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Hepatitis C Virus Translation Inhibitors Targeting the Internal Ribosomal Entry Site

Miniperspective

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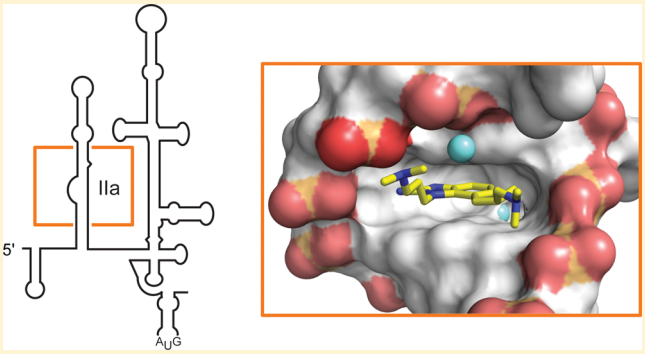
ABSTRACT: The internal ribosome entry site (IRES) in the 5′ untranslated region (UTR) of the hepatitis C virus (HCV) genome initiates translation of the viral polyprotein precursor. The unique structure and high sequence conservation of the 5′ UTR render the IRES RNA a potential target for the development of selective viral translation inhibitors. Here, we provide an overview of approaches to block HCV IRES function by nucleic acid, peptide, and small molecule ligands. Emphasis will be given to the IRES subdomain IIa, which currently is the most advanced target for small molecule inhibitors of HCV translation. The subdomain IIa behaves as an RNA conformational switch. Selective ligands act as translation inhibitors by locking the conformation of the RNA switch. We review synthetic procedures for inhibitors as well as structural and functional studies of the subdomain IIa target and its ligand complexes.



1. INTRODUCTION

Synthesis of the HCV polyprotein depends on an internal ribosome entry site (IRES) in the 5′ untranslated region (UTR) of the viral RNA genome.[1,2] The IRES element is responsible for assembling functional ribosomes at the viral start codon in a mechanism that bypasses canonical translation initiation which depends on the 5′ cap modified terminus of eukaryotic mRNA. The HCV IRES first recruits host cell small (40S) ribosomal subunits and eukaryotic initiation factor 3 (eIF3), which is a large multiprotein complex required to prevent premature association of 40S and 60S ribosomal subunits. In a complex process that is coordinated by the IRES RNA large (60S) subunits join to assemble complete (80S) ribosomes at the start codon and translation is initiated through a 5′ cap-independent mechanism that obviates the need for other initiation factors.[3−6] The IRES RNA is a potential target for HCV translation inhibitors due to its unique function and high conservation in clinical virus isolates.[7−10] While drugs directed at the highly conserved viral IRES promise to benefit from rare occurrence and low fitness of resistance mutants in the treatment-naive population, developing druglike selective inhibitors for an RNA target is an exceptionally challenging endeavor.[11−14]

The HCV IRES element extends from position 40 through 372 of the viral RNA genome, spanning the 5′ UTR and 30 nucleotides beyond the start codon at A342 into the polyprotein coding frame.[15] The IRES contains three independently folding domains (II−IV) connected by flexible linkers (Figure 1).[16] Domain I within the 5′ UTR is not required for IRES-driven translation but participates in viral replication together with the 3′ UTR.[17] Ribosomal recruitment and positioning of the viral mRNA start codon is orchestrated

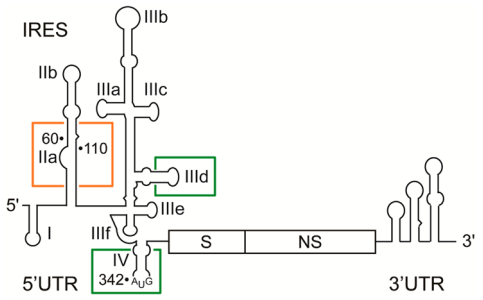


Figure 1. Organization of the HCV RNA genome showing the structured untranslated regions (UTR) including the IRES element in the 5′ UTR. Structural (S) and nonstructural (NS) gene regions are indicated. Boxed subdomains have been extensively studied as targets for small molecule translation inhibitors (IIa) and oligonucleotides as well as peptides (IIId, IV).

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by intermolecular interactions across the central domain III, which also provides the binding site for the eIF3 complex.[18,19] The hairpin loop of domain IV, which includes the start codon and ~13 nucleotides of the coding region, unwinds during 40S binding at the IRES to expose the viral translation initiation site in the ribosomal decoding groove.[19−21]

In addition to scaffolding the assembly of components in the translational machinery, the IRES RNA participates as an active player in dynamic changes during translation initiation. IRES domains affect the catalytic activity and subunit composition of initiation factors. The IRES domain II promotes both eIF2-catalyzed GTP hydrolysis and subsequent eIF2/GDP release from the 48S complex and mediates removal of the eIF3j component before 60S subunit joining.[22,23] The correct orientation of the HCV mRNA at the ribosomal decoding site involves interaction with the IRES subdomain IIb, a completely conserved RNA hairpin loop at the tip of domain II which also contacts ribosomal protein rpS5.[23−25] Positioning of the subdomain IIb hairpin in the ribosomal E-site at the subunit interface relies on the folding of the asymmetric internal loop of subdomain IIa, which adopts a 90° bent conformation.[26−28] The topology of the IRES−ribosome interaction requires subdomain IIb to be removed from the E-site to allow the P-site tRNA to translocate during translation initiation.[20,25] It has been suggested that conformational changes in the subdomain IIa may release the hairpin IIb from the E-site.[29,30] Alteration of the L-shaped architecture of subdomain IIa through mutations or by interference with small molecule ligands that affect the RNA conformation has been shown to disrupt IRES-driven translation and effectively inhibit viral protein synthesis.[22,29−31]

Here, we provide a review of approaches to inhibit viral translation by targeting domains of the HCV IRES with oligonucleotides, peptides, and small molecules. Emphasis will be given to the subdomain IIa, which currently is the most advanced IRES target for small molecule translation inhibitors. The subdomain IIa is an RNA conformational switch capable of forming a deep solvent-excluded binding pocket for small molecule ligands which may serve as leads for the development of IRES-targeting antiviral drugs.

2. STRATEGIES FOR INHIBITION OF THE HCV IRES

HCV translation has been recognized as a potential target for the development of antiviral therapeutics.[7−10] The high conservation of the IRES element suggests that inhibitors will potentially benefit from selection of low-fitness resistance mutants that have reduced frequency of occurrence in the treatment-naive population. IRES ligands that have been considered as potential inhibitors of HCV translation include oligonucleotides, peptides, and small molecules. The following sections discuss briefly the rationale and mechanisms for the various inhibitor classes.

