Bioorganic & Medicinal Chemistry 19 (2011) 4690–4703

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Review

Recent advances in drug discovery of benzothiadiazine and related analogs as HCV NS5B polymerase inhibitors

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ARTICLE INFO

Article history:
Received 20 April 2011
Revised 22 June 2011
Accepted 27 June 2011
Available online 1 July 2011

Keywords:
Hepatitis C
HCV
NS5B
Polymerase
Benzothiadiazine
Benzothiazine
Benzoisothiazole
Tetramic acid
Review

ABSTRACT

Hepatitis C virus (HCV) is a major health burden, with an estimated 170 million chronically infected individuals worldwide, and a leading cause of liver transplantation. Patients are at increased risk of developing liver cirrhosis, hepatocellular carcinoma and even liver failure. In the past two decades, several approaches have been adopted to inhibit non-structural viral proteins. The RNA-dependent RNA polymerase (NS5B) of HCV is one of the attractive validated targets for development of new drugs to block HCV infection. In this review, we report the recent progress made towards identifying and developing benzothiadiazines as HCV NS5B polymerase inhibitors. The substituted benzothiadiazine class was identified by HTS in 2002 as an NS5B inhibitor. Further optimization and modification of the core has improved the potency and pharmacokinetic properties of substituted benzothiadiazines. Research on palm site-binding benzothiadiazine analogs and related derivatives and analogs is discussed in this article.

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doi:10.1016/j.bmc.2011.06.079

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1. Introduction

1.1. HCV and liver diseases

Hepatitis C virus (HCV) was first characterized in 1989 as the major cause of non-A and non-B hepatitis infections.[1] HCV was recognized as the principal etiological agent of hepatitis C infection and the cause of hepatocellular carcinoma (HCC) and chronic liver disease (including liver cirrhosis and liver failure).[2–4] Even two decades since its discovery, HCV remains a burden on public health systems worldwide[5] and a leading cause of liver transplantation. Globally, an estimated 170 million individuals, 3% of the world's population, are chronically infected with HCV and 3–4 million people are newly infected each year.[6–9] HCV is most commonly transmitted[10] by blood transfusions, hemodialysis and intravenous drug use. In developing regions, improper cleaning and disinfection of tools and operation equipment used in clinics and hospitals remain a major source of virus transmission. Only a small percentage of infected people can resolve HCV infection naturally via their immune systems.[11] Symptoms can be mild or non-existent for years after initial infection, and patients can be asymptomatic for decades before developing liver cirrhosis and/or liver cancer.[12] In approximately 70% of cases, HCV escapes the body's immune system and establishes a persistent infection. Patients with this 'chronic carrier status' are at risk of developing life-threatening liver diseases including hepatocellular carcinoma.[13] Currently there are no vaccines available for any HCV genotypes, and evidence suggests that developing effective HCV vaccines will be difficult.[14] An effective broad spectrum therapy with activity against all genotypes of HCV is not available.[15–17] The current standard of care (SoC) for HCV-infected patients consists of a combined therapy of pegylated interferon-α (pegIFN-α) and ribavirin (RBV) for 24 or 48 weeks, depending on the HCV genotype. The success rate for achieving a sustained viral response for genotype 1 patients in the US, Europe and Japan remains low, with a sustained virology response (SVR) of 40–50%.[18] This regimen has the disadvantage of severe side effects[19,20] including headache, fever, depression, myalgia and hemolytic anemia.[21] The treatment market for HCV was predicted to rise from approximately US $3 billion per year to $8 billion by 2010.[22] Accordingly, several different structural motifs of HCV inhibitors have been identified and many pharmaceutical companies are competing to identify new drugs.[23–30] The expanding pipeline of specific HCV drugs in clinical trials is summarized in Table 1.

In this review, we report the recent progress made towards identifying and developing benzothiadiazines as HCV NS5B polymerase inhibitors.

1.2. HCV genome

HCV is a small (40–60 nM diameter), spherical, enveloped, single-strand, positive (+)-sense RNA virus belonging to the *Flaviviridae* family.[31,32] The most closely related human viruses are GB virus C (GBV-C), hepatitis G virus (HGV), yellow fever virus and dengue virus. At least six major genotypes (1–6) of HCV have been recognized with various subtypes (a, b, c, d, etc.). The HCV genome is an uncapped, linear molecule with a length of 9600 nucleotides harboring a single open reading frame (ORF) flanked by 5′ and 3′ non-translated RNA segments (NTR's).[34] The ORF encodes a single polyprotein of approximately 3010 amino acids that is further processed by host peptidases and viral proteases to provide at least 10 proteins: the HCV structural proteins—C, E1, E2—located at the amino-terminal end of the polyprotein, and the nonstructural (NS) proteins NS2, p7, NS3, NS4A, NS4B, NS5A and NS5B. Due to their necessary roles in viral replication, viral enzymes such as the HCV protease NS3/NS4A and NS5B RdRp have been the most studied viral proteins and recognized as one of the most viable protein targets for small molecule HCV drug discovery.[35–37]

HCV infects liver cells at the level of 10^8–10^{11} copies of HCV RNA per gram of tissue. Some recent reports indicate that HCV can infect T-cells, B lymphocytes, dendritic cells and cells of the central nervous system.[33] Similar to other single-stranded RNA viruses, HCV polymerase lacks proofreading ability to correct errors during replication. HCV NS5B replicates the HCV RNA strand with an error rate of 10^{-2}–10^{-3} nucleotide substitutions per site per year.[38] Due to the high replication rate and the lack of proofreading ability by NS5B, the HCV genome has a genetic variability that makes it challenging to develop drugs against HCV.

1.3. NS5B polymerase as an anti-HCV drug target

Many NS5B inhibitors have been discovered recently and several are in clinical trials. The NS5B polymerase of HCV is an attractive target for inhibition of HCV replication and a validated target for antiviral therapy.[35] As an RNA-dependant RNA polymerase, it has no counterpart in mammalian cells and so it is expected that inhibition of the enzyme will not cause target-related side effects.

The HCV nonstructural protein 5B (NS5B) is a 66 kDa RNA-dependent RNA polymerase (RdRp)[36] that resides at the C-terminus of the polyprotein. It plays a pivotal role in the synthesis and replication of viral RNA.[39] NS5B initiates the synthesis of complementary negative-strand RNA using the HCV genome (positive polarity) as a template and subsequently generates positive-strand RNA from the negative-strand RNA template.

The three dimensional structure of NS5B has a right hand architecture with finger, thumb and palm domains (Scheme 1).[40–43] The palm domain contains the active site of the enzyme. In addition to the active site, four allosteric sites have been identified: palm I (palm domain near active site), palm II (overlapping palm I and towards the active site), thumb I (thumb domain near the fingertips) and thumb II (the outer surface of the thumb domain). NS5B inhibitors can be divided into two classes: (a) nucleoside inhibitors (NIs)—active site nucleoside or nucleotide inhibitors that mimic natural polymerase substrates and (b) non-nucleoside inhibitors (NNIs)—allosteric inhibitors that bind to less conserved sites

Table 1
Clinical and launched HCV inhibitors

NS3/4A protease	NS5B polymerase	NS5A replication factor
Launched		
telaprevir (VX 950), boceprevir (SCH 503034)		
Phase-III		
TMC-435350		
Phase-II		
ACH-1625, BI-201335, BMS-650032, GS-9256, R-7227/ITMN191 (danoprevir), SCH 900518 (narlaprevir), MK-7009 (vaniprevir), ABT-450	ANA598, B-207127, BMS 791325, filibuvir (PF-868554), GS-9190 (tegobuvir), PSI-7977, RG7128 (mericitabine), VCH-222, VCH-759, ABT-072, ABT-333	BMS-790052
Phase-I		
VX-500	IDX-375, INX189, MK-3281, PSI-7851, PSI-938, VCH-916	PPI-461

outside the active site and impair the enzyme's catalytic efficiency. A spherical hydrophobic pocket, comprising the amino acid residues P197, R200, L384, M414 and Y448 has been observed within the active site. This pocket is considered an allosteric NNI binding site.[27] The discovery and development of NS5B NNIs have been reviewed.[27–30,44]

The most advanced active site NI (nucleoside inhibitor) in clinical development is R-7128[45–47], the *bis-iso*butyl ester prodrug of PSI-6130 (2′-deoxy-2′-fluoro-2′-*C*-methylcytidine). The compound was discovered by Pharmasset and is currently being developed in collaboration with Hoffmann-La Roche. The results of a combination of R-7128 (500 mg or 1500 mg b.i.d. for 28 days) with peg-IFN-α/RBV showed 85% rapid virologic response and good tolerability.

