4. Exploration of the Two-Rings Hydrocarbon Version of Quinolinones: 1,1-Dialkylnaphthalenones and Derivatives

The replacement of the quinolinone core by a closely related naphthalenone core was firstly patented by Abbott Laboratories in 2005.[34] Since then, the chiral naphthalenone derivatives **20** and **21** were extensively investigated by the company for the development of HCV RdRp non-nucleoside inhibitors (Table VII). In this class of molecules, the replacement of the nitrogen at position 1 by a quaternary, chiral carbon allowed a better fit of the compounds in the hydrophobic subpocket of Palm Pocket I.

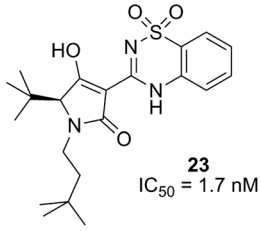
Methyl substitution led to impressive improvement of activity, as seen on **20a** and **21** (IC_{50} = 0.9 and 0.6 nM, respectively).[35–37] The other evaluated unsymmetrical or symmetrical dialkyl compounds were far less active, suggesting sterical concerns.[38–40] Substitution of the alkyl by a hydroxyl or an amino group also gave interesting results (compounds **20b, 20c**, and **20d**, IC_{50} < 5 nM).[41,42] However, as they were probably less suitable for binding into a hydrophobic pocket,

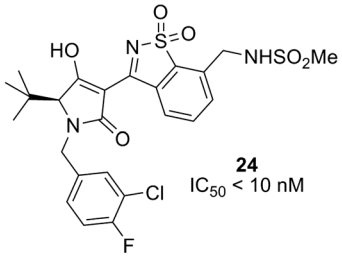
Table VII. Structure and In Vitro Inhibition of 1,1-Dialkylnaphthalenones Derivatives Against 1b HCV RdRp





Compound	*R*	*R′*	IC_{50} (nM)
20a	Me	H	0.9
20b	OH	H	5
20c	NHOMe	H	0.9
21a	Me	Me	0.6
21b	$NHCH_2$-(2,6-dimethylphenyl)	Me	19





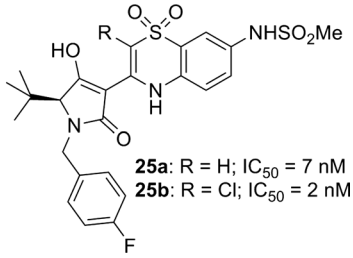


Figure 3. Structure and in vitro inhibition of tetramic acid derivatives against 1b HCV RdRp.

none of these derivatives reached the efficiency of **20a** and **21**. Very recently, the replacement of the benzothiadiazine core of **21** with an analogous carboxylic acid quinoline backbone led to the interesting compound **22** (IC_{50} = 5 nM).[43] Unfortunately, up to date, no X-ray structures of dialkyl inhibitor–RdRp complex are available.

5. Replacement of the Quinolinone Core: Incorporation of the Tetramic Acid Moiety Potency

The evaluation of the biological potency of the tetramic acid structure was undertaken in 2006 by GSK laboratories. Early results showed good inhibition against isolated RdRp (IC_{50} = 1.7–88 nM for the most active compounds, such as **23**, Fig. 3).[44,45] Further studies allowed to improve the efficiency of the subclass by introducing an appropriate substituent at position 7 of a benzothiadiazine core analog (benzoisothiazole) and an aromatic ring bound to the nitrogen of the tetramic acid moiety. The most active compound **24** exhibited good inhibition on the isolated enzyme (IC_{50} < 10 nM).[46] Other trials were conducted to develop new derivatives of this benzoisothiazole-substituted tetramic acid subclass, but cellular replicon inhibition remained higher than 70 nM for these molecules, with a very high serum protein binding effect, a poor oral bioavailability and a rapid clearance, which led to a relative disinterest for these derivatives.[47] Replacement of the benzothiadiazine by a benzothiazine and incorporation of tetramic acid provided more active compounds such as **25a** (1b RdRp IC_{50} = 7 nM, Fig. 3).

However, the low absorption and high clearance remained a major concern. The synthesis of compounds substituted at position 2 afforded additional relevant results (IC_{50} = 2 nM for compound **25b**).[48] However, the contribution of the tetramic acid subclass to the development of Palm Pocket I inhibitors remains anecdotal. X-ray structure of two complexes of HCV RdRp with **24** and a close analog to **25a** (PDB id: 3D5M and 3H59) showed a good superimposition (Fig. 4C). The position of key groups such as the enol, the ketone, and the sulfoxide, is very similar to quinolinone compounds; these groups are involved in crucial hydrogen bonds, while the hydrophobic interactions seem to be very different.

B. Proline Derivatives

1. Acyl Pyrrolidines

Chiral acylpyrrolidines derived from proline is another class of molecules found to bind into Palm Pocket I. These structures were also discovered by GSK following a high-throughput screening process. Among identified active compounds, thiophene-based chiral compound **26a** exhibited promising submicromolar inhibition values (IC_{50} = 190 nM, IC_{50} = 300 nM for the racemic version)[49] and further work led to a slight improvement of their in vitro activity (Table VIII). Thus, compounds **26b** and **26c** (IC_{50} = 30 and 70 nM, respectively) were developed with an alternative thiazole ring, a *tert*-butyl substitution on the phenyl ring, and a modified carboxylic acid moiety. Attempts to replace the carboxylic acid group by a quaternary carbon atom led to complete activity loss.[50] Modifications on the phenyl group led to one-digit nanomolar range inhibition values (IC_{50} = 5 nM for compound **26d**). On the other hand, replacing the carboxylic acid moiety by secondary alcohol derivatives improved the pharmacokinetic profile of the molecules, but at the cost of reduced in vitro activity (e.g., compound **26e**).[51] Exchanging the isobutyl substituent with five-membered heteroaromatic rings such as thiazole, isothiazole, or pyrazole led to several active compounds (IC_{50} < 200 nM).[52,53] Efforts made in this way were rewarded with the discovery of compound **26f** (GSK625433, IC_{50} = 28 nM).[54] This compound was successfully studied in Phase I clinical trials both in healthy volunteers and infected adult patients.[55] Very recently, structures derived from compound **26f** were described, bearing a sulfonamido group instead of the quaternary carbon carboxylic acid substituent. However, activities were not communicated for these molecules.[56] Crystallographic structures of **26a** and **26b** with the protein (PDB id: 2JC0 and 2JC1) revealed the role of the *tert*-butyl or trifluoromethyl substituted phenyl ring, which binds into the small hydrophobic subpocket (delimited by residues Leu384, Pro197, and Tyr415), also occupied by the $N-1$ alkyl or aryl substituent of benzothiadiazines. As the carbonyl of the amido moiety shows a substantially similar orientation to the enol group of benzothiadiazines, a comparable hydrogen bond exists with residue Tyr448. In addition, another hydrogen bond exists between the carboxylic acid and the nitrogen atom of residue Gly449. The isobutyl substituent plays a similar role as the thiophene group in compound **13a**, interacting with the pocket hydrophobic edge. The other part of the pocket is almost empty, as thiophene or thiazole groups do not allow the molecule to fill it as well as 6′-substituted benzothiadiazine derivatives (Fig. 4D).

2. Proline Sulfonamides

Proline sulfonamides were discovered as non-nucleoside RdRp inhibitors in 2006. A single paper has reported the promising activities of these molecules, with a complete study of the substitution pattern. Compounds **27a–d** showed a very large spectrum of activities (Table IX). Indeed, if the substitution of position 1 seemed to be essential for the inhibitory activity (IC_{50} = 770 nM for **27a** and > 20,000 nM for **27b**), the introduction of a bulky group at this position led to inactive compounds (IC_{50} > 20,000 nM for **27** with R_1 = NHCOMe or NHCOPh).

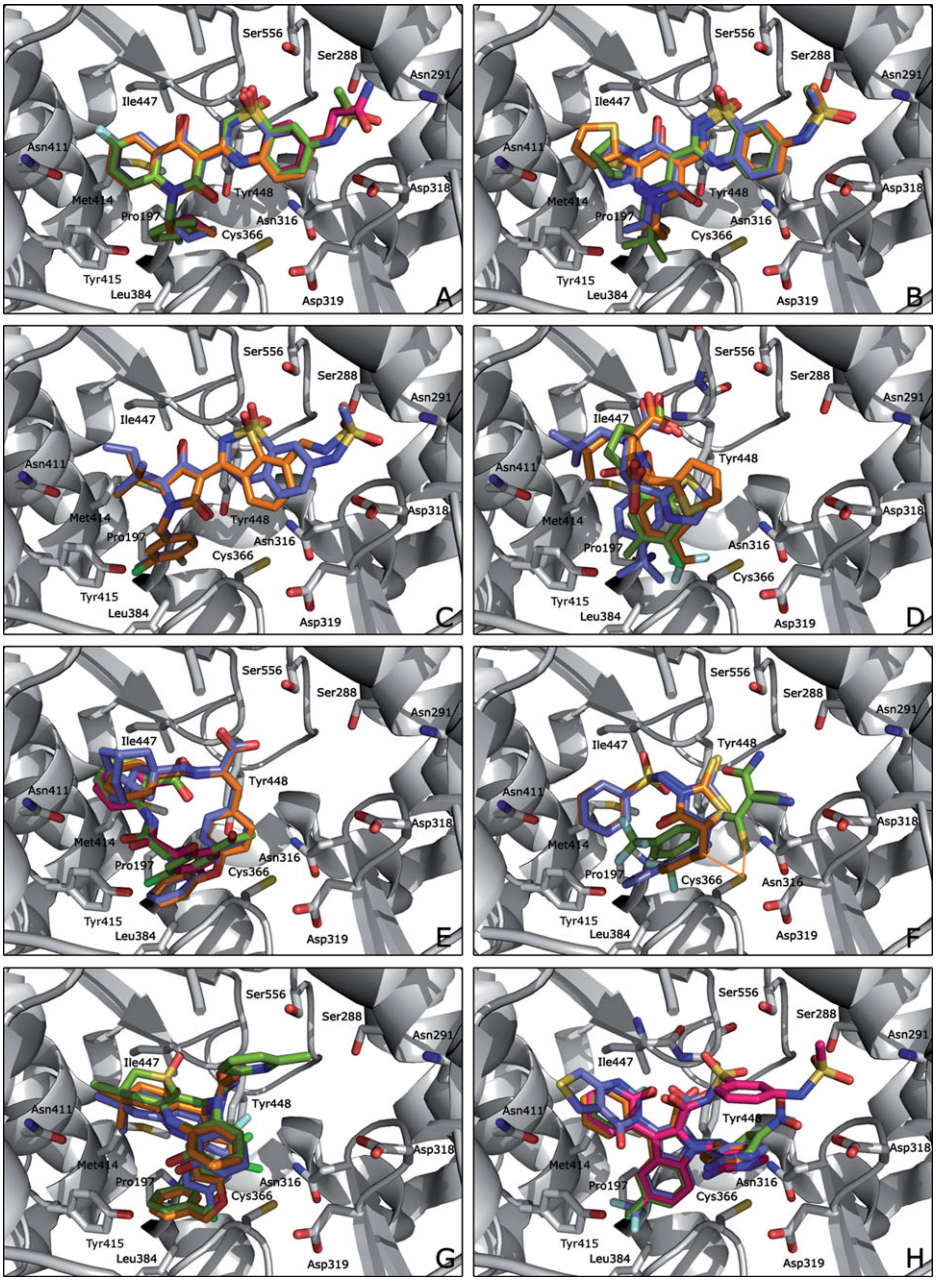
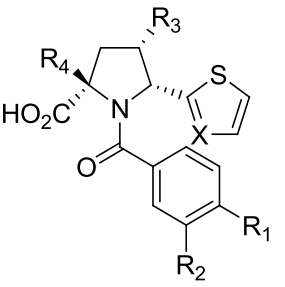


Figure 4. X-ray structures of Palm Pocket I inhibitors–RdRp complexes: (A) Compounds **1a** (2FVC, orange), **3** (2GIQ, purple), **7b** (3HHK, magenta), and a sulfonamide derivative (3G86, green); (B) compounds **13a** (3CDE, orange), **16a** (3H98, purple), and **17** (3CVK, green); (C) compounds **24** (3D5M, orange) and an analogue of **25a** (3H59, purple); (D) compounds **26a** (2JC0, orange), **26b** (2JC1, purple), and **27d** (2GC8, green); (E) compounds **30a** (1YVF, orange), **30d** (1Z4U, purple), **31a** (2QE5, magenta), and **32c** (2QE2, green); (F) analogue of **33a** (2AWZ, orange), compound **34** (2AX0, purple), and an isothiazole derivative (2IJN, green); (G) analogue of **35a** (3CSO, orange), compounds **35b** (3GOL, purple) and **36** (3GNW, green); (H) compounds **38a** (3SKH, orange), **38c** (3SKE, purple), **38i** (3TYQ, green), and **38e** (3U4R, magenta).