2.1. Oligonucleotide Inhibitors. Soon after the discovery of the IRES as the key driver of HCV translation, antisense oligonucleotides targeting the 5′ UTR were studied as inhibitors of viral gene expression.[32−35] In addition to chemically modified oligonucleotides, morpholino, peptide nucleic acid (PNA), and locked nucleic acid (LNA) antisense oligonucleotides were used to suppress IRES function.[36−40] Among the IRES hairpin loop motifs, the subdomain IIId and domain IV proved to be the most responsive targets for antisense approaches (Figure 1). A 20-residue phosphorothioate oligodeoxynucleotide, complementary to the region around the start codon in domain IV,[41] was evaluated in a phase I clinical trial. While a related antisense oligonucleotide (ISIS 6547)[42,43] that targeted the same IRES region had an in vitro IC_{50} value of 0.1−0.2 μM, the ISIS 14803[41] drug showed only weak transient activity in human, with plasma HCV RNA reduction of <2 log(10) at thrice-weekly dosing of 2−3 mg over 4 weeks.[42,43] The insufficient potency accompanied by side effects eventually led to discontinuation of the development effort.[44]

In addition to antisense ligands, RNA aptamer inhibitors were developed for all domains (I−IV) of the IRES. Published aptamers of the HCV IRES recognize apical loops in hairpin domains by hybridization with a fully complementary sequence.[45−51] Aptamer oligonucleotides for domain I interfere with replication rather than translation, as was expected from the function of this IRES domain.[49,51] The most potent inhibitors were found among aptamers of the subdomain IIId, with in vitro activities comparable to those of antisense oligonucleotides.[46,48] In vivo data have not been published for IRES-targeted aptamers.

Inhibition of HCV translation by oligonucleotide-mediated cleavage of the IRES RNA was investigated using ribozymes,[52−58] DNAzymes,[59,60] conjugated chemical nucleases,[61,62] and siRNA and shRNA.[54,63−72] The design principle shared in common by these constructs requires an accessible single stranded sequence in the IRES[73] that provides the target for a complementary oligonucleotide ligand which is either associated with or, in the case of siRNA and shRNA, directs a ribonuclease activity. Among ribozymes tested in vitro were hammerhead constructs targeted at the apical loop of subdomain IIIb (HCV-195)[52,53] or the subdomain IIIf (HH363).[56−58] Conjugated chemical nucleases consisting of RNA-hydrolyzing imidazole derivatives tethered to guide oligonucleotides were shown to inhibit in vitro IRES-driven translation at submicromolar IC_{50} values.[61] However, testing of the conjugates in HCV-infected cells suggested that the inhibition was due to the antisense effect of the guide oligonucleotide and not to cleavage by the attached imidazole.[62]

Combination of ribozymes with siRNAs directed at sequences within the IRES resulted in additive inhibition of viral translation.[54] Targeting of IRES sequences by siRNA or shRNA has resulted in efficient in vitro knockdown of translation by the RNA interference mechanism.[63−72] The inhibition potency of interfering RNAs was essentially related to the accessibility of the target sequences within the IRES structure. For siRNA and shRNA inhibitors as well as other types of oligonucleotide ligands targeting the IRES, efficient cellular delivery remains a confounding challenge.

2.2. Peptide Inhibitors. Only few studies have been reported of targeting the HCV IRES with peptides. A 24 amino acid peptide (LaR2C)[74] derived from the RNA recognition motif 2 (RRM2) of the human lupus autoantigen (La) was shown to moderately inhibit IRES-driven translation by competing with binding of cellular La protein. A similar inhibitory effect was achieved with a peptide (LAP)[75,76] corresponding to the N-terminal 18 amino acids of La.[75,76] La protein is an abundant component in the nucleus where it plays diverse roles in the metabolism of various RNA precursors.[77] HCV translation as well as replication requires the La protein which binds to both the IRES domain IV and the 3′ UTR[78−80] and which itself has been proposed as a potential therapeutic target.[81]

Amphiphilic peptides consisting mainly of lysines and leucines were shown to bind with nanomolar affinity to a hairpin RNA derived from domain IV of the HCV IRES albeit with only moderate (up to 2-fold) discrimination against other RNA targets.[82] Antiviral activity of the peptides was not tested.

In an attempt to create an artificial metallonuclease that site-selectively cleaves the HCV IRES, a Y(D-R)FK tetrapeptide was linked with a copper-chelating GGH tripeptide.[83] Copper-(II) cleaves ribose moieties in proximity by an oxidative mechanism that likely involves peroxide generation. While no rationale was provided for the proposed selectivity of the peptide for the IRES subdomain IIb, the artificial metallonuclease did degrade a hairpin loop IIb model oligonucleotide but not two unrelated RNAs. The cleavage mechanism and site selectivity were not further investigated. However, the peptide metallonuclease showed antiviral activity with an IC_{50} value of 0.58 μM in an HCV replicon assay.

2.3. Sequestration of IRES-Binding Factors. In an approach that does not target the IRES directly but proteins that bind to the HCV 5′ UTR, decoy-like nucleic acids have been used to sequester host cell factors that are required for IRES function. A 60 nt RNA (IRNA),[76,84] which originally had been isolated from yeast, was shown to inhibit HCV translation in extract and cells, likely by sequestration of IRES-binding factors including the La protein. Inspired by these findings, RNA decoys derived from subdomains of the HCV IRES were tested for their ability to inhibit viral translation.[85] RNAs corresponding to the full domain III or just the subdomain IIIef inhibited IRES-driven translation in a dose-dependent fashion. The ribosomal protein S5 was identified as one of the host factors interacting with the subdomain IIIef decoy RNA. Even smaller oligodeoxynucleotides containing nine residues were shown to inhibit IRES-mediated translation by sequestration of two yet to be identified host cell proteins.[86]

2.4. Small Molecule IRES Inhibitors. Both in vitro translation and direct target binding assays have been used to screen for small molecule inhibitors that affect HCV IRES-driven translation.[87−93] High-throughput screening of ∼300 000 compounds by electron spray ionization mass spectrometry (ESI/MS) against an oligonucleotide representing the subdomain IIIe hairpin identified six binders with affinities of <50 μM.[89] Among the hits were four peptides and two aminoglycosides of undisclosed structure. Biological activity in an IC_{50} range of 3−12 μM was reported for the six hits but without a description of the biological assay. An affinity assay that identified small molecule ligands based on stabilization of a structured RNA ("SCAN") had been used to screen ∼135 000 compounds against the subdomain IIId hairpin loop.[91] The SCAN screen of the IIId hairpin loop identified 12 potential hits including one selective small molecule ligand that bound the target with 0.7 μM affinity. Biological activity and structures of the hits were not disclosed.

