

REVIEW



Unmasking the information encoded as structural motifs of viral RNA genomes: a potential antiviral target

Cristina Romero-López* and Alfredo Berzal-Herranz*

Instituto de Parasitología y Biomedicina 'López-Neyra', IPBLN-CSIC, PTS Granada, Armilla, Granada Spain

SUMMARY

RNA viruses show enormous capacity to evolve and adapt to new cellular and molecular contexts, a consequence of mutations arising from errors made by viral RNA-dependent RNA polymerase during replication. Sequence variation must occur, however, without compromising functions essential for the completion of the viral cycle. RNA viruses are safeguarded in this respect by their genome carrying conserved information that does not code only for proteins but also for the formation of structurally conserved RNA domains that directly perform these critical functions. Functional RNA domains can interact with other regions of the viral genome and/or proteins to direct viral translation, replication and encapsidation. They are therefore potential targets for novel therapeutic strategies. This review summarises our knowledge of the functional RNA domains of human RNA viruses and examines the achievements made in the design of antiviral compounds that interfere with their folding and therefore their function. Copyright © 2013 John Wiley & Sons, Ltd.

Received: 14 March 2013; Revised: 23 July 2013; Accepted: 24 July 2013

INTRODUCTION

The appearance of viral mutants resistant to antiviral compounds is a major public health concern. The continuous generation of such mutants is a characteristic of RNA viruses, many of which, for example, hepatitis C virus (HCV), human immunodeficiency virus (HIV) and DENV (all with a single-stranded, positive RNA genome), cause serious diseases. The genome of RNA viruses acts both as a template for replication and as an mRNA. During replication, mutations are introduced into

the genome via errors in the action of viral RNA-dependent RNA polymerase; this can generate mutants that may greatly contribute to viral proliferation—although it can also lead to the disappearance of the virus if such changes leave it non-viable or induce acute pathogenesis in the host, the so-called error catastrophe (for reviews, see [1,2]). Viruses must, therefore, strive to reach an equilibrium that allows for adaptive fitness without compromising their persistence. To accomplish this, RNA viruses have developed an information storage system that does not code for proteins, but for specific, complex and highly conserved, folded genomic regions that play direct, functional roles in the viral cycle. These viral regions present an adaptive behaviour that has evolved with the selective pressure to achieve high genetic robustness [3–5]. As a result, mutations in viral RNA genomes present minor incidence in the functional elements [4].

RNA molecules show two levels of compaction: their secondary and their tertiary structures. The secondary structure is the result of double-stranded and single-stranded regions arrangements, for example, helices closed by loops. The tertiary structure is determined by the relationships established between secondary structure elements.

*Corresponding authors: C. Romero-López and A. Berzal-Herranz, Instituto de Parasitología y Biomedicina 'López-Neyra', IPBLN-CSIC, Parque Tecnológico de Ciencias de la Salud, Av. del Conocimiento s/n, Armilla, 18016 Granada, Spain.
E-mail: cristina_romero@ipb.csic.es; Aberzalh@ipb.csic.es

Abbreviations used

CRE, cis-acting replicating element; DENV, dengue virus; EMCV, encephalomyocarditis virus; FMDV, foot and mouth disease virus; FRD, functional RNA domain; IF, initiation factor; HV, highly variable region; HVR, hypervariable region; IGR, intergenic region; IRES, internal ribosome entry site; ITAF, initiator *trans*-acting factor; JEV, Japanese encephalitis virus; PABP, polyA binding protein; PCBP, poly-rC binding protein; PK, pseudoknot; PTB, polypyrimidine tract binding protein; SELEX, systematic evolution of ligands by exponential enrichment; sp., species; UTR, untranslated region; YFV, yellow fever virus.

The combination of these two folding levels establishes the final shape of the RNA molecules, the so-called RNA structurome [6]. This conformational view has prompted the search of structured RNA motifs as FRDs, not only in the viral genomes [7] but also in the transcriptome of all living organisms (for a review, see [6]).