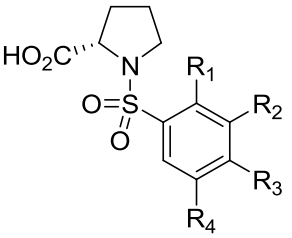
Table VIII. Structure and In Vitro Inhibition Values of Various Acylpyrrolidine Derivatives Against 1b HCV RdRp



26

Compound	R_1	R_2	R_3	R_4	X	IC_{50} (nM)
26a	CF_3	H	CO_2H	*i*-Bu	CH	190
26b	*t*-Bu	H	CO_2H	*i*-Bu	N	30
26c	*t*-Bu	H	$CONH_2$	*i*-Bu	N	70
26d	CF_3	OMe	$CONH_2$	*i*-Bu	CH	5
26e	*t*-Bu	OMe	CH_2OMe	*i*-Bu	N	440
26f	*t*-Bu	OMe	CH_2OMe	Pyrazole	N	28

Table IX. Structure and In Vitro Inhibition of Various Substituted Derivatives of **27** Against 1b HCV RdRp



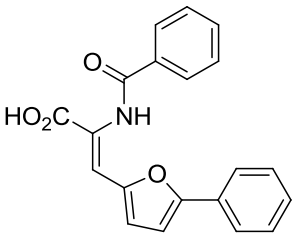
27

Compound	R_1	R_2	R_3	R_4	IC_{50} (nM)
27a	OH	Cl	H	Cl	770
27b	H	Cl	H	Cl	>20,000
27c	OH	Cl	Cl	Cl	80
27d	NH_2	H	Cl	Me	3100

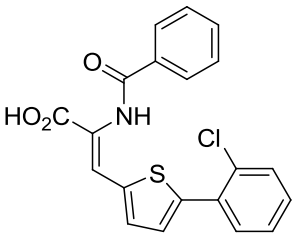
Substitutions of the other positions were pertinent, especially with chloride groups (IC_{50} = 80 nM for **27c** and 3100 nM for **27d**) and unnatural enantiomers were far less active than the natural ones.[57]

The structure of a complex of **27d** with RdRp (PDB id: 2GC8) exhibits very similar features to complexes with acyl pyrrolidine derivatives. The pyrrolidine moieties in the two classes of molecules show very good superimposition, like the phenyl groups in the hydrophobic subpocket (Fig. 4D). The sulfonyl bridge also acts as a mimic of the ketone moiety of acyl pyrrolidines. Finally, the carboxylic acid substituent of proline sulfonamides remains in the same orientation as in acyl pyrrolidines, allowing the binding of residue Gly449 in a similar

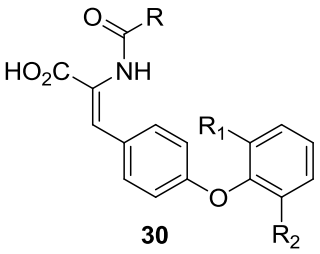
Table X. Structure and In Vitro Inhibition Values of Acrylic Acid Derivatives Against 1b HCV RdRp



28
IC_{50} = 6,700 nM



29
IC_{50} = 660 nM



30

Compound	*R*	R_1	R_2	IC_{50} (nM)
30a	Ph	I	H	40
30b	Ph	Br	F	30
30c	2-EtOPh	Br	H	50
30d	Adamantyl	Br	H	70

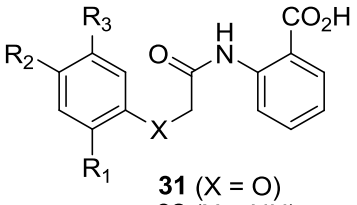
way. However, the proline sulfonamide derivatives bear a far less complex substitution pattern than acyl pyrrolidines. Consequently, the affinity for the pocket seems to be lower, a feature that can explain the difference in terms of activity.

C. Acrylic Acids

Based on the discovery of compound **28** (PNU-248809, $IC_{50} = 6700$ nM, Table X), Pfizer Global Research focused their work on the modification of the 2-phenylfurane part of the molecule. Replacing the furane with a thiophene ring provided compounds with similar activities. The substitution pattern showed that the introduction of bulky substituents on the phenyl ring led to inactive derivatives. Substitution at different positions with smaller groups like fluoride, methyl, chloride, or trifluoromethyl did not provide more active compounds than **28**, except for **29** that exhibited an IC_{50} below 1 μM ($IC_{50} = 660$ nM, Table X). Better results were obtained when replacing the 2-phenylfurane with a substituted phenoxyphenyl moiety. Iodide, bromide, chloride, and fluoride substituents all had beneficial effects on the activity, while bulkier groups failed to reach correct inhibition values.[58] It is worth noting that all active compounds share a Z-stereochemistry at the double bond.

Modifications of the phenylamide part of **28** did not enhance the inhibitory activity. Substituted compound **30c** was the most active one in this series ($IC_{50} = 50$ nM). Interestingly, adamantyl analog **30d** also showed a good activity ($IC_{50} = 70$ nM).[59] The crucial role of the phenoxy group is clear in the crystallographic structure of a bromide analog of **30a** and RdRp (PDB id: 1YVF), as this part of the molecule binds the hydrophobic subpocket (Fig. 4E). This can explain the lack of activity for compounds bearing bulky C-ring substituents. The rest of the molecule acts as a mimic of the quinolinone part of benzothiadiazine inhibitors. Indeed, good superimpositions are seen between the carboxylic acid, the amido carbonyl and the A-ring of acrylic acids with the sulfoxide, the enol moiety, and the thiophene substituent of compound **13a**, respectively, therefore involving hydrogen bonds with the same residues. Additionally, aromatic edge-to-face π–π interactions can be seen between the B-ring of the inhibitor and residue Tyr448. The X-ray structure of a **30d**–RdRp complex (PDB id: 1Z4U) shows a perfect similarity of binding modes. However, the adamantyl group seems to fill the hydrophobic edge of the pocket in a better way.

Table XI. Structure and In Vitro Inhibition Values of Various Anthranilic Acid Derivatives Against 1b HCV RdRp



31 (X = O)
32 (X = NH)

Compound	R_1	R_2	R_3	IC_{50} (nM)
31a	H	Cl	H	1640
31b	Cl	Cl	Cl	30
31c	Br	Cl	Me	110
32a	H	H	Br	870
32b	Ac	F	Br	10
32c	Ac	F	Cl	17

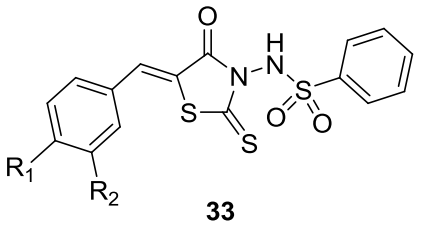
D. Anthranilic Acids

Anthranilic acid derivatives were identified by Wyeth Research as potent RdRp inhibitors. The first lead compound was **31a** (IC_{50} = 1640 nM, Table XI). Modifications of the anthranilic core, for example, shifting the carboxylic acid moiety, dramatically increased IC_{50} of the compounds (> 30,000 nM). It was then found that chloride groups had a positive influence on the activity (IC_{50} = 30 nM for **31b**, 110 nM for **31c**). Replacement of the phenoxy group by a phenylamino was also interesting. Optimizations of this phenylamino derivative revealed an advantageous substitution pattern with an *ortho* methyl acetophenone group (IC_{50} = 10 and 17 nM for **32b** and **32c**, respectively, Table XI).[60] The crystallographic data revealed a different binding mode for anthranilic acids from that of acrylic acid derivatives. However, the complex of **31a** with RdRp (PDB id: 2QE5) points out a similar role for the A-ring of acrylic acids and for the B-ring of anthranilic acids. Both groups are involved in hydrophobic interactions with the pocket edge (residues Met414, Gly410, and Asn411). The carboxylic acid substituent plays the same role as the amido carbonyl of acrylic acids and the enol group of benzothiadiazines, forming an important hydrogen bond with residue Tyr448. The A-ring is intended to bind into the hydrophobic subpocket, and the amido carbonyl allows an extra hydrogen bond with residue Arg386, which does not exist for other classes of inhibitors. Compound **32c** behaves in a similar way (PDB id: 2QE2), and well superimposes to **31a** in the binding pocket (Fig. 4E).

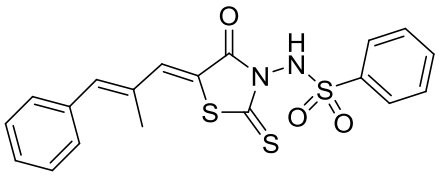
E. Rhodanines

With an initial hit in hand (**33a**, IC_{50} = 1500 nM, Table XII), Amgen Inc. started the synthesis of structurally modified rhodanines to get more active compounds. In an initial optimization array, free hydrazine derivatives ($R_3 = NH_2$) were used and the *para* substitution of the benzylidene moiety was investigated (with amino, methoxy, methyl, halide, or cyano substitutents). This work did not allow for the identification of more active compounds but revealed the importance of the sulfonamide moiety. A combined screening of R_3 and R_4' substituents of **33** also failed to reach submicromolar inhibition values. A thorough study of complete benzylidene substitution patterns yielded di- or trihalide compounds (e.g., **33b**, IC_{50} = 200 nM, Table XII). The good activity of **34** also showed that an increased size of the benzylidene group is well tolerated

Table XII. Structure and In Vitro Inhibition Values of Various Substituted Derivatives of **33** and **34** Against 1b HCV RdRp



33



34
IC_{50} = 200 nM

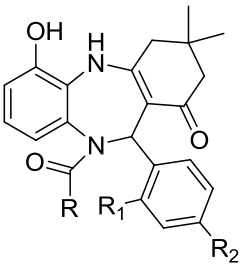
Compound	R_1	R_2	IC_{50} (nM)
33a	Cl	H	1500
33b	Br	Br	200

by the hydrophobic part of the pocket.[61] As part of a very recent virtual screening study, 3-acetic acid derivatives were reported, but with no improvement in activity (IC_{50} = 7700 nM for the best inhibitor).[62] Unlike most of the other Palm Pocket I inhibitors, X-ray structure of a bromide analog of **33a** with RdRp (PDB id: 2AWZ) revealed that this class of molecules is engaged in a covalent bond between the Michael acceptor benzylidene group and the sulfur atom of residue Cys366, although this type of moieties is not especially known as a strong thiol alkylator (Fig. 4F). This anchor allows the close bromophenyl group to bind to the adjacent small hydrophobic subpocket. Besides, the sulfonamine group is mimicking the enol of benzothiadiazine inhibitors and strongly interacts with residue Tyr448. The B-ring plays the same role as the thiophene substituent of **13a**, allowing the formation of strong hydrophobic interactions. Interestingly, compound **34** binds into the pocket in the exact same conformation, indicating a possibility of going deeper into the small hydrophobic subpocket (PDB id: 2AX0).

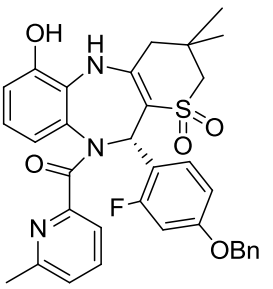
F. Benzodiazepines

Developed by Tibotec Company in 2008, the 1,5-benzodiazepine scaffold was recently explored for its inhibition potential against HCV RdRp. The first report involved only seven derivatives, including the highly efficient inhibitor **35a** (IC_{50} = 40 nM, Table XIII), which was the starting point of further optimization arrays.[63] More recent focus on the phenyl ring modifications and substitutions were unsuccessful, leading to less active compounds. However, the development of chiral compound **35b** allowed the identification of the active configuration of the benzodiazepine core (IC_{50} = 81 nM for (*R*)-**35b**). Five-membered heterocyclic substitutions of the *N*-Ac group were of great interest and provided compounds **35c** and **35d** (IC_{50} = 29 and 21 nM, respectively). The introduction of analogs containing six-membered rings did not reach similar inhibition values (IC_{50} = 51—109 nM).[64] The replacement of the intracyclic carbonyl by a sulfone moiety and the development of a chiral (*S*) isomer of this modified scaffold led to compounds with the same activity range (IC_{50} = 26 nM for **36**).[65] Elucidated X-ray structure of a complex involving an analog of **35a** and RdRp (PDB id: 3CSO) highlighted that benzodiazepine derivatives bind to Palm Pocket I in the same part as acyl pyrrolidine, proline sulfonamide, anthranilic acid, acrylic acid, and rhodanine inhibitors. The position of the benzodiazepine derivative reveals the role of the intracyclic ketone, which is analogous to the enol group of benzothiadiazines. The benzyl group is bound to the Leu384 subpocket, while the *gem*-dimethyl cyclohexane moiety is located near residue Ser407 at the hydrophobic edge of the pocket. Despite important

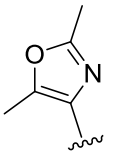
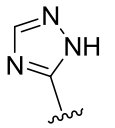
Table XIII. Structure and In Vitro Inhibition Values of Various Benzodiazepine Derivatives Against 1b HCV RdRp



35



36
IC_{50} = 26 nM

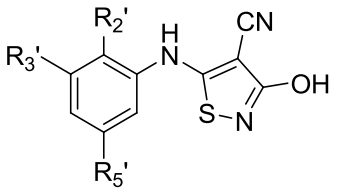
Compound	*R*	R_1	R_2	IC_{50} (nM)
35a	Me	Cl	OBn	40
35b	Me	Cl	Cl	81
35c		F	OBn	29
35d		F	OBn	21

structural differences, compounds **35b** and **36** show very similar binding modes (PDB id: 3GOL and 3GNW, Fig. 4G).

G. Isothiazoles

Amino substituted isothiazoles were discovered as RdRp inhibitors in 2007. However, until now only one report was published on this class of molecules. The phenyl substitution pattern of the general structure **37** was investigated to determine the key features required for efficient inhibition of HCV RdRp (Table XIV). Halide and benzyloxy groups increased the potency of the compounds, reaching micromolar range (IC_{50} = 300 nM for **37a** and **37b**, 200 nM for **37c**). The C-5 position appeared to be crucial for the activity, as 5-unsubstituted compounds were far less active. A modified isoxazole derivative and various sulfonyl substitutions did not show any improvement of activity. However, none of these compounds reached inhibition values below 200 nM.[66] In the same way as rhodanine derivatives, isothiazole-based inhibitors are involved in a covalent bond with Cys366. The crystallographic structure of a complex of a derivative with RdRp (PDB id: 2IJN) shows a disulfur bridge between Cys366 and the sulfur atom of an interesting opened form of the isothiazole ring. Additionally, the opening of the ring leads to a free amido group, which can create hydrogen bonds with residue Ser556. The nitrogen atom of the cyano group allows the formation of another hydrogen bond with Asp318, and it should be noted that isothiazoles are the only inhibitors with benzothiadiazines interacting with this side of the pocket (Fig. 4F).