Screening attempts for IRES-binding inhibitors have been hampered by complications specifically encountered with RNA targets. First, there is a lack of druglike molecules for screening that exhibit bias for RNA binding but are not structurally complex natural products. Second, challenges arise from the need for assays that identify hits based on functional consequences of ligand binding to an RNA target rather than returning binding affinities only. Assessment of functional inhibition is critical for RNA-binding ligands that typically have binding affinities inferior to potent inhibitors targeting well-defined hydrophobic pockets in proteins. Weak binding affinities observed for RNA-binding antibiotics might be related to their unique mechanisms of action that often do not involve direct competition with a cognate ligand. For example, macrolides and ketolides, which are among the most potent RNA-binding antibiotics, bind to the ribosomal peptidyl transferase active site with low nanomolar affinities (erythromycin, ∼14 nM).[94] Aminoglycoside antibiotics, including paromomycin and tobramycin, have weak binding affinities of around 1 μM for their target in the bacterial ribosomal decoding site RNA.[95] For oxazolidinones such as linezolid, RNA target dissociation constants in the range of 20−100 μM have been measured.[96,97]

In vitro translation (IVT) assays test for functional discrimination of compound impact on IRES-driven versus cap-driven translation by using either bicistronic or sets of monocistronic reporters. While IVT assays probe for inhibition of IRES function, ribosomes present in the assay mix at high concentration function both as key functional components and as a source of complex competitor RNA that may interfere with ligand binding to the target under study. The large number of false positive hits reported in a recent IVT screen of the HCV IRES has been attributed to off-target effects, for example, by inhibitors targeting other RNA components of the translation machinery.[92] From a cell-based bicistronic reporter screen of ∼132 000 compounds, amino-substituted phenazines **1** (Figure 2) were reported as IRES inhibitors but lacked significant selectivity for the IRES target.[87] A high throughput IVT screen against a library of ∼180 000 compounds identified biarylguanidines **2**, which were moderately active, highly polar translation inhibitors that proved to be difficult to optimize.[88] In what is probably the most elaborate published IVT screening

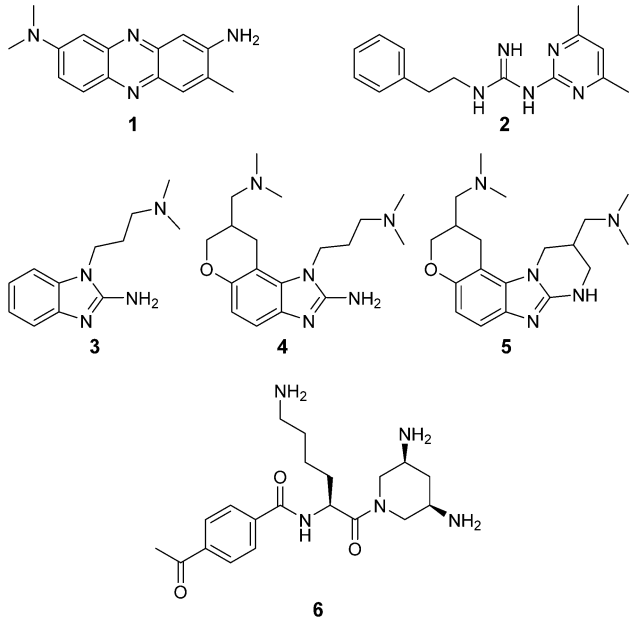

Figure 2. Inhibitors of HCV IRES-driven translation. Phenazines like **1** and biarylguanidines such as **2** were discovered in IRES-translation inhibition screens.[87,88] Benzimidazoles **3**−**5**, which target the IRES subdomain IIa and inhibit HCV replicon, were initially identified by mass spectrometric screening at Isis Pharmaceuticals.[90] The diaminopiperidine **6** also targets the subdomain IIa but inhibits HCV translation through a mechanism that is distinct from that of the benzimidazole ligands.[30] Target binding, translation inhibition, and replicon activity of the inhibitors are summarized in Table 1.

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campaign for IRES inhibitors, interrogation of a library of ~430 000 compounds resulted in ~1700 initial hits that in thorough secondary screening turned out to be luciferase or general translation inhibitors.[92]

Table 1. Target Binding and Biological Activity of IRES Inhibitors

compd	subdomain IIa target affinity (μM)	IRES translation inhibition (μM)	IRES/cap selectivity (fold)	replicon inhibition (μM)	ref
1	na	0.08	10	nd	87
2	na	9.6	2.4	nd	88
3	100[a]/50[b,c]				90[a]
4	0.86[a]/3.4[b]			3.9/2.8	90/101
5	0.72[a]/0.6[b]			5.4/4.0	90/29
6	6.3[b]			75% inhibition at 10 μM	30
23	100[b]	30% inhibition at 100 μM			93

[a] K_D determined in a mass spectrometry assay.[90] [b] EC_{50} determined in a FRET-based assay (Figure 4).[29] [c] Unpublished results of subdomain IIa target affinity determined by a FRET assay[29,93] in the authors' laboratory.

In a seminal contribution to the discovery of HCV translation inhibitors, high throughput screening by ESI/MS of a 29-mer oligonucleotide representing the IRES subdomain IIa led to the identification of **3** (Figure 2), which had a target affinity of 100 μM in the mass spectrometric assay.[90] Medicinal chemistry elaboration of the hit compound, which relied on extensive structure–activity relationship data,[9,90,98] eventually furnished multicyclic derivatives such as **4** and **5** with improved binding to the subdomain IIa fragment at K_D values of 0.86 and 0.72 μM, respectively. Benzimidazoles **4** and **5** were shown to inhibit HCV replicon at EC_{50} values of 4–5 μM without cytotoxic effects up to 100 μM in Huh-7 cells. Binding affinity and replicon inhibition were measured using racemic mixtures of **4** and a mixture of cis and trans diastereomers of **5**. Subsequent testing of **5** in a replicon translation assay established the benzimidazole derivatives as selective inhibitors of IRES-driven viral translation.[29,99]

A second, chemically distinct series of subdomain IIa-binding inhibitors of the HCV replicon was discovered among modular 3,5-diaminopiperidine (DAP) dipeptides such as the lysine derivative **6** (Figure 2).[30] The DAP scaffold had been proposed as a simplified structural mimetic of 2-deoxystreptamine (2-DOS), which is a recurring pharmacophore in RNA-binding natural aminoglycoside antibiotics.[100]

3. IRES SUBDOMAIN IIa TARGET

3.1. Structure, Conservation, and Function of the IRES Subdomain IIa. The HCV IRES is composed of independently folding domains connected by flexible single-stranded regions. This architecture gives rise to an ensemble of conformers for the isolated full-length RNA.[16,102,103] A single defined overall fold has been observed for the IRES element in cryo-EM studies of complexes with the 40S subunit and the 80S ribosome.[20,25] Higher resolution three-dimensional structures have been determined by X-ray crystallography and NMR for individual (sub)domains,[104] including II,[26] IIa,[27,28] IIIabc,[105] IIIb,[106] IIIc, IIId,[107,108] IIIe,[107] and most recently, the central pseudoknot domain IIIe-f.[19] Atomic structures of IRES domains in conjunction with cryo-EM and small-angle X-ray scattering data have been used to construct models of the full-length IRES.[19,103]