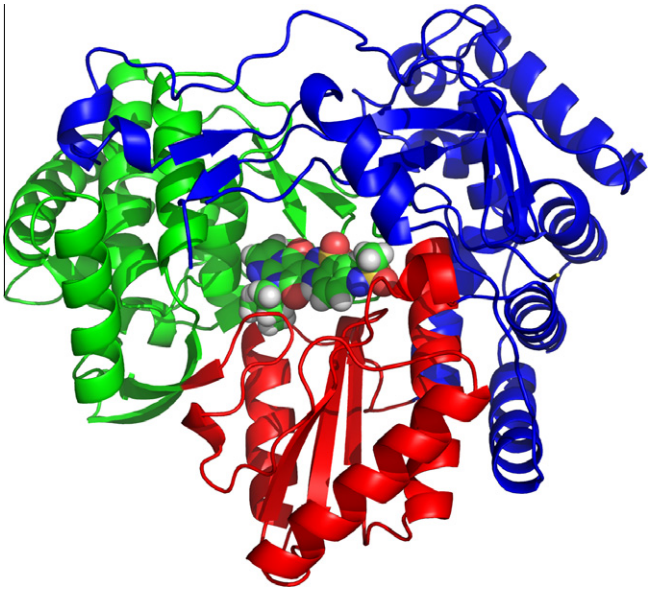
A number of non-nucleoside allosteric site inhibitors are in clinical development. In the late 1990s, benzimidazole-based compounds were identified by Japan Tobacco and Boehringer Ingelheim as 'thumb pocket I' inhibitors of HCV polymerase.[48] JTK-109 (Japan Tobacco Inc.) was advanced into clinical studies.[49,50] This chemotype has been further modified.[51–53] MK-3281 has been described as a potent 'finger loop' allosteric inhibitor of HCV NS5B, and safety and tolerability of the drug has been tested on HCV-infected male patients.[54] The 'thumb II' site binder, filibuvir (FBV; formerly PF 00868554), is a non-nucleoside inhibitor of HCV polymerase, currently in phase II clinical studies.[55,56] VCH-916, VCH759 and VCH-222 (structures not disclosed) are in advanced clinical trials.[57–59] 'Palm site I' inhibitors ABT-072, ABT-333, ANA-598 are in phase II development. GS-9190, a 'palm site II' inhibitor, has been advanced into clinical phase II studies.

The 3-substituted 1,2,4-benzothiadiazine-1,1-dioxides are one of the recently identified NNIs, represented by general structure **1** (Fig. 1). The structure consists of a tetracyclic ring system (labeled A, B, C and D). Usually the A- and D-rings are substituted aromatic rings, fused with B- and C-rings, respectively. The B-ring is linked to the 3-position of the benzothiadiazine C-ring (Fig. 1). A mechanistic study of the benzothiadiazine class of HCV polymerase inhibitors revealed that this class may be very useful for treating HCV.[60]

2. Discovery of the benzothiadiazine core as an NS5B inhibitor

1,2,4-Benzothiadiazine-1,1-dioxide analogs were known as diuretics[61] for many years. Benzothiadiazine analogs are increasingly used in different therapeutic areas including as antiviral agents,[62] ATP-sensitive potassium channel inhibitors,[63–65] anti-tumor agents,[66] and allosteric modulators of AMPA receptors.[67]

High throughput screening (HTS) of the GlaxoSmithKline (GSK) proprietary compound collection using an RdRp assay with oligo(rG)-primed poly(rC) substrate[68] allowed identification of several structurally distinct, putative polymerase inhibitors.[69]



Scheme 1. A ribbon diagram of the X-ray crystal structure of the HCV polymerase with the benzothiadiazine inhibitor **23a** bound to the palm site (PDB ID 3H98, Ref. 89). The inhibitor is shown in a CPK representation. The HCV polymerase palm, fingers, and thumb subdomains are colored red, blue, and green respectively. We consider the palm site to include residues 188–227 and 287–370, the fingers site to include residues 1–187 and 228–286, and the thumb site to include residues 371–563. The figure was generated using PyMOL Molecular Graphics System, Schrödinger, LLC.

Among them, the *N*-butyl benzo-1,2,4-thiadiazine **2a** (R1 = butyl, R2 = H) was selected for additional studies (Fig. 2).[69] The benzo-1,2,4-thiadiazine **2a** demonstrated potent inhibition of the HCVΔ21(C-terminal 21 amino acid deletion) RdRp with an IC_{50} value of approx. 0.2 μM (Table 2). Moreover, preliminary structure activity relationships indicated a critical role for the quinolone N-1 substituent for biological activity. Both the N-unsubstituted **2b** (R1, R2 = H) and *N*-methyl **2c** (R1 = Me, R2 = H) analogs were significantly weaker NS5B inhibitors, while **2a** and *N-iso*pentyl derivative **2d** were potent inhibitors. Compound **2d** showed an IC_{50} of approx. 0.08 μM. The compounds **2a** and **2d** retained activity against the full length enzyme.[69] It was also shown that compound **2a** did not interact with nucleic acid (it was noncompetitive with respect to GTP), and it reduced subgenomic viral RNA replication in a Huh-7 cell-based HCV replicon system. Compound **2d** was identified as a more potent inhibitor. Further mechanistic studies demonstrated that compound **2d** interfered with RNA synthesis and might form a ternary complex with the enzyme and the RNA template.[60,70] The benzo-1,2,4-thiadiazine

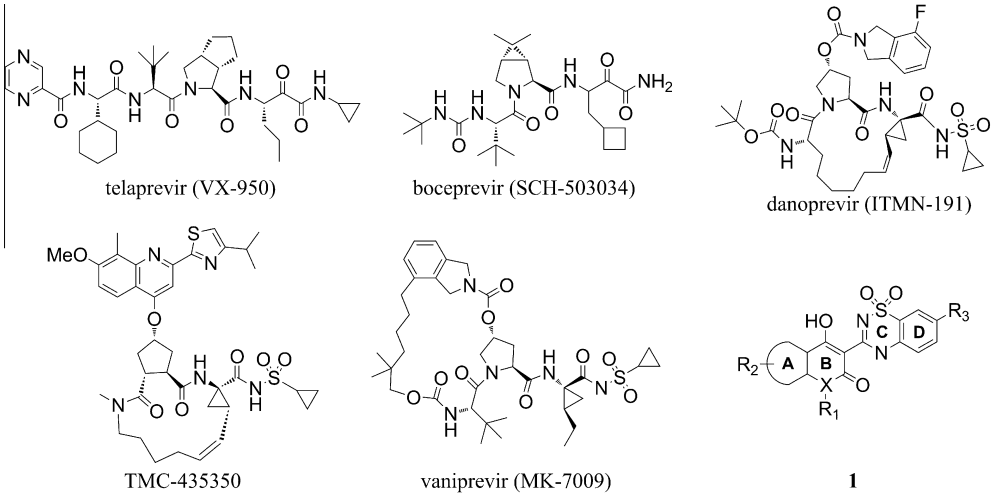


Figure 1. The general structure of benzothiadiazine core and selected advanced HCV protease inhibitors.

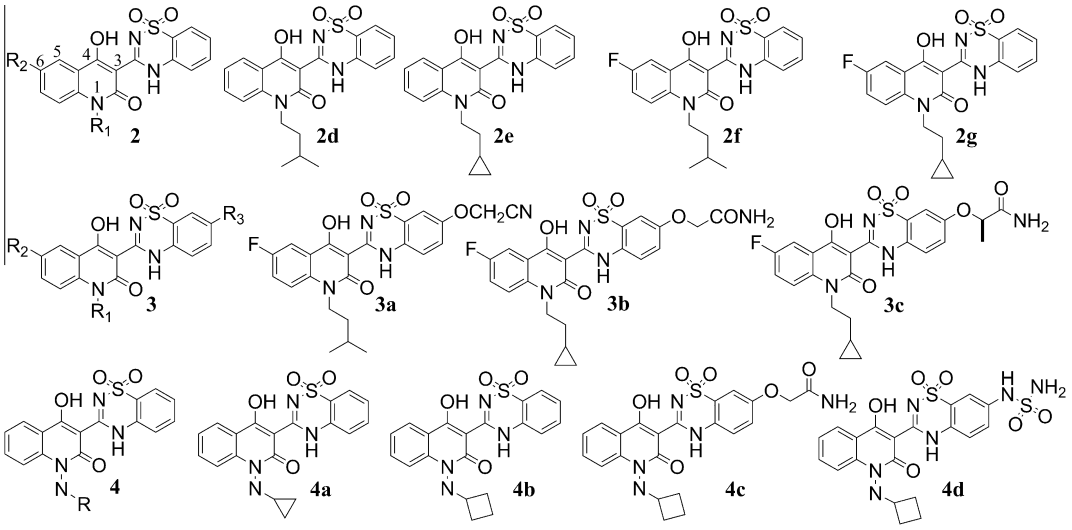


Figure 2. 4-Hydroxyquinolin-2(1*H*)-one analogs.

derivatives were highly selective for HCV-RNA polymerase and failed to inhibit other viral and mammalian polymerases.[69] Selection of HCV RNA replicons resistant to compound **2d** showed that a mutation in the polymerase coding region (methionine to threonine at residue 414) reduced the potency of RdRp inhibitors.[71,72]

After the discovery of benzothiadiazine analog **2d** as a potent inhibitor of NS5B, the general core **1** was studied extensively by several groups to improve the potency and pharmacokinetic properties. The systematic exploration of the core is described below.