Table XIV. Structure and In Vitro Inhibition of Various Substituted Derivatives of **37** Against 1b HCV RdRp



37

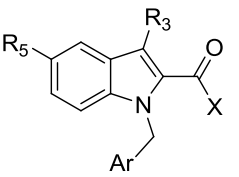
Compound	R_2'	R_3'	R_5'	IC_{50} (nM)
37a	H	F	F	300
37b	OBn	H	Br	300
37c	H	Cl	Cl	200

H. Indole 2-Carboxylic Acids

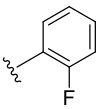
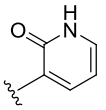
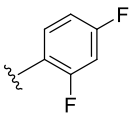
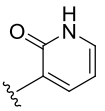
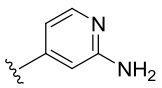
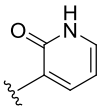
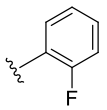
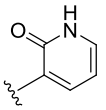
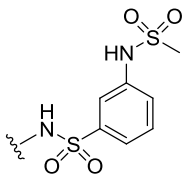
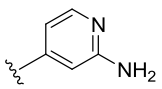
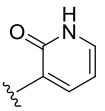
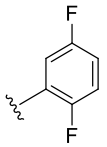
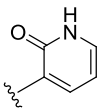
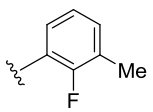
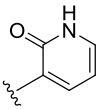
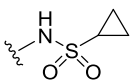
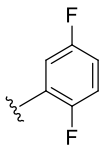
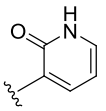
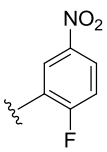
Very recently, indole 2-carboxylic acid derivatives were reported as potent HCV RdRp inhibitors by Merck Laboratories. The initial lead **38a**, a 3-(2-fluoro)phenyl analogue bearing a *N*−1 benzyl substituent, was discovered as a submicromolar inhibitor, exhibiting an IC_{50} of 900 nM (Table XV). Modifying the nature of the *N*−1 substituent, the authors achieved the development of more efficient compounds, that is, the 2,5-difluorobenzyl and 3-aminobenzyl analogues, which reached IC_{50} values of 300 and 200 nM, respectively. Positions 3 and 5 were also meticulously investigated, and provided a large number of tenfold more active inhibitors, in the low nanomolar range. Among them, compounds **38b** and **38c** showed the highest potency (IC_{50} = 25 and 17 nM, respectively).[67] Further variations around the 2-carboxylic group were undertaken and led to five more active sulfonamide derivatives (IC_{50} = 12–41 nM). The methyl sulfonamide moiety was especially impressive, and led to the single-digit nanomolar range inhibitor **38d** (IC_{50} = 6 nM). A series of aryl sulfonamide analogues were successfully developed. Twenty derivatives thus reached IC_{50} between 16 and 49 nM, including compound **38e** (IC_{50} = 39 nM), which was the best inhibitor in the replicon assay (EC_{50} = 11 nM).[68] The further exploration of positions 4, 5, and 6 of the indole core allowed the identification of 5-trifluoromethyl and 5-trifluoromethyloxy moieties as the most beneficial ones for increasing RdRp inhibition (IC_{50} = 16 and 17 nM for compounds bearing these substituents). Starting from these results, the optimization of the compounds was achieved by introducing ad hoc fluorobenzyl *N*−1 substituents, leading to compounds **38f** and **38g**, which shared very high activities (IC_{50} = 4 and 5 nM) among other single-digit nanomolar inhibitors. It is worth noting that position 5 of the indole core was moreover very permissive in terms of nature and size, as methyl, ethyl, bromo, tert-butyl, or cyclopropyl substituents all led to compounds exhibiting IC_{50} below 10 nM. Finally, sulfonamide analogues induced an increase of activity, reaching an IC_{50} of 3 nM for compound **38h**.[69] Interestingly, *N*−1 substitution with 2-fluoro-5-nitrobenyl leads to a reaction between the phenyl ring and residue Cys366. This phenomenon allows the formation of a covalent bond between the sulfur atom of the amino acid and the carbon atom initially bearing the fluoro group, which is removed. The authors hypothesized a presumed SN_{Ar} reaction, leading to potentially irreversible inhibitors of RdRp, such as compound **38i** (EC_{50} = 1 nM).[70]

Several X-ray structures of inhibitor–RdRp complexes were resolved, which highlight the key interactions taking place in the pocket. Compounds bearing a 2-carboxylic acid moiety,

Table XV. Structure and In Vitro Inhibition Values of Various indole 2-Carboxylic Acid Derivatives Against 1b HCV RdRp



38

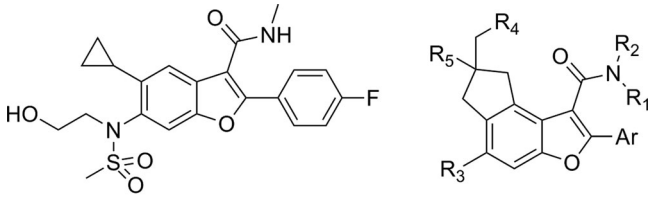
Compound	R_3	R_5	X	Ar	IC_{50} (nM)
38a		Cl	OH	Ph	900
38b		CF_3	OH		25
38c		CF_3	OH		17
38d		CF_3	$NHSO_2Me$		6
38e		Cl			39
38f		CF_3	OH		4
38g		CF_3	OH		5
38h		Et			3
38i		Et	OH		1 (EC_{50})

38a, **38c**, and **38i** (PDB id: 3SKH, 3SKE, and 3TYQ), were found to interact mainly with both hydrophobic subpockets described above, and especially with residues Tyr415, Met414, Gly410, or Asn411. Hydrogen bonds are also observed between the 3-heterocyclic core and the main chain of residues Ile447 and Tyr448, in particular for 3-pyridone derivatives **38c** and **38i**. Interactions exist between the $N-1$ aromatic substituent and residue Cys366, and a covalent bond is even formed for compound **38i** by a nucleophilic substitution reaction on the fluoro position. This leads to an irreversible modification of the enzyme. Crystallographic structures of sulfonamide analogues **38e** and a close derivative of **38h** (PDB id: 3U4R and 3TYV) display the same structural features, but the sulfonamide part of the molecules interacts directly with the other edge of the pocket. Residues Asp318, Asn291, Ser556, or Ser288 especially allow the formation of direct or water-mediated hydrogen bonds with the sulfonamido part of the compounds. In the case of compound **38e**, a face-to-face π-stacking effect between the phenyl group and the aminopyridine promotes the observed conformation, favorable for the hydrogen bond network. These sulfonamido derivatives, together with the previously described benzothiadiazines, are the only Palm Pocket I inhibitors that can interact with this side of the pocket (Fig. 4H).

3. PALM POCKETS II/III INHIBITORS

A. Benzofurans

The benzofuran class of inhibitors was extensively studied to identify promising clinical candidates, but led to very few structure–activity relationship reports. Indeed, almost all studies on inhibition, pharmacokinetics, resistance, or mechanisms studies were done on a single, very promising compound **39** (HCV-796, IC_{50} = 40 nM, Fig. 5).[71–73] Although Phase I clinical trials led to encouraging results, the program was stopped for safety concerns (elevation of liver enzymes) at an early stage of Phase II. Even if the other activities of benzofuran derivatives were often not disclosed, the general structure **40** seemed to be promising (Fig. 5).[74] Despite the lack of information about the benzofuran class of RdRp inhibitors, compound **39** was crystallized with RdRp (PDB id: 3FQL) and the complex allowed to elucidate key interactions with the protein (Fig. 14A).[75] The benzofuran core is anchored into the deep pocket by two major hydrogen bonds: between the amido NH and residue Ser365, and between the oxygen atom of the amide and an amino group from Arg500. The fluorophenyl side of the molecule is maintained by a very hydrophobic environment formed by close residues Ile363, Val370, Leu360, Leu204, Val321, Leu314, and Leu384. The sulfonyl part of the molecule does not make significant interactions with the protein, as it reaches an empty side of Palm Pocket I, partially overlapping it.

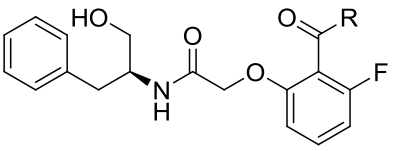


39
IC_{50} = 40 nM

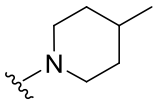
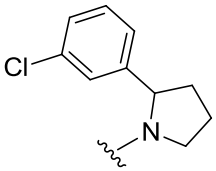
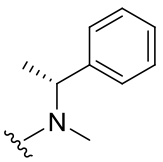
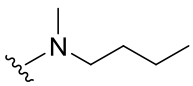
40

Figure 5. Structure and in vitro inhibition of benzofuran derivatives against 1b HCV RdRp.

Table XVI. Structure and In Vitro Inhibition Values of Various Substituted Derivatives of Oxyfluorobenzamides Against 1b HCV RdRp



41

Compound	*R*	IC_{50} (nM)
41a		2300
41b		1900
41c		430
41d		800

B. Oxyfluorobenzamides

Derived from two closely related screenings, the family of 2-oxy-6-fluorobenzamides was investigated with the aim of finding unknown active structures against RdRp. In this context, a large number of compounds based on backbone **41** were evaluated (Table XVI). Introducing a single substituent on the amido nitrogen failed to provide active compounds (IC_{50} > 20,000 nM). Cycloalkyl substituted amides were more potent, especially the methylpiperidine derivative **41a** (IC_{50} = 2300 nM), and the phenylpyrrolidine **41b** (IC_{50} = 1900 nM). The submicromolar range was reached by compounds bearing two alkyl or aryl *N*-substituents. Compounds **41c** and **41d** were representative of these derivatives (IC_{50} = 430 and 800 nM, respectively). The introduction of alkene, cyclopropyl, ether, ester, or pyridine substituents led to less active compounds.[76] Compound **41d** was crystallized with RdRp (PDB id: 3LKH) and was found to bind the enzyme near Palm Pockets I and II. Although the benzyl group of the compound interacts with Met414, as well as Palm Pocket I and Palm Pocket II inhibitors, the rest of the molecule is placed in another cavity named Palm Pocket III, generated by a significant shift of some amino acids, that is, Tyr448, Phe551, Tyr555, or Leu547. An internal hydrogen bond between the carbonyl of the terminal amide group and the nitrogen atom of the central amide group allows a folded conformation for the ligand. Several intermolecular hydrogen bonds are observed, especially between the carbonyl of the central amide and residue Asn316, and between the lateral hydroxyl moiety and Tyr195. A large part of the interactions consists in hydrophobic contacts between the compound and residues Pro197, Tyr415, Ile447, Tyr448, Trp550, and Phe551 (Fig. 14A).

C. Piperazine-2-Carboxamides

Identified by Bristol Myers Squibb in a high-throughput screening study, this class of molecules was reported only very recently as RdRp inhibitors. Exclusively evaluated through the replicon assay, the compounds were not confronted to the isolated enzyme, and thus structure–activity relationships could associate structural elements and biological activity only with hardness. However, it can be highlighted that the best results were obtained from di(4-trifluoromethylphenyl) analogues bearing a piperidine *N*-carboxamidepyridazine substituent, regardless the nature of the carboxamide moiety (EC_{50} = 7–9 nM for **42a**, **42b**, **42c**, and **42d**, Table XVII).[77] Several cocrystals were reported between 1b RdRp and piperazine-2-carboxamide derivatives. They support the binding of this class of molecules near the previously described Palm Pocket III. Interestingly, compound **42e** (PDB id: 3QGD) occupies a hydrophobic pocket where residues Trp550 and Phe551 are located in the apo structure, and both these amino acids are part to a C-terminal regulatory motif that modulates RdRp activity. Strong interactions take place between **42e** and RdRp: in particular, two main π-stacking effects are observed, between the 4-methoxyphenyl ring of **42e** and residue Tyr448; and between the dimethoxypyrimidine and residue Phe193. These interactions are supplemented by the formation of a carboxamide–Tyr195 hydrogen bond. It can be highlighted that only the (*R*) stereochemistry allows suitable interactions, since the evaluation of the (*S*) enantiomer was unsuccessful. The crystallographic structure of a **42d**–RdRp complex (PDB id: 3QGG) displays exquisite superimposition with the one discussed above, as well as similar interactions, despite differences in RdRp construction (Bartenschlager construct or wild-type enzyme).[78]

A series of nicotinamide derivatives displaying several structural similarities with piperazine-2-carboxamides (i.e., the presence of a piperidine core, a carboxamide link, and three aromatic rings) was also developed, with moderate inhibition potency. Indeed, the modulation of the nature and substitution of the aromatic rings led to several compounds exhibiting IC_{50} around 500 nM. A single methylpyrimidine derivative reached a significantly higher activity (IC_{50} = 160 nM for **43**). Interestingly, according to NMR studies and mutagenesis, these compounds bind RdRp near the Palm Pockets I and II, and it can be envisaged that they actually occupy Palm Pocket III. No direct evidences are available through X-ray structures, however.[79]

4. THUMB POCKET I INHIBITORS

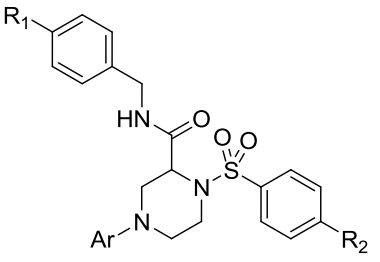
A. Benzimidazoles and Indoles

1. Discovery and Early Structure Optimizations

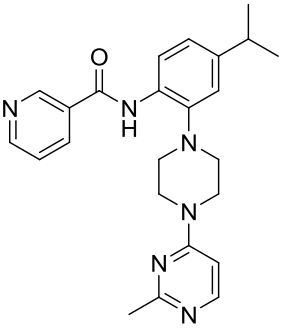
The benzimidazole class of RdRp inhibitors was formerly independently discovered by Japan Tobacco and Boehringer-Ingelheim in the early 2000s.[80,81] Starting from original hits obtained from screening libraries (e.g., **44**, **45** from Boehringer-Ingelheim and **46** from Japan Tobacco, Fig. 14), both companies undertook the synthesis of variously substituted derivatives in order to construct a rational structure–activity relationship.