NMR analysis first established that the metal-dependent folding of the subdomain IIa internal loop (Figure 3A) is

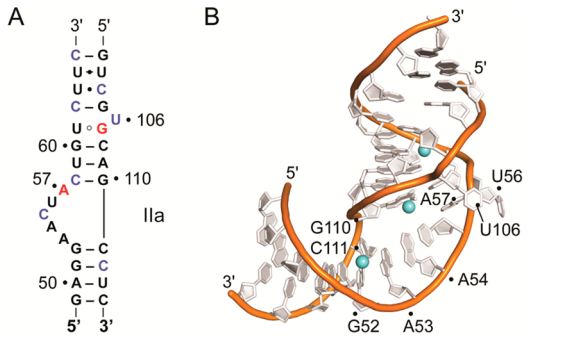

Figure 3. HCV IRES subdomain IIa target. (A) Secondary structure of the subdomain IIa RNA. Conservation of residues in clinical isolates is indicated by font color (black, 100%; blue, >90%; red, <90%). Conservation analysis was performed using 5000 unique clinical isolate sequences identified by BLASTn[109] search against the NCBI nonredundant nucleotide collection database. As the query sequence, we used the HCV genotype 1 Reference Sequence, accession number NC_004102.1. Because of the heterogeneous sequencing coverage within the 5′ UTR of HCV clinical isolates, the internal loop of subdomain IIa (residues 53–57) was present in ~2000 isolates while the 3′ region (residues 105–112) was covered in ~4800 sequences. Conservation percentages were calculated relative to the actual number of sequences available for each position. (B) Crystal structure of the subdomain IIa. The RNA fold is stabilized by three structural magnesium ions (cyan spheres).[27] Image was prepared from PDB coordinate file 2NOK.

responsible for the overall bent shape of domain II[26] which directs the apical hairpin loop IIb toward the ribosomal E-site.[20,25] Crystal structure analysis of subdomain IIa revealed a high resolution picture of the subdomain IIa architecture, which adopts an L-shaped motif that contains three magnesium ions as an intrinsic part of the RNA fold (Figure 3B).[27,28] The nucleotide arrangement in the internal loop, which introduces a 90° bend in the RNA between base pairs G52–C111 and C58–G110, is stabilized by a combination of stacking, hydrogen bonding, and metal ion participation. The bases of A53, A54, and C55 stack continuously on the G52–C111 pair and extend the lower stem into the bend. Connected by the looped out residue U56, the bases of A57 and C58 stack in extension of the upper stem. Coordination with two magnesium ions further reinforces the interfaces of both helical parts with residues of the internal loop. A third structural magnesium site is found locking U106 looped out from the upper stem.

Residues of the subdomain IIa are 99–100% conserved in clinical isolates (Figure 3A) with the exceptions of A57, C104, U106, and G107, which show conservation of 88%, 93%, 98%, and 77%, respectively. For the lowest conserved G107, the exclusively observed exchange is transition to an A (23% of clinical isolates) which retains canonical base pairing with U61. At A57, the impact of the dominating transversion to U (12% of clinical isolates) is not readily explained by the crystal structure. The Hoogsteen edge of the A57 base forms hydrogen bonds to the ribose 2′-OH of C55, which helps to stabilize a

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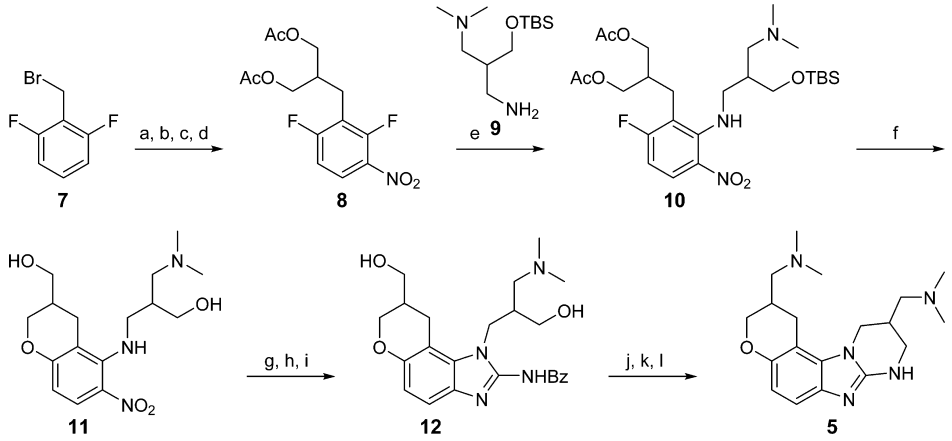
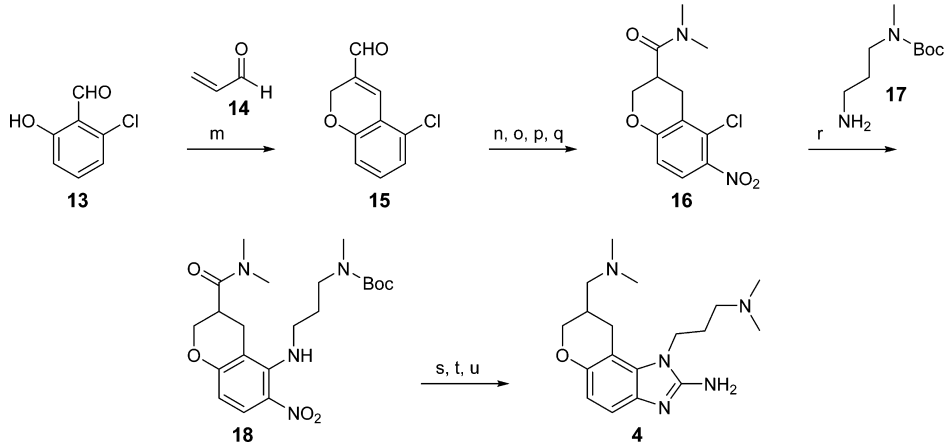
Scheme 1. Original Synthesis of IRES-Binding 5[a,90]



[a]Reagents: (a) NaH, diethyl malonate, THF; (b) $LiAlH_4$, THF; (c) acetyl chloride, DCM, TEA, DMAP; (d) HNO_3; (e) **9**, DCM, $CaCO_3$; (f) DMSO, MeOH, K_2CO_3; (g) Pd/C, H_2, MeOH; (h) BzNCS, DIPEA, DCM; (i) EDC, DCM; (j) HCl, dioxane; (k) mesyl chloride, TEA, DMAP, DCM; (l) Me_2NH/H_2O, DMF.