3. Structure–activity study of A-, B- and D-ring analogs

3.1. 4-Hydroxyquinolin-2(1*H*)-one analogs

The synthesis of 1-alkyl-3-(1,1-dioxo-2*H*-1,2,4-benzothiadiazine-3-yl)-4-hydroxy-2-(1*H*)-quinolinones **2** (R1 = alkyl) was first described by Ukrainets et al.[73] and reported to have diuretic activity. A detailed study of the structure–activity relationships (SAR) of the substituents on both benzo rings of compound **2** and the effects on NS5B was published by GSK scientists. The work led to the discovery of active inhibitors of viral replication with potency in the low nanomolar range.[74] Analogs with short alkyl chains of 1–3 carbons on the quinoline nitrogen atom were weak inhibitors of NS5B. The compounds with unsaturated double and triple bonds of different chain lengths (3–5 carbons) were also weak inhibitors. Incorporation of a branched alkyl chain of 4–5 carbons increased the activity significantly. Branched chain analog **2d** and the linear butyl **2a** have similar levels of inhibitory activity in the replicon assay but a sixfold difference in enzyme inhibition (Table 2). SAR studies on the A-ring with alkyl chains, halogens, nitro-, amino-, and carboxylic acid functionalities at the 5-, 6-, 7- or 8-positions identified **2f** and **2g** (with fluorine atoms at the R2 position) as potent inhibitors against both the isolated enzyme and in the standard replicon assay. All four benzothiadiazines (**2d**, **2f**, **2g**, **2h)** were tested on rat, dog and cynomolgous monkey and had moderate plasma half-lives, low plasma clearance and good oral bioavailability ($\geqslant$35%).[74] Compound **2g** was identified as the best candidate for preclinical development. However, **2g** was highly lipophilic and much weaker in the protein-attenuated replicon assay in the presence of physiological concentrations of human serum albumin (HSA) and alpha acid glycoprotein (AAG). To lower the protein binding and improve the activity in the cell-based and in vivo assays, another set of compounds **3** was prepared (Table 2).[75] Substitution on the D-ring was performed at positions 5, 6,

Table 2
Polymerase inhibition of **2**, **3**[74,75]

Entry	IC_{50} (1b) (nM)	Replicon IC_{50} (1b) (nM)	PA-replicon IC_{50} (nM)[60]
2a (R_1 = butyl, R_2 = H)	200	444	—
2d (R_1 = *iso*-amyl, R_2 = H)	32	417	—
2e	13	152	—
2f	20	261	—
2g	10	38	24,461
2h (R_1 = *iso*-pent. R_2 = Me)	47	180	—
3a	7	81	—
3b	<5	2	1484
3c	<5	3	1017
3d (R_1-*iso*-amyl, R_2 = H, R_3 = NH_2)	13	218	45,000
3e (R_1-*iso*-amyl, R_2 = H, R_3 = OH)	30	>20,000	—
3f (R_1-*iso*-amyl, R_2 = H, R_3 = $CONH_2$)	6	383	—

PA-replicon = protein-attenuated Replicon asssay.

7 and 8. Starting with a 7-hydroxy group on the D-ring, a series of functional groups was incorporated into the side chain. The oxyacetonitrile substituted compound **3a** containing an *iso*amyl side chain at the N1 position was a very potent inhibitor in the enzymatic assay. Optimizing the alkyl chain length at the N1 position and introducing functionality at the R3 position led to the identification of compounds **3b** and **3c** that demonstrated very good potency and in vivo properties. The compounds were evaluated for pharmacokinetics and cytochrome P450 inhibition. Potency in the protein-attenuated replication assay was significantly improved and CYP2C9 inhibition was decreased for **3b** and **3c** compared to **3a**.[75]

A nitrogen-for-carbon replacement at the N1 position yielded 4-hydroxy-quinolin-3-yl-benzothiadiazine **4** as a potent inhibitor of genotype 1 HCV polymerase.[76] The cyclopropyl analog **4a** was approximately threefold more active than **2d** in the replicon system. N-alkyl substituents with halogen or polar groups of the same chain lengths decreased the inhibitory effect. A range of alkyl- and aryl-derivatives were made and evaluated in a cell-based HCV replicon system against genotype 1b. Small rings improved the potency over linear chains and aromatic rings. Compounds **4a** and **4b** were good representatives of the series with potency ~0.1 μM. **4b** (cyclobutyl) exhibited 10-fold better potency than **4a** (cyclopropyl). Substitution on the D-ring of compound **4b** with an ethanecarbamyl group led to compound **4c**, which showed an EC_{50} value of 70 nM with no cellular toxicity up to 63 mM, and a good pharmacokinetics profile in animal studies.[77] The primary sulfonyl urea **4d** and methyl sulfonamide analog potently inhibited HCV replication, with an EC_{50} value of 3 nM.[78]

3.2. Heterocyclic A- and/or D-ring benzothiadiazine analogs

N-Alkyl-4-hydroxy-1,8-naphthyridon-3-yl-benzothiadiazines **5** were prepared by Abbott Laboratories (Fig. 3). The effect of substituents on the D-ring on potency was extensively studied by modification at the 5-, 6-, and 7-positions.[79] Methoxy substitution at the 7-position (**5b**) was well tolerated. Incorporation of methoxy at the 5-position of the thiadiazine ring decreased the potency to IC_{50} = 1.17 μM (1b) and 17.58 mM (1a). Incorporation of a methoxy or hydroxy at the 5- or 6-position exhibited at least a 20- to 30-fold loss in potency compared to no substitution. The 6-position of the D-ring is indeed close to the protein as was revealed from X-ray structures.[74] The Abbott group extensively studied the tolerance of the 7-position of the D-ring of the benzothiadiazine core to substitution.[79] Compound **5i** improved the potency of the series to the nanomolar range (IC_{50} = 6 nM) (Table 3) and exhibited approximately threefold greater potency than **5c** (IC_{50} = 16 nM). **5e** and **5i** were evaluated in the HCV replicon assay containing 40% human serum and showed a loss of approximately 30- to 400-fold compared to the replicon assay performed with 5% fetal calf serum. Subsequent measurements of the compounds **5e** and **5i** in serum plasma demonstrated high protein binding (>99% bound). **5e** was orally administrated to rats as a solution formulation at a dose of 5 mg/kg and exhibited modest pharmacokinetic properties with 35% bioavailability, low maximum concentration (C_{max} = 0.18 μM), and a half-life (2 h) with modest clearance (0.74 L/h/kg).[79]

Following the discovery of the importance of substituents at the C7 position of the D-ring combined with a nitrogen heterocycle on the A-ring (1,8-naphthyridine core), extensive studies on both the A- and D-rings were done to improve the physicochemical properties, plasma protein binding and the potency of this inhibitor class by GSK.[80] 1,8-naphthyridinone systems **5f**, **5g** and **5h** and thienopyridinones **5j**, **5k** and **5l** were extremely potent compounds in

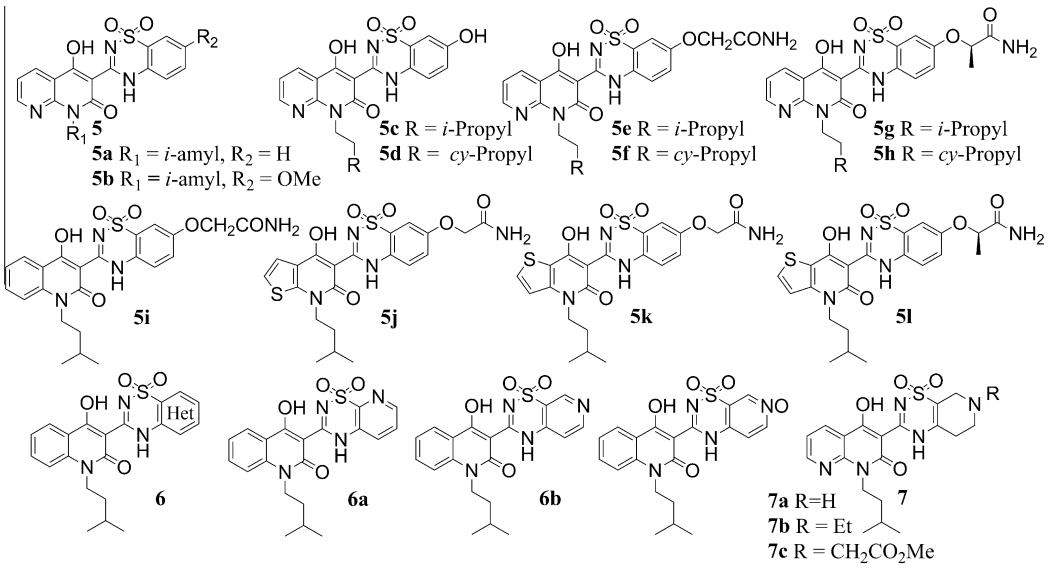


Figure 3. Heterocyclic A- and/or D-ring benzothiadiazine analogs.