Exploration of the minimal benzimidazole backbone substitutions by Boehringer-Ingelheim led to a large variety of compounds, among which interesting activities were helpful to build a clear structure–activity relationship. Evaluation of various compounds bearing carbonyls at position 5 allowed to identify the carboxylic acid moiety as the most optimized substitution for this position (IC_{50} = 4300 nM for **47**, Fig. 6). Since the free amine in position 1 led to a totally inactive derivative (IC_{50} > 500,000 nM), *N*-substitutions were studied and revealed the potency of the cyclohexyl group present in the most active compound of the

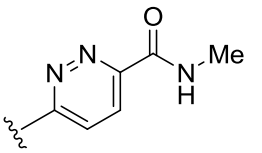
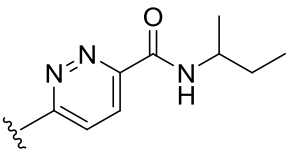
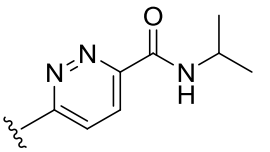
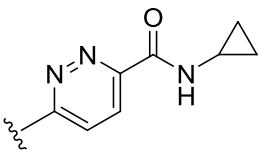
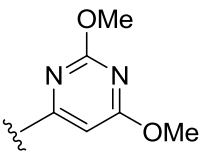
Table XVII. Structure and In Vitro Inhibition Values of Various Piperazinyl Derivatives Against 1b HCV RdRp



42



43
IC_{50} = 160 nM

Compound	R_1	R_2	Ar	IC_{50} (nM)
42a	OCF_3	OCF_3		7
42b	OCF_3	OCF_3		7
42c	OCF_3	OCF_3		8
42d	OCF_3	OCF_3		9
42e	OMe	Et		300

series (compound **47**). Cyclopentyl and methylcyclohexyl groups seemed to retain some affinity with the binding pocket. Contrariwise, aliphatic or aromatic groups led to a dramatic drop of activity (IC_{50} > 100,000 nM for phenyl or isopropyl). Finally, position 2 was also diversely substituted with a series of heterocyclic rings and noncyclic moieties. Among these groups, the introduction of a furan core led to the best activity, indicating potency for this moiety

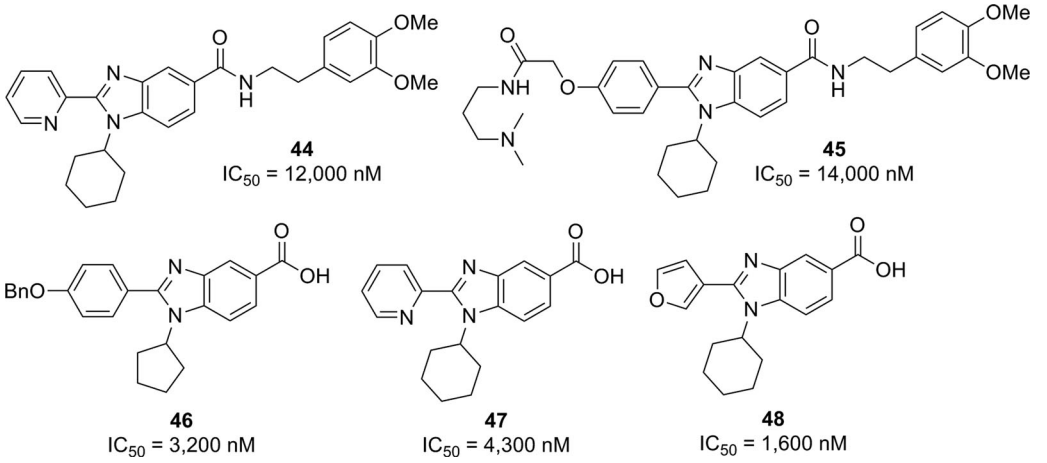


Figure 6. Structure and inhibition values of benzimidazole derivatives **44–48** against 1b HCV RdRp.

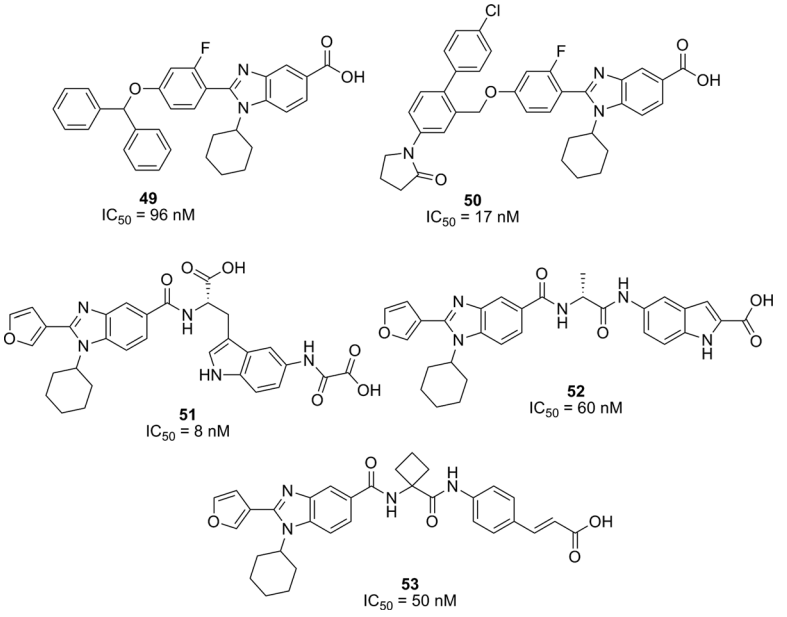


Figure 7. Structure and inhibition values of benzimidazole derivatives **49–53** against 1b HCV RdRp.

(IC_{50} = 1600 nM for **48**). Many other aromatic substituents were suitable, such as pyridine (compound **47**), thiophene, thiazole, or phenyl groups.[82]

2. Further Exploration of the Influence of the 2- and 5-Subtitutions on Activity

While Boehringer-Ingelheim worked on basic optimizations of a minimal scaffold, Japan Tobacco capitalized on their preliminary results and started a study focused on the modification of the benzyloxyphenyl group in position 2. Among all 2-aryl substituted compounds, triaryl derivative **49** was identified through this work as a submicromolar inhibitor of RdRp (IC_{50} = 96 nM, Fig. 7).[83] Substitutions of triaryl cores also led to more active compounds, such as **50** (JTK-109), which exhibited a strong in vitro inhibition (IC_{50} = 17 nM). Additionally, compound **50** showed interesting inhibition values on genotypes 1a and 3a (IC_{50} = 62 and 61 nM,

respectively). A large number of diaryl derivatives reached IC_{50} below 20 nM in this series. The amido-substituted compound was the best one (IC_{50} = 13 nM), but other analogs, similarly substituted by other groups, were also of interest (IC_{50} <30 nM for acetyl, sulfonamido, carboxyl, or benzylamido moieties).[84] Further minor modifications of the structure of **50** did not lead to a higher in vitro inhibition level. However, morpholino-substituted compounds exhibited inhibition values in the same range.[85]

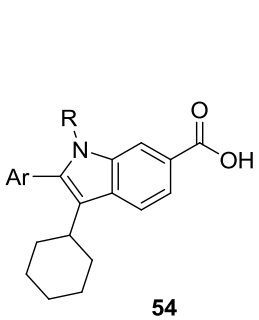
The replacement of the 5-carboxylic acid substituent by various amido moieties was undertaken by Boehringer-Ingelheim. It was found that an amido coupled tryptophan nucleus showed strong influence on the activity, leading to a one-digit nanomolar inhibition value for compound **51** (IC_{50} = 8 nM, Fig. 7). Benzyl derivatives were also evaluated, but with much less impact on activity.[86] Nonetheless, the activity of these compounds against replication was almost inexistent. Additionally, other classes of aromatic substituents, such as *N*-substituted tyrosine derivatives, were also evaluated, but with much lower activities on the isolated enzyme.[87] Introduction of alanine-based spacers between the carboxyl group and the aromatic part of the chain provided compounds with good in vitro activity (IC_{50} = 60 nM for **52**).[88] Combined modifications at positions 2 and 5 confirmed the strong effect of 2-furyl and 5-amido chain substitutions on the biological activity of these molecules. Cyclobutyl derivative **53** was found to inhibit RdRp with an IC_{50} of 50 nM.[89] A part of a very recent report focused on conformational models and biomolecular recognition of long 5-amido chains for RdRp. The presence of the cyclobutyl group in compound **53** was described as a key factor for the activity as it promotes the bioactive conformation of the molecule. Contrariwise, 5- or 6-membered rings seem to disrupt this conformation through the presence of chair/boat structures, and corresponding derivatives were less active (IC_{50} = 130 and 480 nM, respectively).[90]

3. Transposition to the Indole Scaffold and N-Acetamide Substitution Pattern

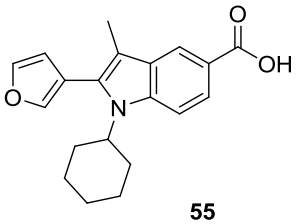
The lack of activity against cellular replication of HCV drove to the search for original modified structures based on the benzimidazole backbone. In order to improve the lipophilicity of these molecules, replacement of a nitrogen atom by a carbon was tried, leading to several indole derivatives. Among them, some compounds showed very good in vitro activities, such as **54a** and **55** (IC_{50} = 16 and 44 nM respectively, Table XVIII). In this study, a number of active alternative cores were also identified but with a slightly less interesting profile (IC_{50} = 81 nM for benzofuran analog of **54a**, 78 nM for benzothiophene analog of **54a**). This indicates the relative insignificance of the nature of the atom in position 3. The 3-cyclohexyl-*N*-methylindole moiety seemed to be of great potency for further optimizations.[91] Studies focused on the *N*-substitution of the indole subclass revealed that acetamide substituents greatly improved the activity. *N*,*N*-dimethylacetamide substituents yielded active compound **54b**, which was an efficient inhibitor of isolated RdRp (IC_{50} = 17 nM). Morpholinyl-subtituted amido moiety led to inhibition values in the same range (IC_{50} = 26 nM for **54c**, Table XVIII). Other *N*-substitutions, with either sulfonyl, amino or benzyl groups did not improve the activity.[92] More complex moieties borne by the *N*-acetamide group afforded better activities. For example, compound **54d**, bearing a pyrrolidine substituent, was a stronger inhibitor of the enzyme (IC_{50} < 10 nM).[93] Another work evaluated many different cyclic or acylic *N*-acetamide substitutions, but with no significant gain of activity.[94] Further optimizations of the 5-amido substitution pattern afforded more active derivatives, such as compound **56**, which strongly inhibited RdRp (IC_{50} = 4 nM).[95]

A large number of compounds were also developed very recently for exploring the 2-heterocyclic and 5-carboxamide moieties of *N*-methylindoles **57** (Table XIX). The most potent substituents at position 2 were 3-aminopyridinyl (IC_{50} = 18 nM for **57a**) and pyrazinyl (IC_{50} = 20 nM for **57b**). As previously described, the introduction of spiro cycloalkyl groups on the

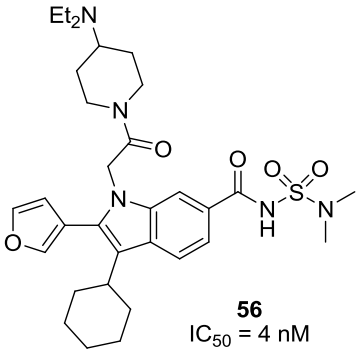
Table XVIII. Structure and In Vitro Inhibition Values of Various Benzimidazole Derivatives Against 1b HCV RdRp



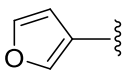
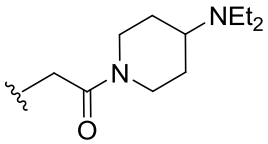
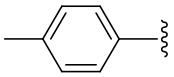
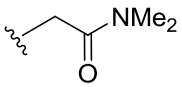
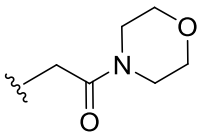
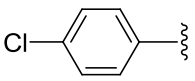
54



55
IC_{50} = 44 nM



56
IC_{50} = 4 nM

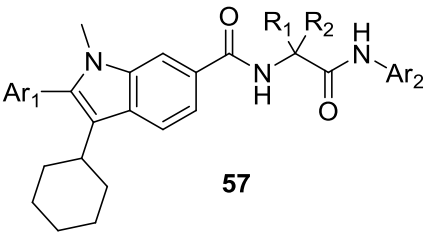
Compound	*R*	Ar	IC_{50} (nM)
54a	Me		16
54b			17
54c		Ph	26
54d			<10

carboxamide side chain induced an improvement of activity. Here, the *N*-methylpiperidine moiety seemed to show the better IC_{50} of the series (IC_{50} = 10 nM for **57c**). Combined with optimized aromatic groups such as benzothiophenes, this structural element surprisingly did not lead to more active derivatives (IC_{50} = 17 nM for **57d**, Table XIX).[96,97] The X-ray structures of complexes of RdRp with compound **54c** and with an analog of **54d** (PDB id: 2BRL and 2BRK) present the binding mode of indoles in the double hydrophobic Thumb Pocket I. The cyclohexyl group binds in the first subpocket, strongly interacting with several hydrophobic amino acids such as Leu425, Ala395, or Ala396. This cyclohexyl moiety seems to be a critical feature, as other substituents give far less active compounds. The 2-aryl group (phenyl or furyl) fills the other subpocket, delimited by residues Leu492, Leu392, or Val23. The 5-carboxylic acid moiety allows to chelate the residue Arg503 and to anchor the molecule in the binding site (Fig. 14B).