Scheme 2. Improved Synthesis of IRES-Binding 4[a,111]



[a]Reagents: (m) acrolein **14**, DABCO, CH_3CN; (n) Ag_2O, NaOH, $EtOH/H_2O$; (o) Na/Hg, NaOH, H_2O; (p) EDC, $Me_2NH{\cdot}HCl$, HOBt, *N*-Me-morpholine, DCM; (q) $NaNO_3$, TFA; (r) **17**, NMP; (s) Pd/C, H_2, EtOH; (t) $BrCN/CH_3CN$; (u) $LiAlH_4$, THF.

right-angled kink in the backbone between residues C55 and A57. Attempts to obtain diffracting crystals for the U57 variant subdomain IIa RNA have failed so far.

While the U57 variant is found in clinical isolates in conjunction with other mutations,[110] introduction of this single mutation in genotype 1b reporter replicon led to reduction of IRES-driven translation activity to about 15% of the wild type level.[29] Deletion of the subdomain IIa internal loop (ΔA53–A57) or parts thereof (ΔA53–A55) eliminates the bend in domain II that is responsible for directing subdomain IIb to the E-site. In these deletion mutants translation is abolished through stalled assembly of 80S ribosomes after 48S complex formation and decreased levels of eIF2 release as well as GTP hydrolysis.[22,23] The effect of the ΔIIa mutation is comparable to that observed after deletion of the entire domain II.

3.2. Translation Inhibitors Targeting the IRES Subdomain IIa. Two classes of translation inhibitors targeting the subdomain IIa have been identified, including benzimidazoles and diaminopiperidines (DAP derivatives) (section 2.4 and Figure 2). FRET studies using a fluorescently labeled target model suggested that the benzimidazole inhibitors capture a straightened conformation of the subdomain IIa that compromises IRES function.[29] Binding of the benzimidazole compounds was not affected by the presence of excess competitor RNA or salt, including magnesium, which indicates that IIa target interaction with the ligands is specific and not governed by electrostatic components. X-ray crystallographic analysis of subdomain II with bound **4** revealed a fully extended RNA structure for the complex.[101] Both the FRET and crystallography studies are described in sections below.

The original synthetic route to constrained benzimidazoles such as **5** suffered from a low overall yield (Scheme 1).[90,98] The chroman scaffold in **5** was constructed over six steps. Polar amino and hydroxy groups were carried through the sequence and complicated purification, negatively affecting the yield of the original procedure. An improved synthetic route was aimed at early generation of the chroman scaffold as well as introduction of polar functional groups in protected form to facilitate normal phase purification of all intermediates (Scheme 2).[111] The improved nine-step synthesis furnished **4** in 10% overall yield.

Discovery of DAP derivatives as ligands of the subdomain IIa relied on a fluorescence assay using target RNA in which A54 in the internal loop was replaced by a fluorescent 2-aminopurine

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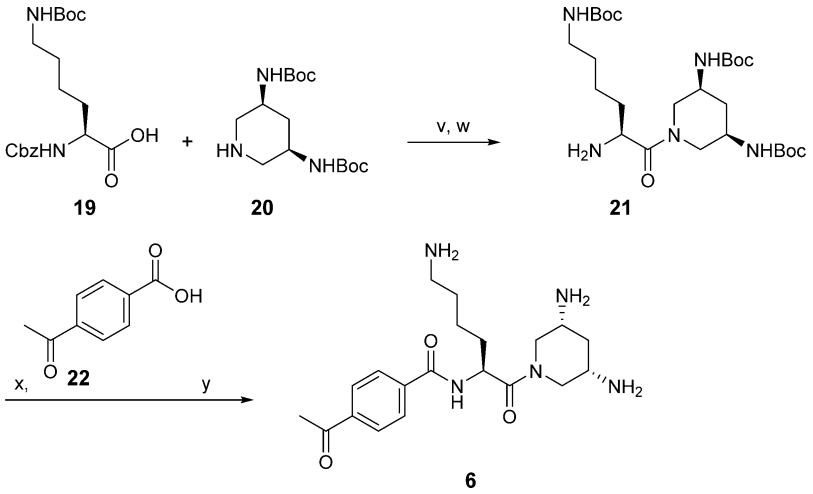
Scheme 3. Synthesis of IRES-Binding 6[a,30]



[a]Reagents: (v) TEA, HATU, HOAT, DCM; (w) Pd/C, H_2, MeOH; (x) **19**, TEA, HATU, HOAT, DCM; (y) HCl/dioxane, MeOH.

(2AP) base analogue.[27,30] Unlike the benzimidazole inhibitors that capture an extended conformation of the subdomain IIa RNA, the DAP ligands seem to stabilize the bent form of the target. Modular RNA-targeting DAP derivatives were readily synthesized by coupling of a protected DAP heterocycle with an α-amino acid followed by a second amide coupling step with a carboxylic acid building block (Scheme 3). Compound **6** and the corresponding arginine derivative bound to the subdomain IIa target with 6–7 μM affinity and inhibited HCV replicon. RNA interaction of the DAP ligands involved a strong electrostatic component as indicated by competition with magnesium binding.

3.3. Subdomain IIa RNA Conformations Captured by Ligand Binding. The bent subdomain IIa plays a key role in positioning the IIb hairpin in the ribosomal E-site. This led us to propose that the subdomain IIa might serve as a target for HCV translation inhibitors that interfere with the conformation of the RNA. To study folding and ligand binding of the subdomain IIa, we developed fluorescence assays that provided insight into the interaction of translation inhibitors with the RNA target. Oligonucleotide models of the subdomain IIa were fluorescently labeled either by replacement of A54 with the base analogue 2AP ("2AP54 construct")[27] or by 5′-terminal attachment of a FRET cyanine dye pair ("FRET construct", Figure 4A).[29] Both constructs were used to monitor magnesium-induced folding of the RNA. In the absence of divalent metal ions, the internal loop of the subdomain IIa is unstructured and flexible, giving rise to a preferentially extended overall shape of the RNA. Magnesium binding induces structural organization of the internal loop, resulting in a dose-dependent increase of FRET signal due to the proximity of the cyanine dyes in the folded L-shaped RNA (Figure 4B).

Fluorescence from the 2AP54 construct, however, decreases upon folding which results in stacking of the fluorescent base analogue between neighboring residues (Figure 3B). Comparative metal ion binding studies, which exploited the differential preference of magnesium versus manganese ions for oxygen over nitrogen coordination, substantiated the role of metal ions observed in the crystal structure as intrinsic constituents of the folded subdomain IIa RNA in solution. The observation that 2AP54 fluorescence is diminished but not fully quenched in the magnesium-stabilized subdomain IIa

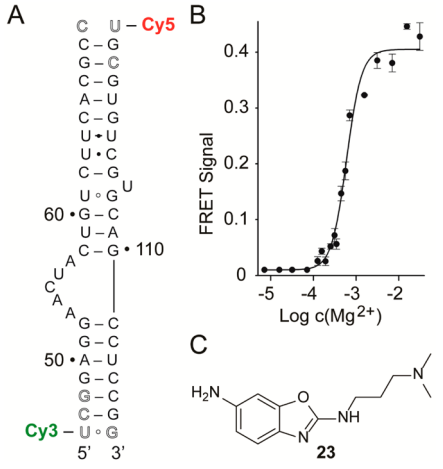


Figure 4. FRET-based assay for the monitoring of conformational changes in the subdomain IIa target during folding and ligand binding.[29,93] (A) Cyanine dye-labeled RNA construct used for FRET measurements. Nucleotides that deviate from the HCV genotype 1b sequence are in outlined font. (B) FRET monitoring of the Mg^{2+} induced folding of the IIa RNA. (C) Benzoxazole derivative **23** identified in a FRET screen for subdomain IIa ligands.[93]

indicates dynamic properties of the internal loop that may allow the RNA motif to explore conformations other than the 90° bend found in the crystal structure.