A main feature of FRDs included in viral genomes is their high genetic robustness, likely derived from their essentiality for the consecution of the infective cycle [3–5]. FRDs are *cis*-acting elements typically involved in RNA–RNA and RNA–protein interactions, required for the correct execution and regulation of essential viral processes [8–10]. One of the FRDs that have attracted the most attention is the IRES. IRES regions direct the recruitment of the cellular translation machinery in a cap-independent manner [11]. Similarly, viral replication and maturation are regulated by the direct binding of viral polymerases and related cofactors to FRDs in the 3' end of the genomic RNA, as well as by direct RNA–RNA interactions between distant regions of the virus genome [12].

The functional importance of structural elements in the viral cycle and their high conservation rate [8,9,13–15] suggest that they might make good therapeutic targets, and the idea of developing novel antiviral tools (mostly RNA based) to attack them has gained currency in recent years. This review briefly summarises what is known about the FRDs of a number of viruses, paying special attention to those infecting humans, and recapitulates the state of the art in the development of therapeutic compounds that might interfere with FRD folding and, therefore, function.

VIRAL IRESs: RNA TAGS FOR EFFICIENT TRANSLATION

Many viral genomes achieve efficient translation initiation by the use of IRESs, which replace IFs by the combined use of several FRDs. These regions present high sequence and structural diversity across different viral groups, which define the mechanisms by which they promote translation. IRESs are therefore divided into four groups [16] according to common IF requirements (Figure 1):

- *Packed IRESs*. This group encompasses the IRES elements located in the IGR of the genome of the *Dicistroviridae* family (whose members infect

- insects). The folding of these elements represents a highly conserved, compact structure that, via the so-called all-RNA-based mechanism, mediates the recruitment of the ribosome with no need of additional protein factors [17]. The IRES structure can be split into three domains (Figure 1 (a)), each defined by a PK motif (PKI–PKIII). This conformation is considered the 'prefolded' isoform of the IRES. Domain PKIII mimics the folding of the initiator transfer RNA (tRNA), allowing it to occupy the P and E sites of the 40S ribosomal subunit. This promotes structural changes in the IRES that finally allow the binding of the 60S particle and the efficient initiation of translation from a non-AUG codon [17–22]. Thus, the conformation of PKIII replaces the need for an initiator tRNA [23].

- *Extended IRES with compact regions*. The prototype of this group, the HCV IRES, folds as an extended region with tightly compact domains. It is mostly contained in the 5'UTR of the RNA genome but also spans a short stretch of the coding sequence (Figure 1(b)) [24,25]. It can be divided into two major domains (II and III) [26] and a short stem-loop containing the start codon (domain IV) [27]. The extended folding of the IRES allows for the proper coaxial alignment of domains II and III on either side of a complex PK structure [28]. This allows easy access for the 40S ribosomal particle; subsequent IRES conformational changes directly position the appropriate start codon in the P site [29]. Then, binding of eIF3 at the IIIabc junction aids the incorporation of the ternary complex eIF2-tRNA_i^{Met} and the joining of the 60S subunit (for a review, see [30]). Interestingly, the requirement for eIF2-tRNA_i^{Met} can be minimised under certain stress conditions [31], providing a selective advantage for efficient HCV propagation even in unfavourable physiological environments. This suggests that an IRES may change its mode of function depending on environmental conditions.