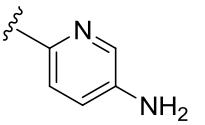
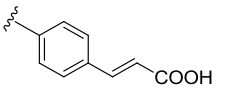
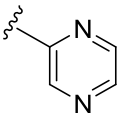
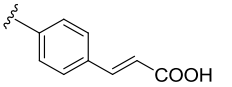
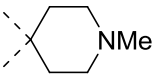
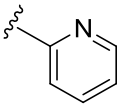
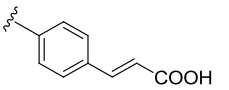
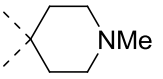
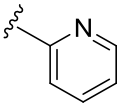
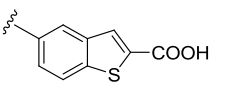
4. Development of Tetracyclic, Pentacyclic, and Macrocyclic Inhibitors

With the aim to develop more active inhibitors of this class, some indole-based tetracyclic derivatives emerged in several studies since 2006. The discovery of the strong inhibitor **58** (IC_{50} = 9 nM, Fig. 8) encouraged to consider the great potency of the cyclized molecules.[98] Further work diversified the structures, involving seven-, eight-, or nine-membered rings, several

Table XIX. Structure and In Vitro Inhibition Values of Various Benzimidazole Derivatives Against 1b HCV RdRp



57

Compound	R_1	R_2	Ar_1	Ar_2	IC_{50} (nM)
57a	Me	Me			18
57b	Me	Me			20
57c				10	
57d				17	

nitrogen positions and different substitutions. It appeared that eight-membered rings led to the most active derivatives, including compound **59** (IC_{50} = 27 nM). The introduction of an intracyclic carbonyl group did not improve the activity.[99] More recent developments around the 5-amido substitution and the 2-heterocyclic core of seven- and nine-membered rings bearing intracyclic amido, amino, or ether moieties did not led to higher activities (IC_{50} = 20–90 nM for 15 derivatives), thus supporting a limited potential associated with these ring sizes.[100,101] However two compounds, combining a seven-membered ring with an intracyclic ether moiety and a pyridine core, reached lower IC_{50} of 14 and 15 nM.[102] Contrariwise, the potency of this eight-membered ring moiety was confirmed recently with the development of substituted ether analogues. The obtained inhibition values were in the single-digit nanomolar range, especially for diamino-substituted derivatives, such as **60** (IC_{50} = 8 nM, Fig. 8). Compound **61**, a pentacyclic spiro analogue, exhibited even higher activities against RdRp (IC_{50} = 4 nM).[103] In the same way, evaluation of the influence of an additional five-membered ring introduced on the tetracyclic structure led to the identification of more active pentacyclic derivatives with good inhibition values such as compound **62**. The inhibition activity of the latter reached 4 nM (IC_{50}) for RdRp. A significant influence of the stereochemistry was also observed, as the enantiomer of **62** was 80-fold less active (IC_{50} = 312 nM). Various substitutions of the intracyclic nitrogen atom showed a minor influence for this position. Indeed, all the compounds obtained in the same configuration as **62** were within a similar range of activity (IC_{50} = 8–31 nM for 12 aminoalkyl or amidoalkyl compounds).[104] Very recently, more complex cyclopropane-based

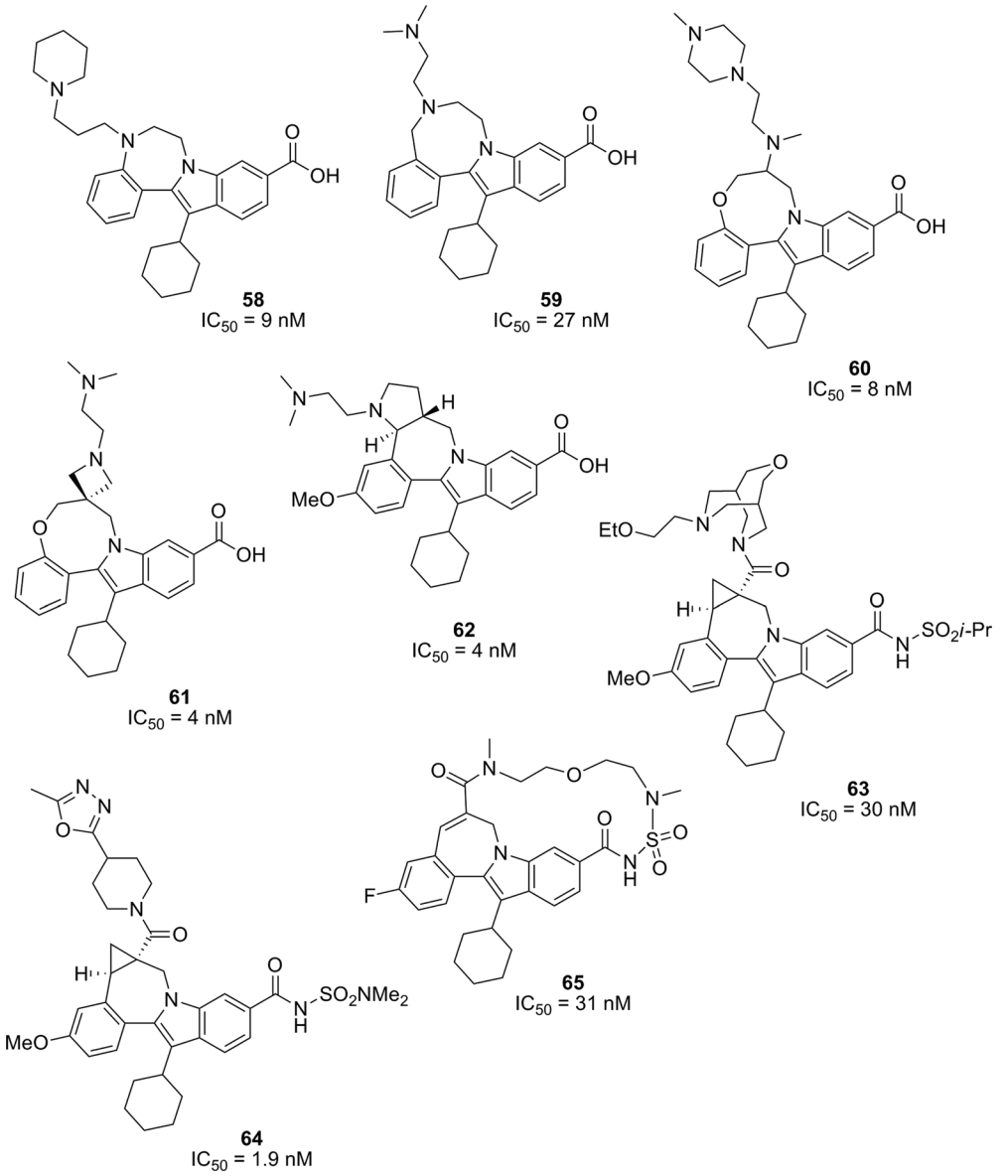


Figure 8. Structure and inhibition of benzimidazoles **58–65** against 1b HCV RdRp.

molecules bearing long side chains, for example, **63** and **64**, were synthesized and evaluated. Compound **64** especially was of impressive potency, with very low inhibition values ($IC_{50} = 1.9$ nM).[105,106] Macrocyclic derivatives did not share this potency but led to interesting compounds such as **65** ($IC_{50} = 31$ nM).[107] A crystallographic structure of a tetracyclic compound bound to RdRp (PDB id: 2DXS) confirmed the high correlation between the rigid tetracyclic backbone and the hydrophobic Thumb Site I pocket. The conformation of the molecule in the pocket is closely related to that for previously described *N*-acetamide indoles. Another tetracyclic derivative (PDB id: 3Q0Z) also displays very similar interactions inside the pocket. Interestingly, the amido substituent at position 5, bearing a terminal phenylpropanoic acid group, allows the formation of an additional hydrogen bond between the carboxylic acid moiety and the guanidine

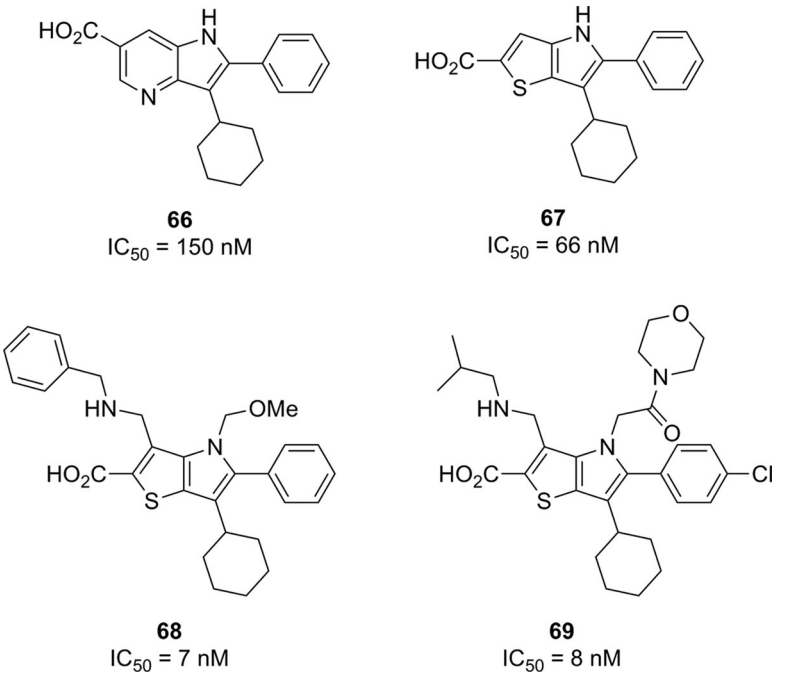


Figure 9. Structure and inhibition of azaindole and thienopyrrole derivatives **66–69** against 1b HCV RdRp.

of residue Arg498, located at ~20 Å away from the hydrophobic pocket. This highlights the importance of introducing long amido chains at position 5 of indoles and benzimidazoles (Fig. 14C).

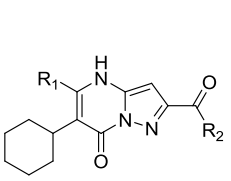
5. Replacement of Indole/Benzimidazole Ring by Alternative Cores

A number of alternative structures containing thienoimidazole, quinoline, indolizine, or other fused bicyclic rings were evaluated against NS5B. Most of these heterocyclic cores led to far less active compounds, exhibiting IC_{50} of 5000–77,000 nM. However, azaindole and thienopyrrole derivatives showed higher potencies, with submicromolar IC_{50}. Compounds **66** and **67** were especially of strong interest (IC_{50} = 150 and 66 nM respectively, Fig. 9). This could be explained by a high level of superimposition for these compounds with indole derivatives.[108] Starting from these results, the authors focused on the development of more active thienopyrrole derivatives. Modulation around positions 3, 4, and 5 of the thienopyrrole ring, as well as the introduction of a tetracyclic scaffold, led to single-digit nanomolar inhibitors, such as **68** and **69** (IC_{50} = 7 and 8 nM, respectively).[109] A crystallographic structure of a thienopyrrole–RdRp complex (PDB id: 2WCX) was revealed, which displays similar interactions than with indole-*N*-acetamides. A good superimposition is also observed between these two classes of compounds (Fig. 14C).