Table 3
Polymerase inhibition of **5**–**7**[80]

Entry	RdRp IC_{50} 1b (nM)	Replicon IC_{50} 1b (nM)	HSA (% bound)
5a	212	417	>99.9
5c	16	473	99.0
5d	12	542	98.8
5e	<5	52	98.4
5f	<5	51	97.8
5g	<5	47	98.3
5h	<5	—	97.8
5i	6	15	99.2
5j	<5	238	98.7
5k	<5	126	98.3
5l	<5	114	98.3
6a	20	226	99.0
6b	31	743	98.7
6c	18	2377	97.7
7a	604	12,219	98.2
7b	679	40,000	97.9
7c	879	—	97.6

the enzymatic assay and maintained good levels of potency in the replicon system while reducing plasma protein binding. Pyridines, thiophenes and pyrazoles were investigated as replacements of the benzofused D-ring of the thiadiazine system.

All the pyridine derivatives were equipotent with the parent compound and showed decreased plasma protein binding. Complete reduction of the heteroaromatic ring **6b** led to **7**. Although the protein binding of compound **7** decreased to 97.6%, the potency dropped significantly.[80]

3.3. *Gem*-dialkyl-4-hydroxynaphthalenone derivatives

Abbott laboratory postulated that the π-stacking of the aromatic rings in the benzothiadiazine analogs might be the cause of their lower solubility. Rosen[81] improved the solubility of fluoroquinolone antibacterial agents by replacing the nitrogen atom of a dihydroquinolinedione ring system with a carbon atom. Following a similar approach, dialkyl chains were introduced in the B-ring of benzothiadiazines and a series of compounds of general structure **8** were prepared (Fig. 4).[82–85] The racemic compounds **8a** and **8b** showed nanomolar potency with IC_{50} = 7 and 4 nM for genotype 1b and 1a respectively. The potency changed slightly (EC_{50} = 17 nM) in the replicon 1a assay. Although a series of dialkyl derivatives were prepared with substituted and unsubstituted D-rings, the only potent compound in the series was the di-*iso*amyl compound **8c**, with an IC_{50} = 68 nM (genotype 1a) and 390 nM (genotype 1b) and weak replicon 1a potency (EC_{50} = 10.1 mM).[84] The promising results with the asymmetric dialkyl derivatives[83] implied that the two groups are binding at two different pockets or environments within the HCV polymerase. Preparation of the enantiopure compounds revealed that (*R*)-isomers **8d** and **8e** were the more active stereoisomers. Further analysis showed that in vivo oxidation of the *neo*-*iso*-amyl moiety on compound **8d** led to compound **8f**. The possibility of oxidation was eliminated by introducing a methyl group in **8e**. The pharmacokinetics properties of **8d** and **8e** and their corresponding sodium salts **8d′** and **8e′** were studied in detail.[85] The results were promising and the (*R*)-*iso*-amyl and *neo*-hexyl substituted compounds **8d′** and **8e′** were identified as lead candidates. By achieving high concentrations in the liver, good bioavailability and a long half-life after oral dosing, compounds **8d′** and **8e′** also demonstrated a desirable pharmacological profile.[85] Compound **8d′** provided the first in vivo validation of the antiviral efficacy of dialkyl-hydroxynaphthalenoyl-benzothiadiazine series in a chimpanzee model of HCV infection.[86,87]

B-Ring alkyl and aminoalkyl analogs were also studied. Benzylamine analogs **8g** and **8h** were found to be potent inhibitors with low nanomolar inhibition. Pharmacokinetic studies in rat revealed that the in vivo performance of *neo*hexyl analog **8j** was significantly improved relative to *iso*amyl analog **8h**. Following a 5 mg/kg iv dose, the plasma half-life increased to 3.1 h for **8j**, versus 1.7 h for **8g**. Furthermore, **8j** was cleared from the plasma more slowly (0.7 L/h/kg) than **8g** (1.16 Lh/kg). Plasma exposure of **8j** following a 5 mg/kg oral dose was greatly enhanced relative to **8j**, with a sevenfold increase in both C_{max} and overall exposure (AUC) for **8g**. The oral bioavailability for **8j** was 55.9%, a fourfold increase relative to **8i**.[88]

3.4. Hydroxynaphthalene derivatives

In search of potent allosteric inhibitors of HCV polymerase, InterMune and WuXi explored several series of benzothiadiazine compounds.[89] The naphthalene benzothiadiazine compounds

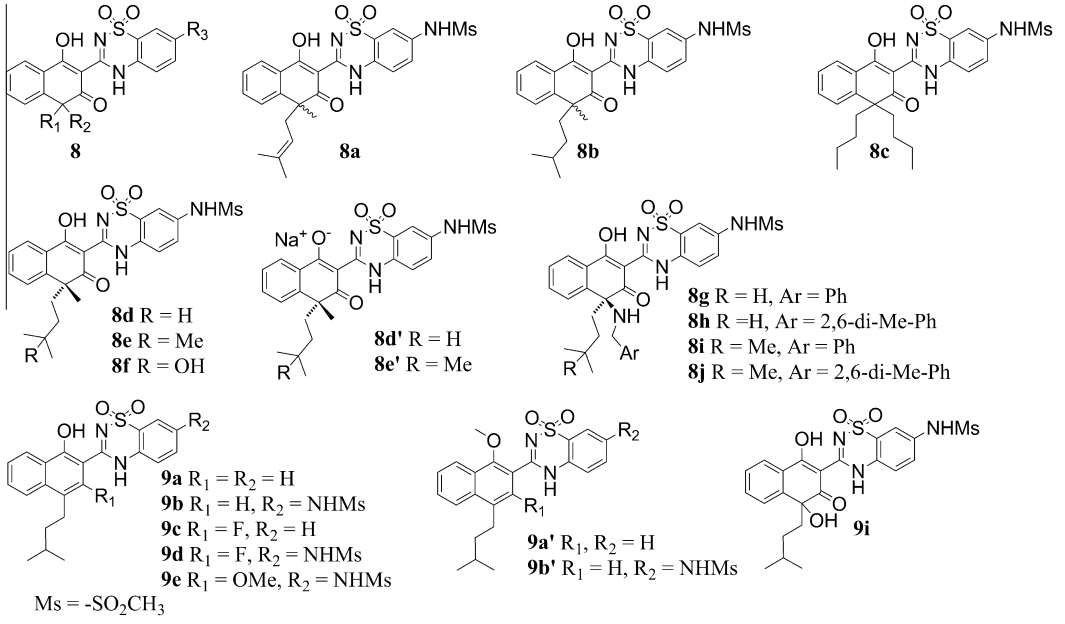


Figure 4. 4-Hydroxynaphthalenone and hydroxynaphthalene analogs.

Table 4
Polymerase inhibition of **9**[89]

Entry	IC_{50} 1b (μM)	EC_{50} 1b (μM)	CC_{50} (μM)
9a	8.4	26	37
9b	0.15	4.3	>100
9c	0.11	19	12
9d	0.031	2.7	>100
9e	0.037	2.9	120
9a′	27	9.5	13
9b′	0.048	21	51
9i	<0.005	0.019	>100

9a–**e**, **9i**, **9a′** and **9b′** were synthesized and tested in NS5B genotype 1b polymerase and replicon assays (Table 4). Fluoro- and methoxy-groups at the R1 position showed more potent inhibition than the unsubstituted analog (R1 = H). The compounds **9b** and **9d** with the methylsulfonamide moiety were significantly more potent than **9a** and **9c** in the NS5B enzyme-inhibition assay (Table 4). In the cellular assay, compounds **9b** and **9d** (EC_{50} = 4.3 and 2.7 μM respectively, both CC_{50} >100 μM) exhibited not only higher potency, but also a higher selectivity window over **9a** and **9c** (EC_{50} = 26 and 19 μM; CC_{50} = 37 and 12 μM, respectively). The enhanced potency and decreased cytotoxicity achieved with the methylsulfonamide moiety at the R2 position were likely due to both enhanced binding to and selectivity for NS5B. The replacement of the C1-hydroxy group on the naphthalene ring by a 3-methoxy group was tolerated, as demonstrated by **9a′** and **9b′** (IC_{50} = 27, 0.048 μM, respectively). Interestingly, tertiary hydroxy-substituted **9i** was a highly potent compound (IC_{50} = 5 nM).

4. Removal of fused A-ring

The X-ray co-crystal structure of **2d** bound to the NS5B polymerase showed that the inhibitor bound at the palm site and had a relatively planar conformation, with a 20° rotation between the quinolinedione (A–B) and benzothiadiazine (C–D) rings governed by intramolecular H-bonding.[74] Increasing the polarity of the molecules was attempted by removing the fused ring and introducing a nitrogen in the quinolinedione core.