Titration of Isis benzimidazole translation inhibitors such as **4** and **5** to the folded IIa FRET construct resulted in dose-dependent fluorescence quenching which indicated a conformational change in the RNA that separated the cyanine dye labels beyond the Förster radius. The 2AP54 construct, on the other hand, responded with a signal increase suggesting exposure of the fluorescent label upon ligand binding. We concluded that the benzimidazole translation inhibitors either capture or trigger a straightened conformation of the subdomain IIa and thereby disrupt correct positioning of the IIb hairpin at the ribosome. Support for this hypothesis comes from studies of a subdomain IIa variant carrying the A57U mutation that occurs in HCV clinical isolates. The A57U exchange weakens by 7-fold binding of benzimidazole **5** to the target RNA as measured by

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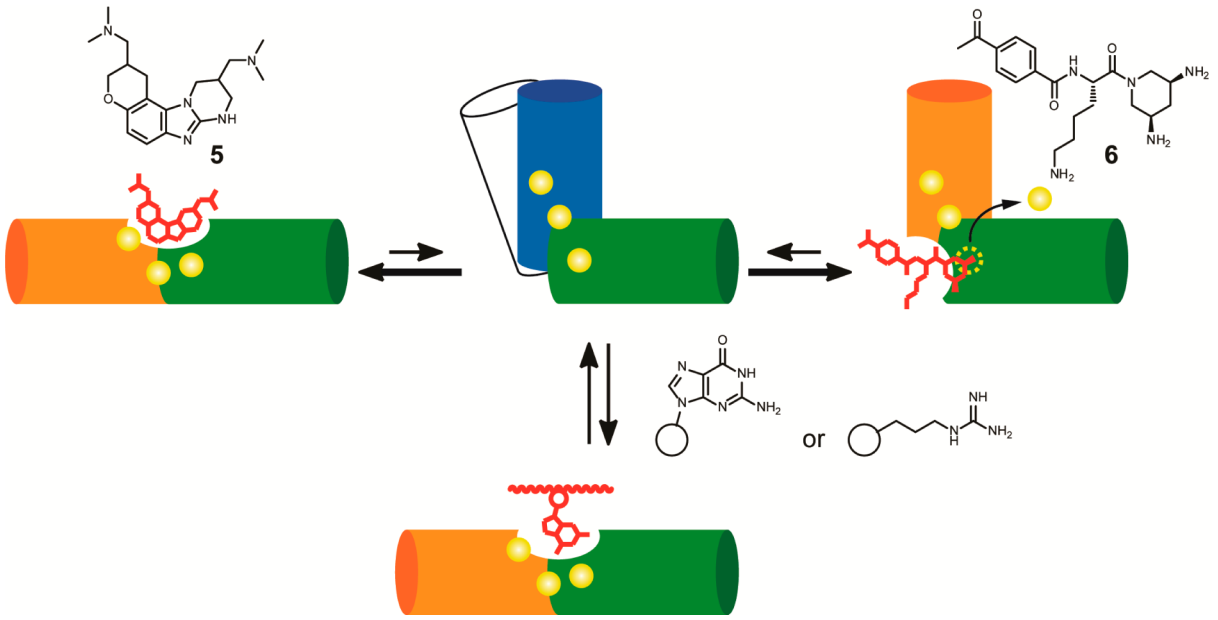

Figure 5. Model for the mode of action of translation inhibitors on the subdomain IIa conformational switch in the HCV IRES. The subdomain IIa RNA adopts a bent fold that is stabilized by magnesium ions and is required for the correct positioning of the IRES on the ribosome. Release of the ribosome after translation initiation might involve dynamical changes in the L-shaped RNA structure, which might be captured by a cognate ligand such as an arginine of a cellular protein or a guanosine in an RNA (center). Benzimidazole IRES inhibitors such as **5** (left) capture an extended state of the IIa RNA switch which leads to disruption of IRES function and inhibition of viral protein synthesis.[29] The diaminopiperidine derivative **6** (right) binds in competition with structural Mg^{2+} ions and locks the IIa RNA switch in the bent conformation, which inhibits IRES function perhaps by preventing ribosome release.[30]

FRET and, as a consequence, reduces the inhibitory effect of **5** on translation in HCV replicon systems.[29]

Binding of **6** to the subdomain IIa was originally discovered using the 2AP54 target construct.[30] Residual fluorescence of the 2AP label, which likely originates from the dynamic conformation of the internal loop, is quenched upon binding of **6**. These data in conjunction with the observed competition of DAP ligands with magnesium ions led to the proposal that compounds such as **6** bind to the subdomain IIa target by displacing structural metals and rigidifying the dynamic internal loop RNA.

Concluding from the fluorescence studies of conformational dynamics and impact of ligand binding, a model was proposed for the subdomain IIa target as a conformational switch that seeks to integrate the role of RNA flexibility for IRES function as well as the mode of action of HCV translation inhibitors (Figure 5).[30] The notion of IRES-targeting inhibitors as allosteric effectors of RNA conformation bears resemblance to the mechanism observed for aminoglycoside antibiotics that target a conformational switch in the decoding site RNA of the bacterial ribosome.

3.4. Screening for Translation Inhibitors Targeting the Subdomain IIa. Previously, affinity screening of IRES fragment targets by mass spectrometry had been successful in the discovery of benzimidazole HCV translation inhibitors (section 2.4 and Figure 2).[90] Target selectivity of the ligands was tested by differential screening of subdomain IIa RNA in the presence of an unspecified competitor RNA of similar size and, presumably, structural complexity. Detection of selectivity for a specific RNA such as an HCV IRES domain over other cellular nucleic acids may be achieved by monitoring a ligand-induced capture or triggering of a unique event in the target. The paradigm for such a conformational RNA target is the ribosomal decoding site in which a change in the orientation of two adenine residues is triggered by the binding of aminoglycoside antibiotics.[112] Outside the ribosome, such conformational RNA targets for small molecule ligands had been largely elusive.