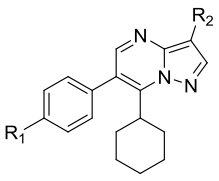
B. Pyrazolopyrimidines, Quinoxalines, and Pyrazolylmethylacrylic Acids

Pyrazolo[1,5-*a*]pyrimidines were developed by Schering-Plough as mimics of previously identified benzimidazole and indole scaffolds. While docking studies on compound **70a** showed a good superimposition with an indole derivative, further optimizations were undertaken with the aim to reach nanomolar inhibition values. Position 6 was then investigated and showed that aromatic, aliphatic, or polar groups were less beneficial than the former cyclohexyl. Interestingly, a cyclohexylethyl moiety allowed to reach the submicromolar inhibition range (IC_{50} = 600 nM for

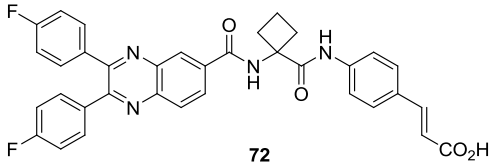
Table XX. Structure and In Vitro Inhibition Values of Various Pyrazolopyrimidine and Quinoxaline Derivatives Against 1b HCV RdRp



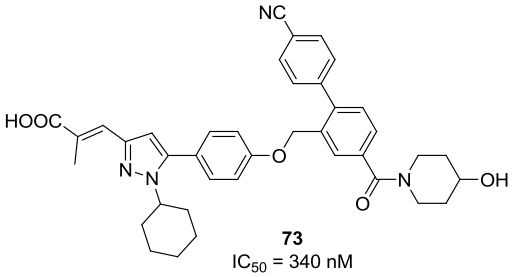
70



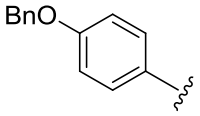
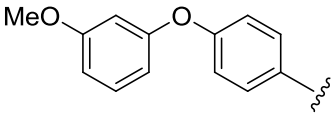
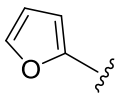
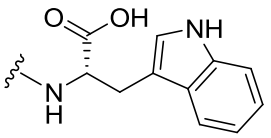
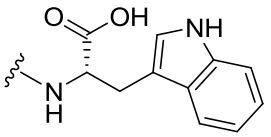
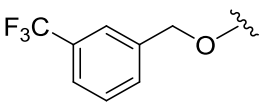
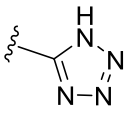
71



72
IC_{50} = 690 nM

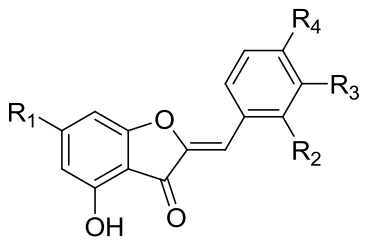


73
IC_{50} = 340 nM

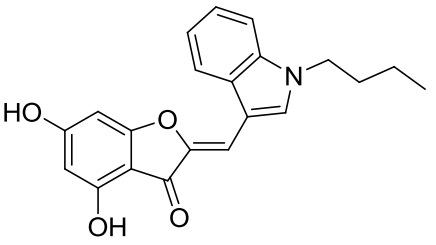
Compound	R_1	R_2	IC_{50} (nM)
70a		OH	1500
70b		OH	900
70c			50
71a	OBn		90
71b			42

70 with an ethylene bridged cyclohexyl group, Table XX). The 5-aryl group was also modified. Although the importance of this group appeared to be less crucial (all the tested compounds showed IC_{50} between 1000 and 6000 nM), **70b** was more active than the 5-benzyloxyphenyl compound **70a** (IC_{50} = 900 nM for **70b**). Inspired by the studies on benzimidazoles, modifications were made on the carboxylic acid moiety in position 2. Tryptophan-based side chains gave the best results and led to compounds like **70c** (IC_{50} = 50 nM, Table XX).[110] Modifications of the substitution pattern were undertaken with structure **71**, in which the key substituents were

Table XXI. Structure and In Vitro Inhibition Values of Various Aurone Derivatives Against 1b HCV RdRp



74



75
IC_{50} = 2,200 nM

Compound	R_1	R_2	R_3	R_4	IC_{50} (nM)
74a	OH	H	OH	OH	5400
74b	H	OH	H	OH	2600
74c	OH	H	H	*c*-Hx	2300
74d	OH	H	H	*n*-Bu	2500

shifted. Indeed, the presence of the cyclohexyl group was still crucial, but in position 7. The amido side chain was also shifted to position 3, and showed no gain in activity, compared to tryptophan moieties. Finally, the aryl substitution in position 6 led to similar results to those obtained with the 5-aryl pattern. This led to compounds **71a** and **71b** (IC_{50} = 90 and 42 nM, respectively).[111]

Quinoxaline derivatives were developed as another class of benzimidazole mimics. In these more anecdotal series, the presence of long side chains inspired by benzimidazole developments provided the best results in terms of activity. Compound **72** showed the only submicromolar IC_{50} of the class (IC_{50} = 690 nM, Table XX).[112] X-ray structures of complexes between these benzimidazole mimics and RdRp are not available, but molecular docking predicts in all cases a binding to the Thumb Pocket I. Hydrogen bonds between Arg503 and the carboxyl group, and hydrophobic interactions of the aromatic and cyclohexyl cores with the double pocket seem to rule the fixation mode of these compounds in the same way as benzimidazole ones. Finally, monocyclic pyrazolylmethylacrylic acid derivatives were synthesized in order to develop another class of indole mimics. Besides the lack of crystallographic structures for these compounds with RdRp, some derivatives showed a good inhibition of the enzyme. Especially, compound **73** shared IC_{50} values of 300–500 nM with several analogues.[113]

C. Aurones

Very recently, we discovered a novel class of naturally occurring flavonoids as promising RdRp inhibitors. This was the first report of a natural product targeting an allosteric site of RdRp. Indeed, although no X-ray structures of aurone–RdRp complexes were available, mutagenesis studies allowed us to identify Thumb Site I as the binding site for this class. Starting from the hit aureusidin **74a** (IC_{50} = 5400 nM, Table XXI), several more active aurone derivatives were found. Aureusidin analogue **74b** especially improved the potency by reaching an IC_{50} of 2600 nM. Introducing an alkyl, bulky substituent at position 4′ led to compounds with similar activities (IC_{50} = 2300 and 2500 nM for **74c** and **74d**, respectively). Finally, replacing the benzylidene group with an indole analogue had a slight beneficial impact on the inhibition, as shown by compound **75** (IC_{50} = 2200 nM).[114] Docking studies were performed around this

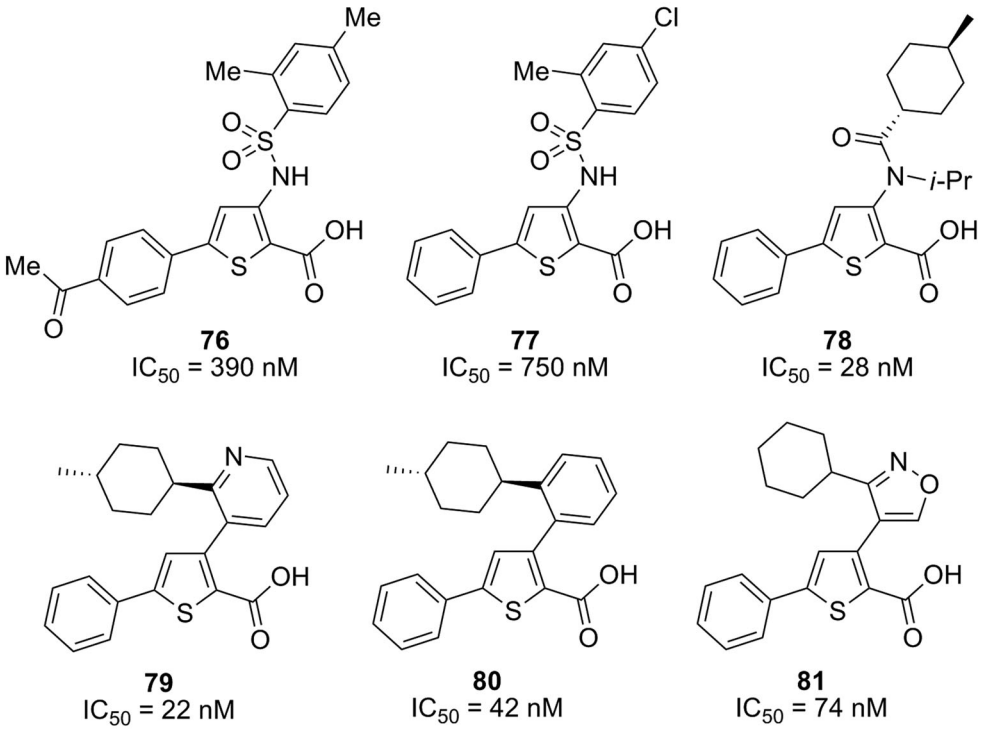


Figure 10. Structures and inhibition activity of thiophene-2-carboxylic acid derivatives against 1b HCV RdRp.

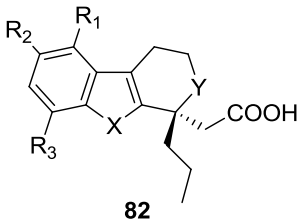
subclass of flavonoids, and the results suggest several key interactions with Thumb Pocket I. The benzofuranone moiety of aurones would especially have a strong impact in ligand anchoring, since the ketone and the 4-hydroxy groups could chelate residue Arg503 via hydrogen bonds, and the 6-hydroxy moiety could interact with Gly493. Inside the pocket, the B-ring of aurones was found to fill the hydrophobic cavity, in particular the indole moiety of compound **75**.

5. THUMB POCKET II INHIBITORS

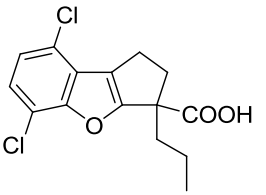
A. Thiophene-2-Carboxylic Acids

A family of thiophene-based compounds was identified through a screening of chemical libraries from Shire BioChem Company. Although the initial hit had a 2-amido substituent, it appeared quickly that carboxylic acid analogs displayed increased potency against RdRp. A series of 3-sulfonamido-5-phenyl derivatives was evaluated, leading to the discovery of the first submicromolar inhibitor of the family (IC_{50} = 390 nM for **76**, Fig. 10). Among the modifications made on the phenylsulfonamide substituent, the introduction of methyl and chloride groups on the ring revealed a low significance of the phenyl substitution pattern (IC_{50} = 1000–13,000 nM for nine derivatives). However, compound **77** seemed to bear the most beneficial substitution (IC_{50} = 750 nM).[115] Studying the possibility of a replacement of the sulfonamido moieties by tertiary amide analogs did not originally provide any improvement of the activity. On the contrary, none of the tested compounds showed submicromolar inhibition values.[116] However, a second very recent report revealed that significant gains of activity in this subclass of compounds can be achieved by inserting an isopropyl substituent, such as in compound **78** (IC_{50} = 28 nM, Fig. 10). Further modifications were made, such as the replacement of the

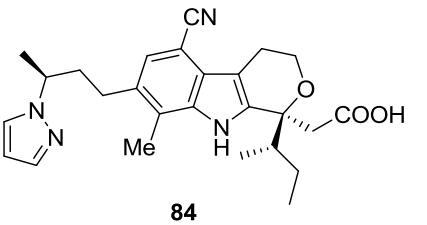
Table XXII. Structure and In Vitro Inhibition Values of Various Pyranoindole Derivatives Against 1b HCV RdRp



82



83
IC_{50} = 550 nM



84
IC_{50} = 2 nM

Compound	R_1	R_2	R_3	X	Y	IC_{50} (nM)
82a	Et	H	Cl	NH	O	3000
82b	Cl	H	Cl	NH	O	60
82c	CN	F	Me	NH	O	80
82d	Cl	H	Cl	O	O	1490
82e	CN	F	Me	S	O	50
82f	CN	H	F	NH	CH_2	2000

tertiary amido moiety by various six-membered aromatic groups. Phenyl and pyridine substitutions led to compounds **79** and **80**, which were strong inhibitors of RdRp (IC_{50} = 22 and 42 nM, respectively). Interestingly, the introduction of a pyridazo moiety dramatically decreased the activity of the compounds (IC_{50} = 3300–92,000 nM). Five-membered analogs did not allow reaching similar inhibition values. Indeed, the most active compound in these series was **81** (IC_{50} = 74 nM).[117] In a X-ray structure involving compound **78** and RdRp (PDB id: 2GIR), it firstly appeared that the Thumb Pocket II can be divided into three subpockets: two subpockets forming a large cleft at the surface (subpockets A and B) of the enzyme, and one deeper hydrophobic subpocket (subpocket C, Fig. 14D).[7] In the complex, the acid group allows the formation of hydrogen bonds with Ser476 and Tyr477. These features exist for all compounds in this class and play a key role in the anchoring of the ligands. While the phenylthiophene group binds to the cleft subpocket A, delimited by hydrophobic residues Met423, Val485, Ile482, and Leu419, the methylcyclohexyl group is deeply anchored to subpocket C, interacting with amino acids Leu474, Arg422, Trp528, and Tyr477. Furthermore, the amide carbonyl is engaged in a water-mediated hydrogen bond with residue Trp528, which maintains the molecule inside the cleft. Compound **80** binds the pocket in a similar way (PDB id: 3MF5), but the lack of hydrogen bond with Trp528 is not detrimental, as the planar structure of the phenyl linker is highly fitted between Leu497 and His475. The subpocket B is not exploited by the thiophene-2-carboxylic acid class of inhibitors.

B. Pyranoindoles and Derivatives

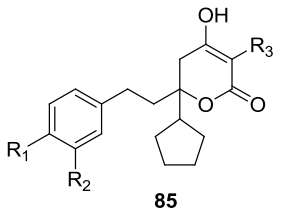
Starting from an initial hit with an IC_{50} of 3000 nM (compound **82a**), a study formerly focused on various substitution positions led to widely aryl-substituted compounds and 1,1-dialkyl derivatives. The first results indicated two key features: both the replacement of the propyl group of **82a** by a corresponding ethyl group and the esterification of the carboxylic acid moiety led to almost inactive compounds (IC_{50} > 30,000 nM, Table XXII). Among the evaluated aryl substituents, cyano, halide, and methyl groups were found to exhibit the best effect on biological activity (IC_{50} = 60 and 80 nM for **82b** and **82c**). It can be highlighted that the *R*-enantiomer of the tested compounds were always better inhibitors than the *S*-enantiomer.[118] The authors pursued

their investigations with the modification of the indole group in benzofuran and benzothiophene analogs. While the first did not provide better compounds than the indole core ($IC_{50} = 1490$ nM for **82d**, the best compound in this subclass), the latter was more interesting. Indeed compound **82e**, the thiophene-based analog of **82c**, shared its potency ($IC_{50} = 50$ nM). Many other diversely substituted derivatives of benzothiophene showed inhibition values below 200 nM.[119] Further modifications made on the pyran ring were undertaken. Replacement of the oxygen atom by a methylene group failed to reach submicromolar inhibition values ($IC_{50} = 2000$ nM for the most active compound **82f**, Table XXII). Slightly better results were obtained from five-membered ring derivatives. Compound **83** was the most active of the subclass, with an IC_{50} of 550 nM.[120] With the introduction of longer substituents of the aryl group of pyranoindoles, better activities were observed, especially pyrazole-ethoxy derivatives. Compound **84** was representative of these more active molecules ($IC_{50} = 2$ nM).[121] Further investigations around the C_7 substitution of pyranoindoles were followed by a rational explanation, supported by X-ray structures. Indeed, a large hydrophobic shelf exists near the Thumb Pocket II, and C_7 substituents interacts with this subpocket, leading to compounds with a higher affinity for the enzyme and stronger activities ($IC_{50} = 2$–10 nM).[122]