4.1. Des-A-ring benzothiadiazines

To reduce bulkiness, the benzothiadiazine A-ring, which fits snugly into a small hydrophobic pocket in the binding site, was removed and several substitutions on the B-ring were evaluated. The potency was dramatically decreased when the A-ring was completely removed (**10a** R = H; IC_{50} = 274 nM) (Fig. 5). Substitution on the B-ring with hydrophobic moieties maintained potency in nanomolar range in both isolated enzyme and cell-culture assay systems, as demonstrated by Abbott Laboratories.[90] A decrease in potency was observed for **10b** (R = Ph; IC_{50} = 425 nM), acetyl substitutions and other long chain substitutions. Five-membered aromatic rings were well tolerated and maintained potency: **10c** (R = 2-thiophene) and **10d** (R = 2-furyl) showed good potency (IC_{50} = 11 and 8 nM, respectively for genotype 1a). The potency of **10d** in the replicon assay was maintained (EC_{50} = 2.5 nM, 1b replicon 5% FCS). Des-A-ring compounds exhibited relatively less protein binding.[90]

4.2. 5-Hydroxy-3(2*H*)-pyridazinones

To improve the physicochemical properties, reduce lipophilicity and improve solubility, Anadys Pharmaceuticals removed the fused A-ring, added one more nitrogen in the B-ring and designed a series of 5-hydroxy-3(2*H*)-pyridazinone structures **11**. Compound **11a** (IC_{50} = 33 μM) was identified as the lead compound. The co-crystal structure of **11a** bound to NS5B revealed that the phenyl ring filled a shallow cavity proximal to the Met414 side chain by rotating up to 45° out of the plane relative to the pyridazinone ring.[85] A smaller thiophene ring was suggested and a systematic variation of the R1, R2 and R3 substituents was performed.[91–94] Substitution on the C7 position of the D-ring influenced potency. Inhibitory potency was dramatically improved by replacing the $-OCH_2CONH_2$ in **11b** with $-NHSO_2Me$ in **11c** (Table 5). The size of the substituent on the sulfonamide had a large impact on the potency, and $-NHSO_2Me$ and $-NHSO_2NH_2$ groups were identified as the optimal R3 moieties. The $-NHSO_2Me$ R3

Table 5
Polymerase inhibition of **11**–**12**[94,95]

Entry	IC_{50} (1b) (μM)	EC_{50} (1b) (μM)	CC_{50} (μM)	HLM $t_{1/2}$ (min)
11b	0.07	0.41	78	23
11c	<0.01	0.005	>33	>60
11d	<0.01	0.007	>1	>60
11e	0.045	0.098	>33	>60
11f	0.027	0.043	>1	>60
11g	0.031	0.36	>1	38
11h	<0.01	0.047	0.34	31
11i	0.01	0.048	>33	>60
11j	0.01	0.016	>1	>60
12a	0.014	0.017	>1	10
12b	0.015	0.62	>33	>60

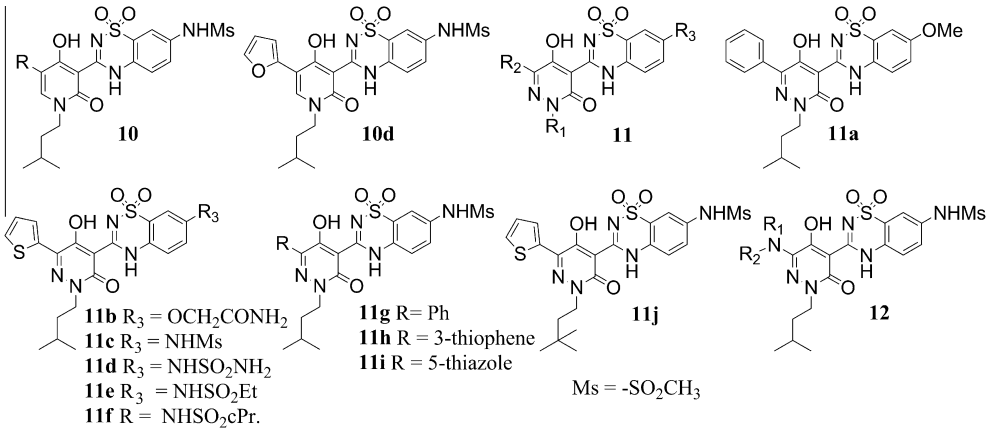


Figure 5. Des-A-ring and 5-hydroxy-3(2*H*)-pyridazinone analogs.

moiety of **11c** formed H-bonds with NS5B residues Asn291 and Asp318. Similar H-bonding interactions were also observed in the co-crystal structure between the acetamide moiety of compound **11b** and NS5B.[86] In addition, the $-NHSO_2Me$ of **11c** formed a third H-bond network with residue Ser288 via a water molecule that was absent in the **11b**–NS5B co-crystal structure. This additional H-bond network, which complements a similar interaction from the $-SO_2$ moiety in the benzothiadiazine ring of **11c**, is likely responsible for improving the potency from **11b** to **11c** (Table 5).[79] A wide range of aryl and aliphatic groups was studied at the R2 position of **11**, and the 2-thiophene group was shown to be optimal. Although the potency of 3-thiophenyl in **11h** was similar to **11c**, the stability of compounds with substituents at the R position exhibited moderate to long half-lives ($t_{1/2}$ >30 min) towards human liver microsomes (HLM). The potency of cyclic four-, five-, and six-membered rings was lower than that of aromatic substituents, with HLM half-lives <25 min. Both oral (po) and intravenous (iv) pharmacokinetic properties of two very potent and metabolically stable compounds **11c** and **11j** were reported in monkeys.[94] The bioavailability of both compounds **11c** and **11j** after oral administration to cynomolgus monkeys was low (%*F* = 1–2). Since the molecules displayed low in vivo clearance values and reasonable solubility in H_2O at pH 7.4, it was believed that their undesirable oral PK properties primarily result from poor gut permeability. Accordingly, both inhibitors displayed very low Papp values in Caco-2 permeability assessments. Poor permeability properties are consistent with the highly polar nature of both **11c** and **11j**, which have polar surface area (PSA) = 203 Å.[96,97] Despite the poor bioavailability of compound **11j** (1b EC_{50} = 16 nM), the C_{12h} in plasma was close to its EC_{50} value. The ratio of liver to plasma concentrations in rat of **11c** (1b EC_{50} = 5 nM) 12 h after a single oral dose of 3 mg/kg was high (64-fold). Since liver is the major site of HCV replication, increasing the concentration of inhibitor enhances the chance of eradicating the virus.

Pyridazin-3-ones bearing 6-amino substituents **12** were synthesized and found to be potent inhibitors of HCV NS5B polymerase. The pyrolidine (**12a**) and morpholine (**12b**) displayed good potency but the stability of **12b** was better. While many of the compounds displayed antiviral activity in cell culture experiments, the in vitro stability and the toxicity data were not promising.[95]

5. A-ring and A–B-rings modifications

5.1. Pyrrolo[1,2-*b*]pyridazin-2-one

It was thought that poor cell permeability was likely caused by the high polar surface area (PSA), which was outside the normal range typically correlated with good absorption.[96,97] Reducing the PSA and increasing the lipophilicity might improve the cell permeability and bioavailability. Toward this end, pyrrolo[1,2-*b*]pyridazin-2-one derivatives **13** were designed by Anadys Pharmaceuticals through a fusion of the five-membered aromatic ring with the pyridazinone (Fig. 6, Table 6).[98] A series of compounds was investigated by varying the R1, R2, and R3 substituents on **13**. Methyl sulfonamides (–NHMs) and cyclopropyl sulfonamides were best at the R3 position, although a reduction in potency was observed for the –NHMs moieties on the D-ring. The changes on the R1 moiety were generally well tolerated. The 4-fluorobenzyl analog **13a** retained good enzymatic and antiviral potencies. Aliphatic chains on **13b** and **13c** showed comparable activities but **13b** showed better stability (half-life = 42 min) than **13c** (half-life = 10 min) towards HLM and was found to be the optimal compound in the series. Introduction of a fluorine atom on the pyrrole ring **13d** improved the microsome stability ($t_{1/2}$ >60 min) and reduced the metabolism of the pyrrole ring but led to a loss of potency (⩾3-fold) compared to **13b**. Both compounds **13a** and **13b** exhibited low nanomolar activity (Table 6) in both biochemical and replicon assays and good stability towards HLM. However, poor oral bioavailability was observed in an in vivo PK study (cynomolgus monkeys). Minimal change in oral bioavailability was observed in changing from open chain aryl **11c** (%*F* = 2) to fused aryl **13a** (%*F* = 1),[98] perhaps due to limited change in the PSA.