The large conformational change exhibited by the subdomain IIa RNA provides an ideal mechanism for the establishment of a target-selective combined binding and functional screen. The FRET assay outlined in the previous section was readily adapted to a high-throughput screening format in which ligands were identified based on their ability to reduce the FRET signal by induction of a widened interhelical angle in the cyanine dye labeled subdomain IIa target.[93] As a control for nonspecific fluorescence quenching, emission from the Cy3 donor was recorded which for true ligands increased concurrently with FRET quenching because of the diminishing resonance energy transfer efficiency at growing distances of the cyanine dyes. Feasibility of the assay format was demonstrated by screening a small set of molecules biased for binding to RNA targets from which **23** (Figure 4C) was identified as a selective ligand for the subdomain IIa RNA. The benzoxazole **23** quenched FRET with an EC_{50} value of ~100 μM, comparable to the affinity of the hit **1**, and selectively inhibited IRES-initiated translation by 30% at 100 μM while not affecting the cap-dependent process. The structural similarity of **23** compared to previously discovered benzimidazoles such as **1** readily explains the activity of the benzoxazole as a ligand of the subdomain IIa target and an IRES inhibitor. The lower basicity as well as reduced hydrophilicity of the benzoxazole scaffold compared to benzimidazoles may lend superior druglike properties to subdomain IIa binding translation inhibitors emerging from future optimization of the hit **23**.

3.5. Structure of a Subdomain IIa Translation Inhibitor Complex. Structural investigations of the subdomain IIa RNA in complex with benzimidazole translation

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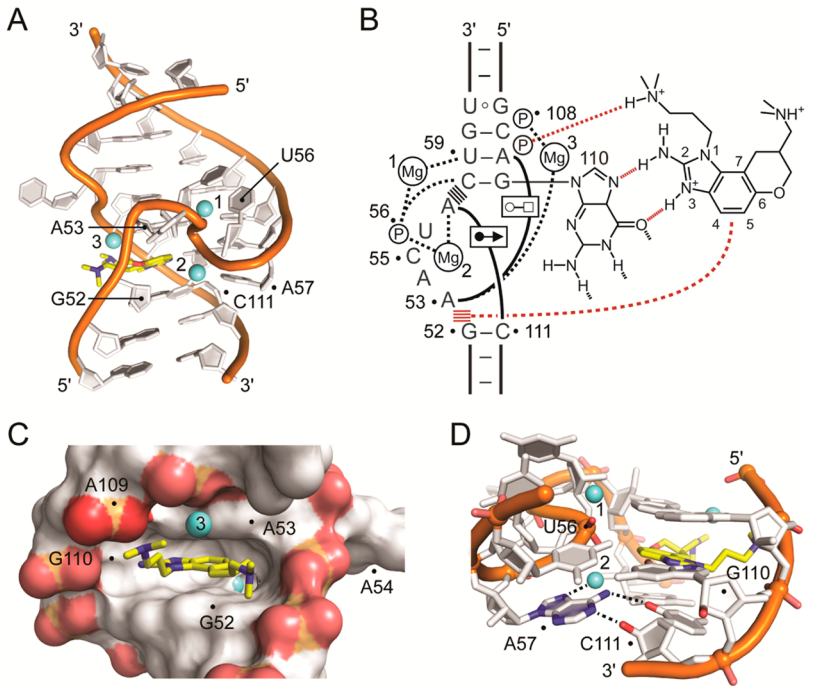

Figure 6. Crystal structure of the subdomain IIa target in complex with **4**, adapted from ref 101. (A) Overall view of the binding site. The ligand **4** is shown in yellow sticks. Mg^{2+} ions are indicated by light blue spheres. (B) Interactions in the ligand binding site. Hydrogen bonds are shown as dashed lines. Stacked lines (≡) indicate stacking of bases and intercalation of the ligand. Formation of non-Watson−Crick base pairs is indicated with solid lines and symbols according to Leontis and Westhof. (C) Surface representation of the binding site in a front side view (rotated by 90° from panel A), highlighting the deep ligand pocket. (D) Backside view of the complex showing hydrogen bonding interactions of the A57 base whose exchange to a uridine weakens binding of the benzimidazole translation inhibitors. Images were prepared from PDB coordinate file 3TZR.

inhibitors have been performed by NMR and X-ray crystallography. An NMR study of the target bound with a racemic benzimidazole derivative related to **4** found an overall extended conformation of the RNA ligand complex, supporting the FRET experiments.[31] However, the NMR structure did not reveal details of the binding interaction. An X-ray crystallographic analysis revealed at 2.2 Å resolution the structure of the target IIa RNA bound with **4** (Figure 6).[101] In the complex the RNA adopts an extended architecture with the internal loop refolded from its curved conformation in the free target and flanked by coaxially stacking helices (Figure 6A). A deep cavity is formed that encapsulates the ligand that docks by hydrogen-bonding to the guanine heterocycle in the C58−G110 base pair and stacking interactions with A53 as well as the G52−C111 pair (Figure 6B,C). An additional intramolecular hydrogen bond occurs between the protonated dimethylaminopropyl side chain of the benzimidazole inhibitor and an RNA phosphate group. Formation of the binding pocket upon adaptive ligand recognition further involves RNA base triples that engage in cross-bracing interactions along the RNA helix (Figure 6B). The participation of magnesium ions in the stabilization of the subdomain IIa target is maintained for the RNA ligand complex. The free RNA as well as the complex each contains three magnesium ions as intrinsic structural components that undergo adaptive reorganization upon ligand binding.

While cocrystallization of the subdomain IIa RNA was performed with a racemate of **4**, the complex structure did not unambiguously reveal the ligand stereochemistry. However, X-ray crystallographic analysis of racemic **4** by itself showed that the structures of the enantiomers are sufficiently similar to allow for RNA target binding of either form through the observed hydrogen bonding and stacking interactions.[113] Titrations of pure enantiomers of a benzimidazole related to **4**, in which the tetrahydropyran ring was replaced by a tetrahydrofuran, with a 2AP fluorescently labeled target RNA[27] suggested that one isomer bound about 3.5-fold tighter than the other.[31] However, the absolute configuration of the tighter binding enantiomer was not determined. Structure−activity relationships for related benzimidazole derivatives of **4** were conclusively explained by the crystal structure.[101] For example, benzimidazoles that lack the amino substituent at the 2-position or the *N*,*N*-dimethylaminopropyl chain off the N1, both of which participate in hydrogen bonds with the target, did not bind to the subdomain IIa RNA. Improved binding was observed for derivatives carrying modifications at the 6-position with basic substituents producing the best activities and with larger nonpolar groups adding no benefit. Substitution at the 4- and 5-position of the benzimidazole was not tolerated because of the tight fit of the ligand into the substrate binding site, and derivatives with such modifications were inactive.