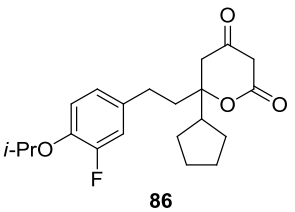
C. Dihydropyranones

The dihydropyranone class of RdRp inhibitors was discovered through compound **85a**, which was an interesting initial hit from high-throughput screening ($IC_{50} = 930$ nM, Table XXIII). First synthesized analogs shared a minimal backbone with **85a**, and allowed to test several cycle sizes and *meta*, *para* phenyl substituents. It clearly appeared that five-membered rings were far more active than four- and six-membered equivalents ($IC_{50} = 8200$ nM vs. 93,000 and 52,000 nM). The introduction of several phenyl substituents revealed a beneficial effect of *meta*-fluoro, *meta*-chloro, and *para*-alkoxy groups ($IC_{50} = 530$ nM for **86**, 790 nM for the ethoxy analog, and 890 nM for the methoxy analog). Contrariwise, the replacement of one of these substituents by a hydrogen always led to less active compounds ($IC_{50} > 1700$ nM). Further substitution of the α-position of the lactone with sulfur groups increased the potency of the molecules. Six- and five-membered aromatic rings provided good results ($IC_{50} < 500$ nM) but *N*-methyl triazole compound **85b** was the best one, with an IC_{50} of 38 nM.[123] Bicyclic ring-based derivatives such as triazolo-pyrimidine compound **85c** ($IC_{50} = 20$ nM for **85c**, 36 nM for the sulfur equivalent) were also found as efficient inhibitors of HCV RdRp. The introduction of a cyanoalkyl group provided better results and a one-digit nanomolar IC_{50} was reached ($IC_{50} = 2$ nM for **85d**).[124] *Meta*-ethyl and *para*-hydroxymethyl groups were also identified for their positive effect on activity, and some pyridine compounds reached the same range of IC_{50} ($IC_{50} = 3$ nM for **85e**, 7 nM for **87**, Table XXIII). The position of the nitrogen atom in the pyridine derivatives did not induce significant impact on activity ($IC_{50} = 7$–10 nM for *ortho*-, *meta*-, and *para*-pyridine groups).[125] The X-ray structure of a **85a**–RdRp complex (PDB id: 1OS5) indicated that the cyclopentyl moiety plays the same role as the methylcyclohexyl group of thiophenes, interacting with the deep subpocket C (Fig. 14E). The enol is involved in crucial hydrogen bonds with backbone NH of residues Ser476 and Tyr477, and the lactone carbonyl oxygen atom forms an additional, water-mediated hydrogen bond with Arg501. In the subpocket A, the phenol group interacts with Leu497 through another hydrogen bond. Finally, the phenyl sulfur substituent positioned in front of the B-subpocket, forms only limited interactions with this pocket. The phenyl sulfur group of compound **85a** allows a π–π interaction with His475 and probably contributes to reinforce the affinity of the molecule for the protein. However, the role of this substituent is more equivocal, as some active compounds do not bear a substituent at this position. Compound **86**, for example, forms a very similar complex with RdRp (PDB

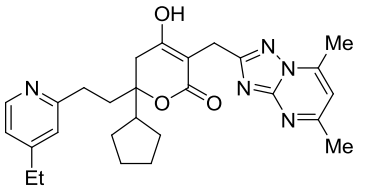
Table XXIII. Structure and In Vitro Inhibition Values of Various Dihydropyranone Derivatives Against 1b HCV RdRp



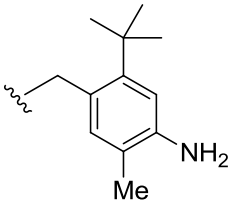
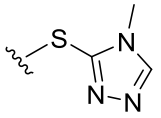
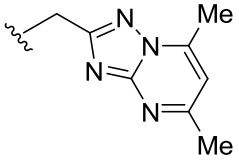
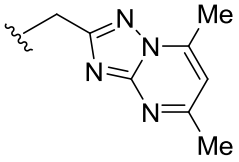
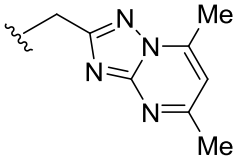
85



86
IC_{50} = 530 nM



87
IC_{50} = 7 nM

Compound	R_1	R_2	R_3	IC_{50} (nM)
85a	OH	H		930
85b	OMe	Cl		38
85c	OMe	Cl		20
85d	$C(CH_3)_2CN$	Cl		2
85e	CH_2OH	Et		3

id: 2HAI) without these supplementary interactions, but exhibits a higher in vitro biological activity against the enzyme.

D. Thiazolones

Based on the preliminary discovered compound **88**, which showed an IC_{50} of 3000 nM, several structural modifications were attempted on the thiazolone core (Fig. 11).[126] Introducing various substituents on the amino acid moiety systematically led to less active compounds, whatever the nature of the group, for example, substituted aryl, diaryl, heteroaryl, or cycloalkyl (IC_{50} =

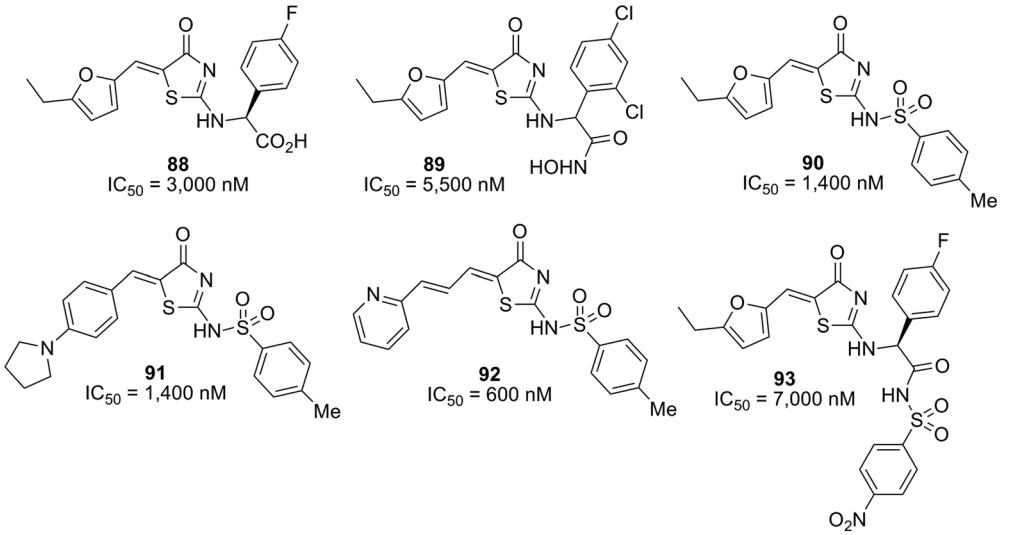


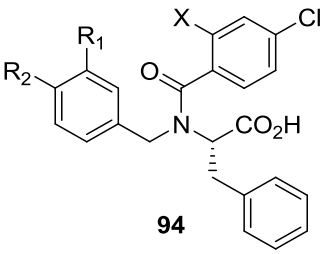
Figure 11. Structure and in vitro inhibition of thiazolone derivatives against 1b HCV RdRp.

6000–100,000 nM). Compound **89** was the best analog of **88** but with moderate efficiency ($IC_{50} = 5500$ nM).[127] The X-ray structure of a cocrystal of **88** with RdRp (PDB id: 2HWI) points out the role of the chiral fluorophenyl substituent: this group binds into the deep hydrophobic subpocket C (Fig. 14F). The thiazolone and furan cores are maintained at the surface of the A-pocket by several hydrogen bonds, involving Tyr477 and Ser476 with the carbonyl and the nitrogen atom of the thiazolone ring, which play the same role as the carboxylic acid in thiophene-based inhibitors. The oxygen atoms of the carboxylic acid moiety of **88** form weaker ionic hydrogen bonds with the charged nitrogen atoms of Arg501 and Lys533. It can be noted on this complex that the empty annex pocket B near His475 and Ala376 could be successfully filled in with appropriate derivatives of the carboxylic acid moiety. Aryl sulfonamine **90** showed better results against RdRp ($IC_{50} = 1400$ nM) and the sulfonamine substituents were used for extra thiazolone optimizations (Fig. 11). Sulfonamine and furan substitution were investigated, but provided only moderate interest. Modifications of the furan ring led to a couple of more active compounds, such as **91** and **92** ($IC_{50} = 1400$ and 600 nM, respectively), but a large number of analogs did not share this potency ($IC_{50} > 5000$ nM).[128] A new design based on a sulfonamide link between two aromatic groups was considered. Among synthesized derivatives, compound **93** showed one of the best activities, which remained in the same range as previous compounds ($IC_{50} = 7000$ nM for **93**).[129] The sulfonamido group of this compound reached the annex pocket B in a complex with RdRp (PDB id: 2O5D, Fig. 14F). In addition to a π-stacking effect with His475, this new group induces a favorable conformation of the sulfonamido moiety to form stronger hydrogen bonds. Indeed, the two oxygen atoms of sulfonamide effectively chelate the amino group of Lys533.

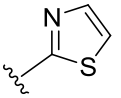
E. Phenylalanine Derivatives

Phenylalanine derivatives were developed by Shire BioChem following an initial hit identification through a screening process. For example, compound **94a**, showed an IC_{50} of 5700 nM (Table XXIV). Optimization of **94a** led to a series of more active derivatives. The 2,4-dichlorophenylamido group appeared to have an important influence on activity, as the removal of either 2- or 4-chloro substituents led to less active compounds ($IC_{50} = 11{,}000$ and 15,000 nM). Modifications of the benzylamino group substitution pattern were more beneficial.

Table XXIV. Structure and In Vitro Inhibition Values of Various Phenylalanine Derivatives Against 1b HCV RdRp



94

Compound	R_1	R_2	X	IC_{50} (nM)
94a	H		Cl	5700
94b	NO_2	H	Cl	700
94c	CN	H	Cl	800
94d	Br	H	I	2700

Indeed, compounds **94b** and **94c** reached the submicromolar range for IC_{50} with the introduction of *meta* electron withdrawing, nitro and cyano substituents (IC_{50} = 700 and 800 nM, respectively). Other substitution positions and other groups in *meta* led to less active derivatives.[130] Further modifications on the phenylamido group were undertaken. The phenyl substituents were investigated, leading to a large number of less active compounds, except for **94d**, which was slightly more active (IC_{50} = 2700 nM). Modification or removal of the carbonyl group caused total loss of activity.[131] According to X-ray structure of a complex between a derivative and RdRp (PDB id: 1NHU), the key dichlorophenyl group is buried in subpocket C and the phenylalanine moiety is maintained in the surface subpocket A.[132] Contrariwise, the *N*-benzyl group seems to have a minor role in the ligand–protein interactions. In this case like with thiophene inhibitors, the carboxylic acid moiety is well positioned to form two hydrogen bonds with Ser476 and Tyr477 (Fig. 14G).

F. Benzoisoquinolines

The identification and optimization of benzo[*de*]-isoquinolines as inhibitors of HCV RdRp were briefly described in 2009. The formerly discovered lead **95**, bearing an aminoalcohol side chain and an *N*-phenyl substitution, was already a very strong inhibitor with an IC_{50} of 20 nM (Fig. 12). Removal of the side chain as well as replacement of the carbonyl oxygens by carbon atoms caused complete loss of activity (IC_{50} > 50,000 nM) and modifications of the *N*-phenyl ring bromide substituent led to a severe decrease of the inhibitory effect (IC_{50} = 1700 nM). The replacement of the alcohol moiety of the side chain by a hydrogen atom or a methoxy group had no major influence on activity (IC_{50} = 29 and 24 nM, respectively), indicating the probable lack of hydrogen bonding between Thumb Pocket II and this part of the inhibitors. Further modifications of the side chain gave more active compounds, such as **96**, a benzylpiperidinyl derivative (IC_{50} < 14 nM, Fig. 12).[133] Starting from these elements and in order to overcome cytotoxicity issues, a structure-based design study was undertaken and provided several analogous chemical classes as inhibitors of RdRp. Especially, the presence of the benzylpiperidinyl fragment conferred higher activities, whereas modifications of the

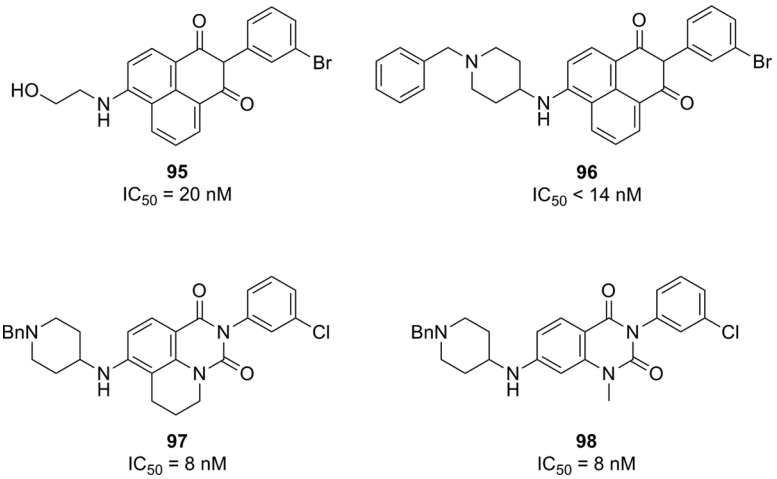


Figure 12. Effect of benzoisoquinoline derivatives against 1b HCV RdRp.