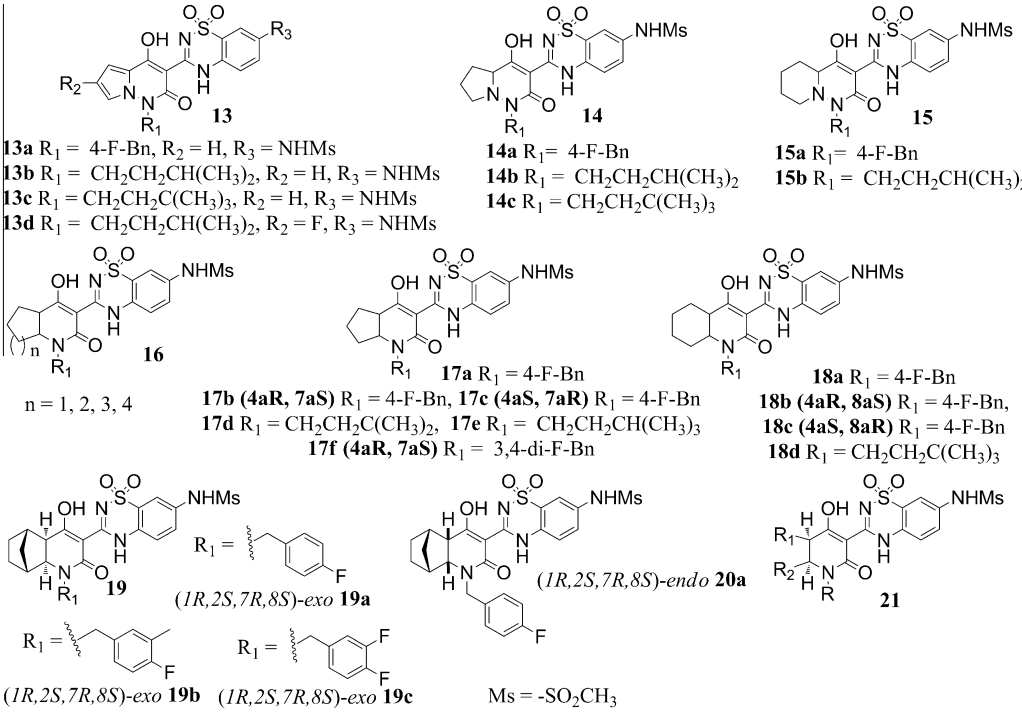


Figure 6. A-ring modifications.

Table 6
Polymerase inhibition of **13**–**20**[98,100,102]

Entry	IC_{50} (1b) (μM)	EC_{50}(1b) (μM)	CC_{50} (μM)	HLM $t_{1/2}$ (min)
13a	<0.01	0.012	>1	>60
13b	<0.01	0.0085	>1	42
13c	<0.01	0.005	>1	10
13d	0.027	0.019	>1	>60
14a	<0.01	0.19	>1	>60
14b	<0.012	0.16	>1	>60
14c	<0.01	0.034	>1	59
15a	0.016	0.3	>1	>60
15b	0.041	0.11	>33	52
17a	<0.01	0.023	>1	>60
17b	<0.01	0.016	>100	>60
17c	0.031	0.22	>33	>60
17d	<0.01	<0.06	>1	10
17e	<0.01	0.024	>1	7
17f	<0.01	0.055	>1	>60
18a	0.022	0.023	>1	>60
18b	0.027	0.12	>1	>60
18c	0.047	0.35	>33	>60
18d	0.043	0.033	>33	16
19a	—	0.003	>100	>60
19b	—	0.006	—	>60
19c	—	0.007	—	>60
20a	—	0.27	>33	>60

5.2. Hexahydro-pyrrolo and hexahydro-1*H*-pyrido[1,2-*b*]pyridiazin-2-ones

To improve the drug-like properties, hexahydro-pyrrolo and hexahydro-1*H*-pyrido[1,2-*b*]pyridiazin-2-ones **14** and **15** were prepared by reducing the pyrrole or pyridine rings.[99] All the compounds displayed good solubility (>100 μM) but many showed only low to modest stability towards monkey liver microsomes. Although the potency of the series was maintained, poor bioavailability was observed. The best compound of the series **14c** exhibited poor bioavailability (%*F* = 7), potentially due to low intestinal permeability.[99]

5.3. 5,6-Dihydro-1*H*-pyridine-2-ones

To reduce the PSA, the ring nitrogen of **14** was removed and 5,6-dihydro-1*H*-pyridine-2-ones of general structure **16** were prepared.[100] Extensive SAR studies of the racemic and chiral compounds with different aliphatic ring sizes (five-, six-, seven- and eight-membered) were conducted. The five-membered ring compounds were the most active, with potency deceasing with increasing ring size, suggesting that larger ring size was not accommodated properly in the binding pocket. The in vitro results of some selected compounds are given in the Table 6. These compounds displayed low to moderate in vivo clearance. Most of the compounds bearing aliphatic chains showed low stability towards monkey liver microsomes (MLM), while those with benzylic substituents (**17b**, **17c**, **17f**, **18b** and **18c**) were stable in the assay.[100] The enantiopure inhibitor (*4aR*, *7aS*)-**17b** showed a good combination of replicon potency and in vitro/in vivo DMPK properties and exhibited plasma levels in monkeys that significantly exceeded its replicon EC_{50} value for at least 12 h following oral dosing. **17b** displayed potent inhibitory activities in biochemical and replicon assays IC_{50} (1b) = 10 nM; IC_{50} (1a) <25 nM, EC_{50} (1b) = 16 nM, good in vitro DMPK properties and moderate oral bioavailability in monkeys (%*F* = 24).[100]

Further work provided tricyclic 5,6-dihydro-1*H*-pyridin-2-ones **19** (*exo*-) and **20** (*endo*-).[102] An extensive SAR study was done investigating chirality and substitutions on the R1 position. The inhibitors containing a bicyclo[2.2.2]octane moiety exhibited greater in vitro antiviral activity against a broad range of R1 substituents. All compounds were very stable with half-lives typically greater than 60 min in a HLM assay. The (2*S*,7*R*)-bicyclo[2.2.2]octane-containing molecules exhibited greater in vitro antiviral potencies against both genotypes 1a and 1b across a broad range of R1 substituents. Inhibitors containing the (1*R*,2*S*,7*R*,8*S*)-*exo*-bicyclo[2.2.1]heptane moieties and (2*S*,7*R*)bicyclo[2.2.2] octane moieties were typically more potent than the corresponding analogs containing the (1*S*,2*S*,7*R*,8*R*)-*endo*-bicyclo[2.2.1]heptane structural motif. The best compounds were evaluated in vivo, and the in vivo clearances were different for isomers **19a**–**c** and **20a**. The slowest clearance was observed for (1*R*,2*S*,7*R*,8*S*)-*exo*-**19a**. The oral bioavailabilities of *exo*-**19a**, **19b**, **19c** (%*F* = 52, 35, 45, respectively) were higher than *endo*-**20a** (%*F* = 26).[102] In addition, the plasma exposure of **19a**, **19b** and **19c** improved significantly compared to **17b**.

5.4. 5,5′-and 6,6′-Dialkyl-5,6-dihydro-1*H*-pyridin-2-ones

To improve the PK properties, a series of aliphatic chain-substituted compounds with chirality on the pyridine 2-ones was prepared with the general structure **2**.[101] The SAR studies identified a number of compounds potent in both biochemical and cellular HCV assays. Oral bioavailability of one of the best compounds **21a** was 16%. However, fast in vivo clearance led to undetectable amounts of **21a** at 12 h post-dosing.

In summary, Anadys Pharmaceuticals demonstrated that using a strategy to reduce PSA resulted an improvement of bioavailability (Fig. 6). Key analogs in the optimization include the fused pyrrole ring **13a** (PSA = 167 Å, %*F* = 1), the saturated five member ring **14c** (PSA = 165 Å, %*F* = 7) and the analog derived from the removal of nitrogen to **17b** (PSA = 162 Å, %*F* = 24). Bioavailability was further improved by increasing the bridged ring size in the molecule. The *exo* compounds showed better bioavailability than the *endo* compounds, with the best bioavailability observed with **19a** (%*F* = 52).

5.5. Azolo[1,5-*a*]pyridine, azolo[1,5-*a*]pyrimidine and 4-hydroxy-2*H*-quinolizin-2-one derivatives

Towards identifying a new selective palm site inhibitor, we shifted the N-atom from the B-ring to the junction of A–B rings and made a series of azolo[1,5-*a*]pyridine-benzothiadiazines **22** (Fig. 7). An additional nitrogen on the five-membered pyrrole ring led to azolo[1,5-*a*]-pyrimidine-benzothiadiazines **23**.[103] Most of the compounds derived from these scaffolds demonstrated very potent NS5B inhibition (<5 nM) and exhibited promising replicon potency values (Table 7). Compounds **22a**, **23a**, **23b** were identified as the most potent compounds in the series, and the X-ray structure of **23a** bound to the polymerase was described.[104] Similarly, 3-(1,1-dioxo-2*H*-[1,2,4]benzothiadiazin-3-yl)-4-hydroxy-2*H*-quinolizin-2-one derivatives **24** were also explored.[104] All of the compounds exhibited very potent inhibition of NS5B, with all IC_{50} values less than 5 nM (Table 7). The most active compound in the replicon system was compound **24c** with an EC_{50} value of 2.3 nM and CC_{50} value of greater than 100 μM. Other compounds **24a, 24b** also exhibited excellent replicon activity with EC_{50} values of 10–17 nM. It is interesting to note that compounds **25a** and **25b**, in which the A-ring was saturated, exhibited good activity in both NS5B and replicon assays. Overall, the quinolizin-2-one benzothiadiazine ring system was demonstrated to be a promising scaffold for further derivatization and optimization.[104]

6. Modified benzothiadiazine general structures

To improve the pharmacokinetic properties, several laboratories have modified the original thiadiazine core **1** to provide new series of inhibitors.