The U57 variant, which conferred HCV replicon resistance to the related translation inhibitor **5**, displayed weakened binding for **4** as well, which had a ∼3-fold lower affinity for the variant target. While the residue at position 57 is not involved in direct contacts with the bound ligand, A57 which occurs in 88% of HCV clinical isolates plays an important role in stabilizing the back side of the benzimidazole pocket in the complex by forming hydrogen bonds with C111 and coordinating a key structural magnesium ion (Figure 6D). We speculate that transversion to U57 increases the flexibility of the subdomain IIa internal loop and thereby interferes with both the ability of the IRES to initiate translation and benzimidazole inhibitor binding. The position 57, which is one of the less conserved residues in the HCV IRES, may very

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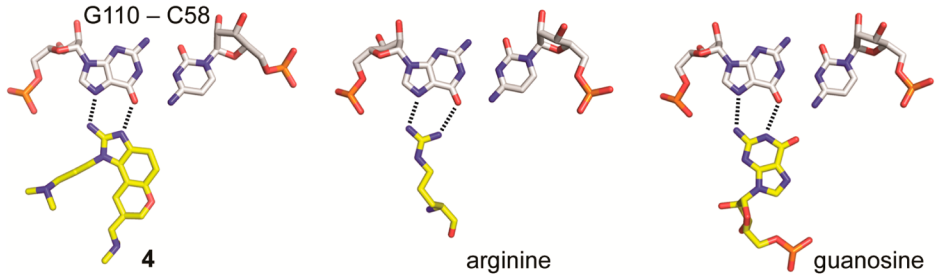

Figure 7. Examples of Hoogsteen edge recognition of G-C pairs by arginine or guanosine compared to the G110−C58 interaction with **4** in the subdomain IIa complex. The image of the benzimidazole complex was prepared from PDB coordinate file 3TZR.

well represent the Achilles' heel of the subdomain IIa target. Further structural studies on the U57 variant RNA are required to devise ligand design strategies for inhibitors that retain activity in this mutant.

Key hydrogen bonds of the subdomain IIa benzimidazole complex interaction are solvent-excluded inside the binding pocket, which together with the stacking interactions lends a strong hydrophobic component to ligand recognition. Both the depth and hydrophobicity of the ligand pocket in the subdomain IIa are unusual features for an RNA target which render the benzimidazole docking site more similar to inhibitor binding pockets in protein targets. Hence, development of druglike HCV translation inhibitors directed at the subdomain IIa RNA seems a feasible endeavor, supported in particular by guidance from the high resolution structure for the RNA target complex.

4. OUTLOOK

Approaches to inhibit HCV translation by targeting the highly conserved IRES RNA have resulted in a diverse set of ligands including oligonucleotdies, peptides, and small molecules that block IRES function by distinct mechanisms. Currently, the subdomain IIa is the only IRES target for which a binding site for small molecule translation inhibitors has been identified. From investigations of the mechanism of inhibitors targeting the subdomain IIa the picture is emerging that this RNA domain functions as a conformational switch whose state may be locked by ligand binding. Structural studies by electron microscopy of IRES−ribosome complexes have led to the conclusion that after assembly of 80S complexes the bent topology of the domain II would prevent the progression of the ribosome from initiation to elongation.[20,25] Domain II has to be moved out of the E site to make room for deacylated tRNA during the transition to productive translation. On the basis of findings of ligand-dependent conformational switching in the highly conserved subdomain IIa, it was proposed that a conformational change triggered by adaptive recognition of a cognate ligand may facilitate the release of the ribosome from the IRES-bound complex (Figure 5).[101] Benzimidazole translation inhibitors may be fortuitous ligands of the subdomain IIa conformational switch perhaps due to their ability to mimic interactions of arginine or guanosine at the Hoogsteen edge of the G110−C58 base pair while stacking between neighboring residues G52 and A53 (Figure 7). Arginine side chains have been found in isostructural interaction with G−C pairs in numerous peptide and protein complexes of both RNA and DNA.[114−117] Guanosine docking at the Hoogsteen edge of G in a Watson−Crick G−C pair accounts for one of the most common base triples found in RNA architectures.[118−121]

While the high resolution crystal structure of the subdomain IIa benzimidazole complex points the way for structure-guided discovery of HCV translation inhibitors, the hunt for potential cognate triggers of the RNA switch has just begun. At least two proteins, including ribosomal protein S5 (rpS5) and heterogeneous ribonucleoprotein D (hnRNP D), have been identified by UV cross-linking and immunoprecipitation as direct binding partners of the HCV domain II RNA.[24,122] It remains to be tested, however, whether these candidate proteins, or any other yet to be discovered factors, interact at the subdomain IIa or affect the conformation of the RNA switch. Alternatively, it is conceivable that either the viral mRNA itself or rRNA may trigger release of the IRES by insertion of a G residue into the subdomain IIa switch. Recently, direct interactions between the IRES domain II and 18S rRNA have been confirmed by chemical probing. It could also be envisioned that the viral mRNA emerging from the ribosome upon translation may provide a guanosine residue for participation in a base triple interaction at the ligand binding pocket of the subdomain IIa switch.

The discovery of the HCV IRES subdomain IIa as a conformational switch that is a target for small molecule translation inhibitors paves the way for the development of drugs directed at the viral RNA. The high conservation of the subdomain IIa in clinical isolates promises that mutations around the ligand binding pocket will be difficult to reconcile with IRES function. Inhibitors directed at this RNA target will potentially benefit from selection of low-fitness resistance mutants with reduced frequency of occurrence. The availability of a target specific functional binding assay combined with high resolution structural data for the free RNA and an inhibitor complex will provide tools for overcoming the Achilles' heel of the subdomain IIa in the U57 target variant and enable the discovery of druglike HCV translation inhibitors.

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Thomas Hermann earned a Chemistry Diploma from the University of Ulm, Germany. He performed thesis research at the Max-Planck Institute for Biochemistry and received a Ph.D. from the Ludwig-Maximilians University in Munich, Germany, in 1996. As a postdoc, he worked with Prof. Eric Westhof at the CNRS in Strasbourg, France, and with Prof. Dinshaw Patel at the Sloan-Kettering Cancer Center in New York. Subsequently, he joined Anadys Pharmaceuticals in San Diego, CA, in 2001, where he built the structural chemistry group and led discovery teams working on ribosome-targeted antibiotics and HCV polymerase inhibitors, laying the foundation for the development of setrobuvir. In 2005, he joined University of California, San Diego, where he currently is an Associate Professor and serves as a founding director of the Center for Drug Discovery Innovation.

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■ ABBREVIATIONS USED

2AP, 2-aminopurine; DABCO, 1,4-diazabicylclo[2.2.2]octane; DAP, diaminopiperidine; DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethylamine; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; 2-DOS, 2-deoxystreptamine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; eIF3, eukaryotic initiation factor 3; FRET, Förster resonance energy transfer; GTP, guanosine triphosphate; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HCV, hepatitis C virus; HOAT, 1-hydroxy-7-azabenzotriazole; HOBt, hydroxybenzotriazole; IRES, internal ribosome entry site; mRNA, messenger RNA; NMP, *N*-methylpyrrolidone; rRNA, ribosomal RNA; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; UTR, untranslated region

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