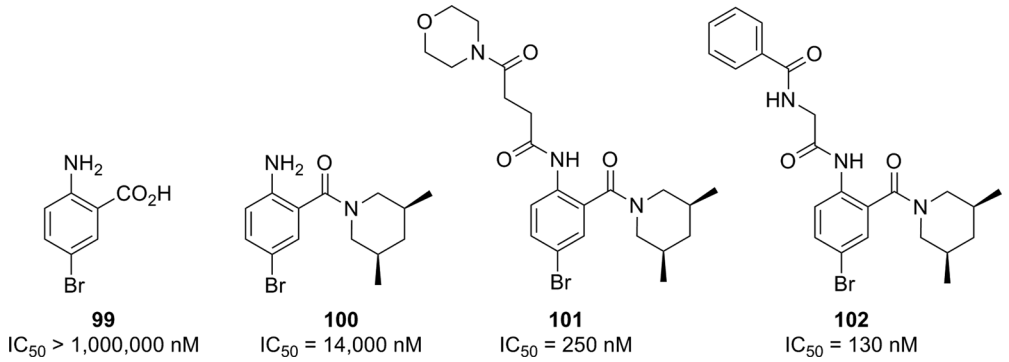


Figure 13. Structure and in vitro inhibition of 1b HCV RdRp by bromoanthranilic acid derivatives.

benzoisoquinoline core exhibited a lesser impact (IC_{50} = 8 and 75 nM for **97** and **98**).[134] The crystal structure of **95**–RdRp complex (PDB id: 2WHO) clearly showed a binding of the bromophenyl group in the deep hydrophobic pocket C. The tricyclic core seems to fill the subpocket A, with a large number of van der Waals interactions with the edges of this subpocket. Water-mediated hydrogen bonds exist between the oxygens of the tricyclic backbone and residues Arg501, Ser476, and Tyr477. The primary alcohol of the side chain allows the formation of another water-bridged hydrogen bond with Arg498 (Fig. 14G).

G. Bromoanthranilic Acid Derivatives

A number of bromoanthranilic acid derivatives have shown a good potency for RdRp inhibition. Using a fragment-based approach for the lead discovery, Antonysamy et al. relied on the former backbone **99** to diversify and improve the activity of derivative inhibitors (Fig. 13). The amidification of the carboxylic acid led to more potent molecules, with IC_{50} switch from millimolar to micromolar range. The dimethylpiperidine substituent in particular seemed to have the best influence (IC_{50} = 14,000 nM for **100**). Combination of the introduction of the amido side chain with the substitution of the aniline nitrogen was even more beneficial. Although, *N*-succinate derivatives led to limited activities (IC_{50} = 1000–500,000 nM), amido

compounds were much more encouraging. Indeed, some compounds reached low IC_{50} values, like **101** or **102** (IC_{50} = 250 and 130 nM, Fig. 13).[135] The X-ray structure of **101**–RdRp complex (PDB id: 3CJ5) pointed out the binding of the bromophenyl group in the deep subpocket C, while the key dimethylpiperidine moiety fills the pocket A with the formation of a hydrogen bond between the nearby amide carbonyl and the NH of Arg501 (Fig. 14G). The morpholino part of the molecule is anchored in front of channel B through a hydrogen bond between the acetaniline carbonyl and the backbone NH of Ser476.

H. Quinolones

Another heterocyclic scaffold was very recently discovered as HCV RdRp inhibitor. Quinolones were indeed identified in 2011 via a high-throughput screening, from compound **103a** as the initial hit (IC_{50} = 1150 nM, Table XXV). Successively, the nature of the *N*-aryl substituent and the moiety at position 3 of the quinolone core were investigated, in order to build a structure–activity relationship. First, the replacement of the *N*-(4-isopropyl)benzyl substituent with alkyl and sulfonyl groups led to a slight gain of activity (IC_{50} = 500–850 nM). Interestingly, the introduction of a 2-fluoro-4-trifluoromethyl pattern in the *N*-benzyl group was more beneficial (IC_{50} = 410 nM). Derivatives bearing *N*-cycloalkyl substituents were far less active. *N*-(4-chloro)benzyl analogues were then modulated at position 3. Although sulfonyl, amido, or urea groups linking the heterocyclic core at position 3 and an aromatic moiety failed to inhibit RdRp, the ester derivative **103b** reached an IC_{50} of 20 nM. Combining these elements allowed the authors to provide more active compounds with IC_{50} = 8–20 nM, such as the 4-tolyl derivative **103c**.[136] Several alternative heterocyclic rings as 3-substituents were also evaluated, with mixed results. Whereas oxazole or tetrazole moieties showed only a moderate potential (IC_{50} > 1800 nM), some oxadiazole isomers were strong inhibitors of RdRp, with IC_{50} below 20 nM. Quinolone **103d** reached in particular an IC_{50} of 14 nM.[137] Two X-ray structures of RdRp–ligand complexes were disclosed, with compound **103d** (PDB id: 3UDL), and with a close analogue (PDB id: 3PHE). These structures reveal a high degree of superimposition. If the quinolone core sits at the edges of the Thumb Pocket II, crucial hydrogen bonds are observed between the 4-carbonyl group and Tyr477; and between residue Ser476 and either the 3-ester carbonyl or the 3-oxadiazole nitrogen atom, which have thus the same role. The benzyl group off the ester or oxadiazole link, as well as the *N*-benzyl substituent occupy small hydrophobic pockets situated inside the cleft. The first, namely subpocket C, is constituted by residues Leu419, Arg422, Met423, and Trp528, and the second one, namely subpocket A, by residues Leu419, Val485, Ala486, Leu489, Leu497, and Met423 (Fig. 14H).

6. CONCLUSION

Chronic hepatitis C has become the main indication for liver transplantation in industrialized countries and will soon be the leading cause of HCC in the Western world. Worldwide mortality related to HCV exceeds 300,000 per year. Faced with this urgent and growing medical need, research into novel therapeutic compounds for the treatment of HCV is rapidly growing. Several novel compounds are at advanced stages of clinical development, including inhibitors of the HCV NS3/4A protease that have been approved for patients infected with HCV genotype 1 only and have side effects and limitations; HCV polymerase inhibitors (including both nucleoside/nucleotide analogues and non-nucleoside inhibitors), NS5A inhibitors, and host-targeted agents such as cyclophylin inhibitors. Over the past 10 years, a tremendous amount of work has been achieved to unravel the structure and function of the HCV RdRp, which have led in particular to the characterization of a number of allosteric sites. These sites are the target

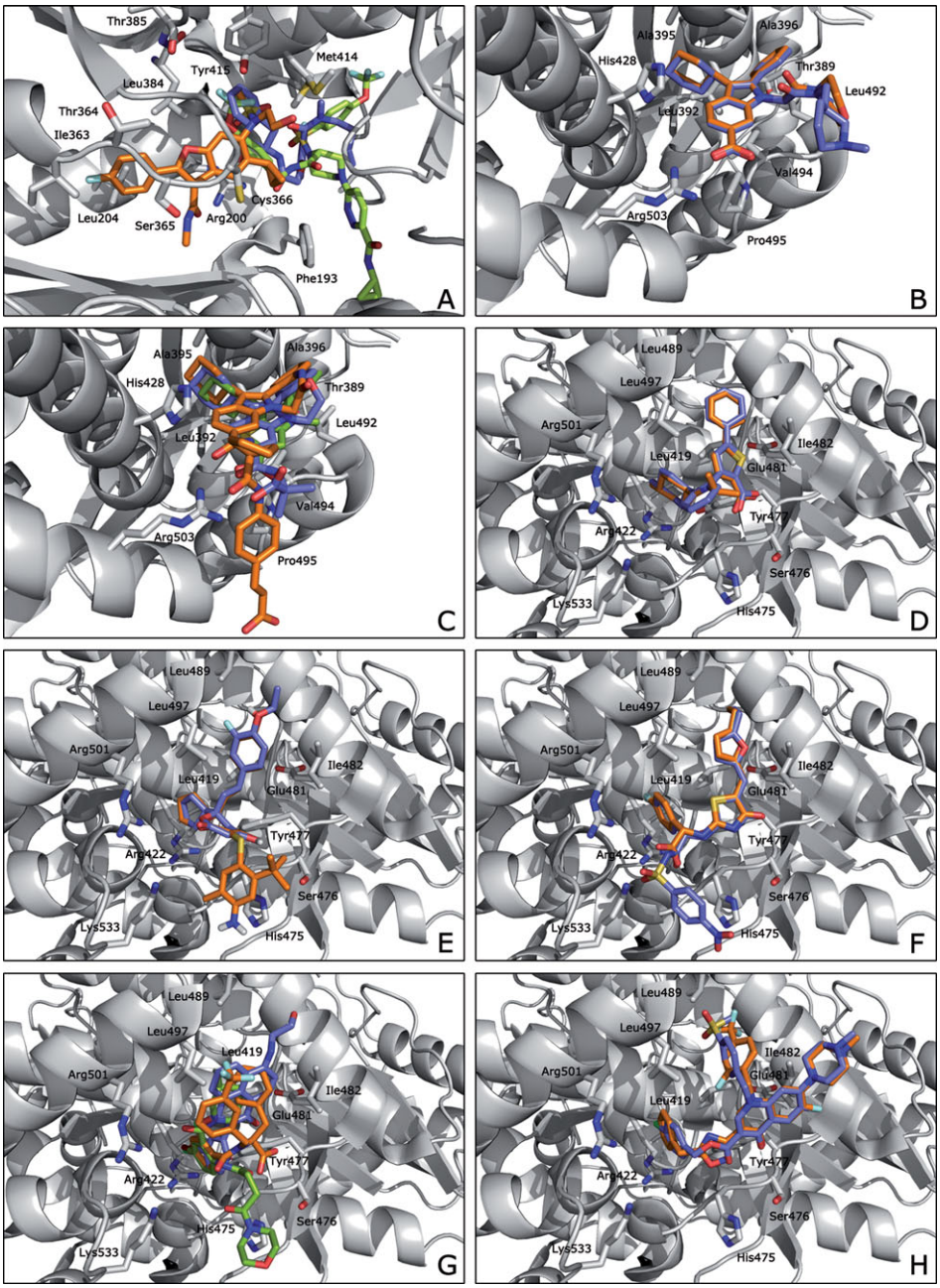
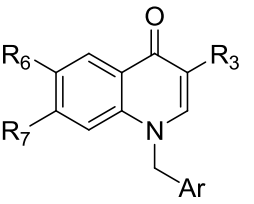
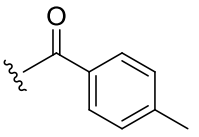
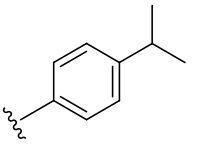
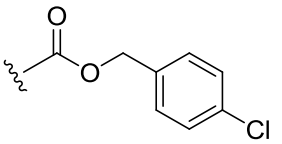
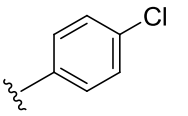
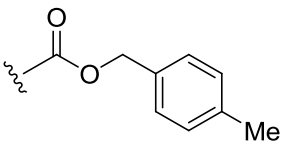
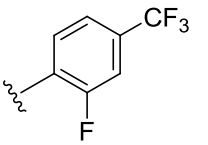
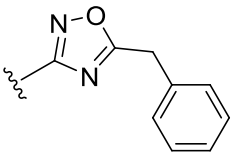
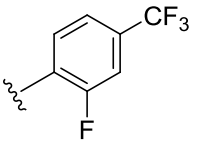


Figure 14. X-ray structures of Palm Pocket II and Thumb Pockets I and II inhibitors–RdRp complexes: (A) Compounds **39** (3FQL, orange), **41d** (3LKH, purple), and **43d** (3QGG, green); (B) compound **54c** (2BRL, orange) and an analogue of **54d** (2BRK, purple); (C) two tetracyclic indole derivatives (3Q0Z, orange and 2DXS, purple) and a thienopyrrole derivative (2WCZ, green); (D) compounds **78** (2GIR, orange) and **80** (3MF5, purple); (E) compounds **85a** (1OS5, orange) and **86** (2HAI, purple); (F) compounds **88** (2HWI, orange) and **93** (2O5D, purple); (G) a phenylalanine derivative (1NHU, orange) and compounds **95** (2WHO, purple) and **101** (3CJ5, green); (H) compound 103d (3UDL, orange) and a quinolone analogue (3PHE, purple).

Table XXV. Structure and In Vitro Inhibition Values of Various Quinolone Derivatives Against 1b HCV RdRp



103

Compound	R_3	R_6	R_7	Ar	IC_{50} (nM)
103a		OMe	OMe		1150
103b		OMe	OMe		20
103c		OMe	OMe		8
103d		F	*N*-methyl-piperazyl		14

of a number of inhibitors of HCV replication with potencies within the nanomolar range. Our analysis of literature from the past 10 years revealed that the most suitable molecules are heterocyclic compounds with a molecular weight in the range of 400–500 g/mol. The chemical characteristics of these inhibitors meet the criteria identified for most drugs (Lipinski's rule). Access to the main part of molecules is generally straightforward and not hampered by rate limiting reactions. The structural diversity of identified inhibitors provides a unique opportunity to perform global quantitative structure–activity relationship studies (QSAR). This study can be done for each class of compounds acting at the same allosteric site.

Some non-nucleoside inhibitors have already reached clinical development, such as for instance tegobuvir (GS-9190, Gilead), filibuvir (Pfizer), or setrobuvir (ANA598, Roche). The large number of highly potent inhibitors obtained thus far is a source of optimism that more potent inhibitors of RdRp will reach clinical development in the near future. However, in spite of their anti-HCV effectiveness, toxicity has already led to halting the development of several compounds and non-nucleoside RdRp inhibitors suffer from a narrow genotypic coverage and a low barrier to resistance, making that they will need to be used in combination with other anti-HCV drugs. Clinical needs will ultimately drive the future of these classes of drugs in the HCV armamentarium.

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