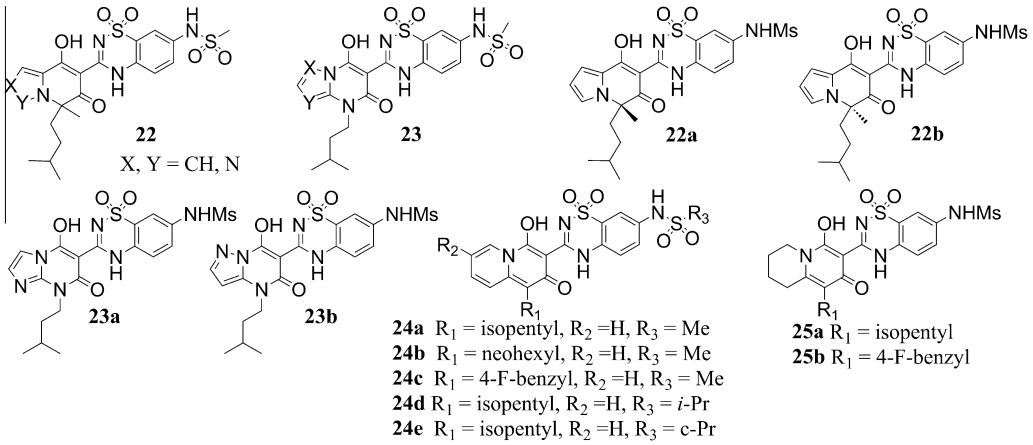


Figure 7. Azolo[1,5-*a*]pyridine and azolo[1,5-*a*]pyrimidine and quinolizin-2-one derivatives.

Table 7
Polymerase inhibition of **22–25**[103,104]

Entry	IC_{50} 1b (μM)	EC_{50} 1b (μM)	CC_{50} (μM)
22a	<0.005	0.145	19.5
22b	0.154	4.92	170
23a	<0.005	0.55	>1
23b	<0.005	0.55	>100
24a	<0.005	0.017	20
24b	<0.005	0.010	>100
24c	<0.005	0.0026	>100
24d	<0.005	0.13	>10
24e	<0.005	0.057	>10
25a	<0.005	0.057	>10
25b	<0.005	0.036	>1

6.1. Tetramic acid analogs

Tetramic acid, a well known heterocyclic marine natural product, showed good biological activity as an antibiotic.[105] In 2006, GSK reported a series of tetramic acid benzothiadiazine analogs as potent HCV polymerase inhibitors.[106] **26a** was the best compound in the series (Fig. 8). These analogs bind to the 'palm' region of NS5B in one of the three binding sites where non-nucleoside inhibitors typically interact with the protein. It was suggested that the tetramic acid moiety could serve as a mimic of the quinolinone moiety of the previously discovered structure **2d**. A series of enantiopure tetramic acid analogs were prepared via a solid-phase approach,[107] and **26b** was identified as the best compound of this series.

6.2. Benzo[*d*]isothiazole-1,1-dioxides

To improve the PK properties, a novel series of inhibitors in which the benzothiadiazine in structure **1** was replaced with a benzo[*d*]isothiazole-1,1-dioxide moiety to give **27**.[108–110] The compounds demonstrated potent inhibitory activities against both HCV polymerase genotypes 1a and 1b. Structure-based design and molecular modeling were employed to guide the optimization.[108] The most potent compound **27a** in this series contained an R2 *tert*-butyl group, an R1 3-chloro-4-fluorobenzyl group, and an R3 methyl methanesulfonamide group. 4-fluorobenzyl **27b** was another potent compound. However, poor oral bioavailability values (%*F* = 1 and 4, respectively) and inadequate plasma concentrations were observed.[108] **27c** exhibited good potency against the genotype 1 NS5B (IC_{50} = 0.069 μM) and the HCV subgenomic replicon (EC_{50} = 0.648 μM). Introduction of the methyl sulfonamide group at the 7-position of the benzo[*d*]isothiazole-1,1-dioxides **27d** provided an excellent boost in potency (IC_{50} = 0.005 μM) but showed poor replicon inhibition (EC_{50} = 4.924 μM). A one-atom extension of the methyl sulfonamide group retained good potency against the enzyme (IC_{50} = 0.003 μM) and showed dramatically improved replicon potency (EC_{50} = 0.1 μM).[110] Improved oral exposure in analogs bearing the lipophilic 3-methyl-4-fluorobenzyl group was observed. Compound **27e** showed low clearance in vivo and modest levels of exposure and oral bioavailability in rat and monkey (%*F* = 17, 11, respectively). The N-methylated sulfonamide **27f** also showed encouraging levels of exposure in rat (%*F* = 24). Changing the substitution to the 6-position in **27g**[110] was tolerated but did not provide a boost in replicon potency (IC_{50} = 0.043 μM,

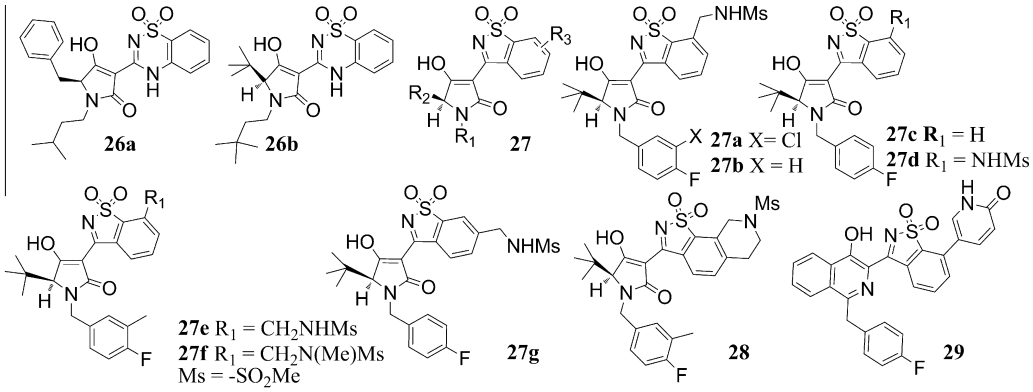


Figure 8. Tetramic acid and benzo-isothiazole-1,1-dioxide analogs.

EC_{50} = 3.844 μM). Another novel cyclic sulfonamide analog **28**[110] was shown to be the most potent analog in replicon assays, with an NS5B inhibition activity of 0.005 μM.[110] 3-Hydroxyisoquinoline thiadiazines were prepared by Roche Palo Alto and Array BioPharma with different aryl groups on the benzothiazine ring, and **29** was identified as the most potent compound in the series.[109]

6.3. Thiazine analogs: hydrogen bond analysis and modification of structures

The low in vivo exposure (%*F* ~10) and poor solubility (<1 μg/mL) of **2g** was attributed to the extensive internal hydrogen bonding between the quinolinedione and benzothiazine rings. A new series of benzothiazine analogs **30** was designed and prepared by the Roche Palo Alto and Array BioPharma group (Fig. 9).[111–113] In these compounds, the benzothiadiazine ring's internal hydrogen bonds were disrupted by exchanging N atoms with –CH groups. Initial study of the core **30a** showed micromolar potency, slightly improved solubility and significantly improved pharmacokinetic properties (%*F* = 79) compared to **2g**. The quinolinedione and benzothiazine rings form a shallow U-shape and fit well in the enzyme active site.[111] Extensive SAR studies on **30** showed that the substituted benzyl at the R1 position **30f**–**h** led to improve potency (Table 8). Small alkyl chains (cyclohexylmethylene **30i**, *iso*amyl chain **30j** or *tert*-pentyl **30k**) were almost equal to the benzyl analogs in potency, but they were not metabolically stable.[112] Compound **30h** was identified as the lead structure for PK studies.

The planar quinolinedione ring was replaced with the non-planar, non-polar, chiral tetramic acid ring in analogs **31**.[113] **31b** was identified for PK studies. The *iso*amyl group **31d** was equipotent with the *p*-fluorobenzyl **31b**, though loss of potency was observed in the presence of human serum. The replacement of the *iso*propyl group of **31d** by a *tert*-butyl group in **31e** enhanced the replicon potency and reduced the potency shift in the presence of human serum (Table 8). Compound **31b** displayed a moderate oral bioavailability in rat (%*F* = 22).

The pyridazine core of general structures **32** and **33** was studied (Anadys Pharmaceuticals), however, the bioavailability of the series was not encouraging.[114]

6.4. Quinoline derivatives

The importance of the sulfonyl moiety on the C- or CD-rings for increasing potency was investigated by Abbott Laboratories.[115] A series of quinoline derivatives of the general structure **34** (Fig. 10) was prepared without the sulfonyl moiety on the C-ring. **34a** showed nanomolar activity in both enzymatic and cell culture assays but was approximately fourfold less active in enzymatic assays than was the benzothiadiazine compound **8e**. A 4-position carboxylate **34b** showed similar enzymatic activity to **8e** but less activity in cell culture assays. The quinoline derivative **35** without the sulfonamide moiety on the C- and D-rings showed poor activity in enzymatic assays.[115]

Table 8
Polymerase inhibition of **30**–**31**[112,113]

Entry	IC_{50} 1b (μM)	EC_{50} 1b (μM) 5%FBS	EC_{50} 1b 40% HSA (μM)
30d	0.005	0.014	0.46
30f	0.007	0.044	0.406
30g	0.014	0.015	–
30h	0.013	0.023	0.088
30i	0.018	0.1	–
30j	0.041	0.009	–
30k	0.012	–	0.24
31a	0.008	0.032	0.139
31b	0.007	0.012	0.033
31c	0.003	0.012	0.122
31d	0.005	0.03	0.66
31e	0.008	0.016	0.065

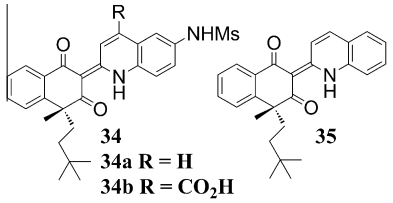


Figure 10. Quinoline derivatives.

7. Advancement of benzothiadiazine analogs and other series as NS5B inhibitors

Intense drug discovery efforts have focused on achieving potent and selective inhibitors of NS5B in recent years. Several structurally distinct cores have been identified for binding to specific NS5B subdomains, namely the palm, thumb, and finger. The inhibitors identified for allosteric binding in the thumb pocket I are benzimidazole,[116] indole,[117,125] quinoxaline[118] and thieno[3,2-*b*]pyrrole.[119] Thumb pocket II site inhibitors include thiophene-2-carboxylic acid,[120] pyranoindole,[121] dihydropyranone[122] and thiazolidin-4-one.[123] The inhibitors for palm I are benzothiadiazine,[69,74] benzylidene,[124] anthranilic acid[126] and acylpyrrolidine[127,128] derivatives. Benzofuran analogs bind NNI site 4. Comparative Molecular Field Analysis (CoMFA) and Comparative

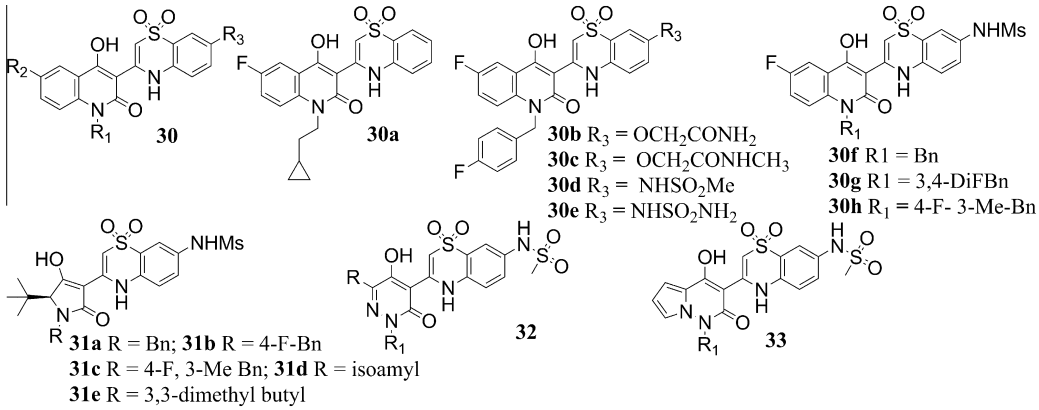


Figure 9. Benzothiazine analogs.

Table 9
Concentration of **8d** and **8e** in rat liver after oral dosing[85]

Compd	Plasma concentration after oral dosing (5 mg/kg)		Liver concentration after oral dosing (5 mg/kg)	
	6 h (nM)	12 h (nM)	6 h (nM)	12 h (nM)
8d	445	135	7340	2860
8e	2240	320	39300	7550

Molecular Similarity Index Analysis (CoSIA) for 239 promising molecules provided important guidelines in the design of the novel benzothiadiazine analogs as NS5B inhibitors.[129]

A number of polymerase inhibitors in clinical trials have demonstrated efficacy.[30,130] Among the nucleoside analogs, R7128[47] is currently the most advanced. HCV-796 was the first non-nucleoside inhibitor that demonstrated targeting to the palm site,[131,132] however, this compound was abandoned for safety reasons. Abbot's benzothiadiazine analog A-782759[77] demonstrated potent activity against HCV replicons in tissue culture. The rat, dog and monkey PK profiling of A-837093 (**8d**) and A-848837 (**8e**)[85,133] showed that both drugs were found at high concentration in rat liver tissue. The liver to plasma ratios at the 6 h time point were 17-fold for both compounds and increased slightly to 20-fold at the 12 h time point (Table 9). A-837093 (**8d**) was also tested in a chimpanzee HCV model.[87] ABT-072 and ABT-333 (structures not disclosed) are advanced clinical candidates. Both compounds have good oral bioavailability in rats and dogs, good in vitro metabolic stability and low potential for drug interactions, predicting favorable pharmacokinetics in humans.[134] ABT-333 is a potent inhibitor with enzyme inhibition IC_{50} levels of 2.2 nM against HCV genotypes 1 and 2. The pharmacokinetic profile of ABT-333 in rat, monkey and dog is characterized by a wide range of plasma clearance values with high volumes of distribution in all species. The results from single ascending dose/multiple ascending dose (SAD/MAD) with ABT-333 at doses 200–400 mg twice-daily b.i.d. to healthy volunteers were promising.[135] ANA598[136,137] (structure not disclosed) was dosed at two doses levels: 200 mg given b.i.d. and 400 mg b.i.d. Anadys reported data from this study for the 400 mg b.i.d. dose level, showing a favorable safety profile through 12 weeks and that 75% of patients at this dose level achieved undetectable levels of virus at week 12, known as complete Early Virological Response (cEVR). These data confirmed the positive profile previously reported for ANA598 at 200 mg b.i.d. through 12 weeks in this study.[137]

In vitro combination studies of HCV protease inhibitor danoprevir with benzothaidiazine ITMN-8020 showed enhanced antiviral activity and suppression of drug-resistant variants.[138] The combination of these two agents may represent a viable therapeutic approach, resulting in antiviral activity greater than that observed with danoprevir alone. ITMN-8020 (**22a**) was a prototype member of a series of non-nucleoside inhibitors of NS5B. Although ITMN-8020 showed inferior oral exposure in primates compared with site II benchmarks, the newer prototype ITMN-8244 (structure not disclosed) displayed superior oral exposure with AUC up to 12-fold higher than site II benchmarks (Table 10).

Table 10
Plasma exposure in primate (5 mg/kg PO)[138]

	8d[a]	**11c**[b]	ITMN-8244[b]	ITMN-8020 (**22a**)[b,103]
EC_{50} 1b (nM)	12	32	3	145
C_{max} (ng/mL)	193	7.63	1577	5.61
T_{max} (h)	0.8	12	4	4
AUC (ng h/mL)	652	103	8341	76.9
$T_{1/2}$ (1 mg/kg IV)	3.8	—	4.0	ND
%*F*	8.6	0.5	—	1.7

[a] 0.5% methylcellulose.
[b] 20% solutol/DI water.

8. Conclusion

HCV NS5B polymerase has both active and allosteric sites for inhibitor binding. Apart from the catalytic site, each allosteric site can be targeted by a separate class of inhibitors. The development of a potent NS5B polymerase inhibitor is the research focus of many groups in academics and the pharmaceutical industry.

In the past several years, GSK, Anadys, Abbott, InterMune, Roche and other pharmaceutical companies have discovered potent benzothiadiazine HCV NS5B polymerase inhibitors with good PK properties. Due to the lack of a "proof reading" function in the NS5B polymerase, the HCV RNA genome can rapidly mutate to escape antiviral drug therapy. The mutations and consequent viral resistance will likely be significant problems for the development of small molecule HCV inhibitors. Thus, a combination of therapies with multiple drugs against different targets may be necessary to inhibit HCV replication effectively. The knowledge from benzothiadiazines and related analog discovery and development, as well as from the mutation studies, will help guide the design and optimization of more potent and promising drug candidates in the future.

Acknowledgment

The authors thank Beth A. Wurzburg for editing.

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