- *Flexible IRESs*. These are mainly represented by picornavirus type II IRESs (FMDV and EMCV among others; Figure 1(c)) [9]. Their overall 3D structure reflects a greater degree of flexibility than that shown by IGR or HCV-like IRESs before ribosomal recruitment [32–35]. Nonetheless, they retain compact self-folded stem-loops and a GNRA sequence motif included within a tetraloop that directs the structural organisation of central domain 3 [32]. Flexible IRESs cannot

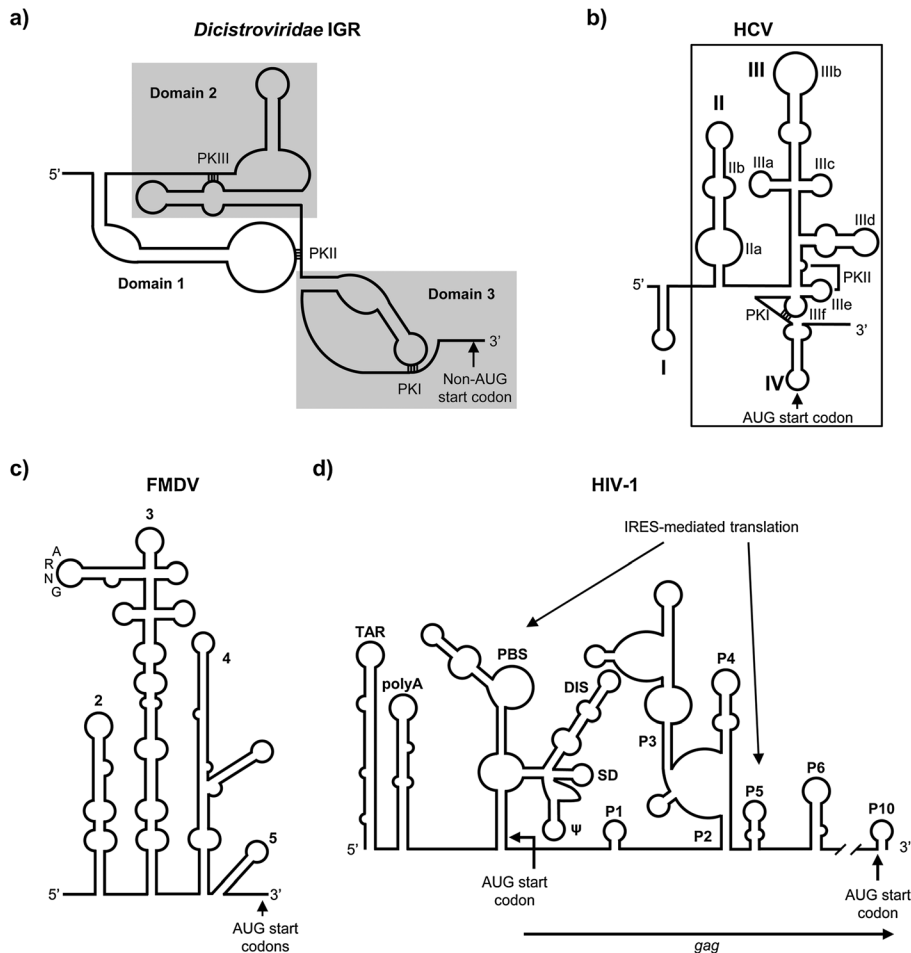


Figure 1. Diagram showing the secondary structure of different types of viral internal ribosome entry sites (IRESs). (a) *Dicistroviridae* intergenic region (IGR) IRES. (b) Hepatitis C virus (HCV), with the minimum IRES region boxed. (c) Foot and mouth disease virus (FMDV) IRES. (d) HIV-1 IRES elements. Functional domains are shadowed or indicated as noted. Start translation codons are marked by arrows. PK, pseudoknots

directly recruit the 40S ribosomal subunit; rather, they first bind eIF4G, eIF4A, eIF4B and eIF3 at domains 4 and 5 [36,37] to finally induce conformational rearrangements that promote the recruitment of the 43S complex. The presence of domains 2 and 3 is crucial because they bind additional ITAFs, such as the PTB, PABP and ITAF45 [38–41]. ITAFs are thought to provide chaperone activity but may also help overcome cell restrictions under stress conditions. Hence, the flexible folding provides specific biophysical properties that define a characteristic mode of action.

– *Inducible IRES*. In lentiviruses, the use of IRES elements is restricted to certain environmental conditions, the initiation of protein synthesis normally being accomplished by a cap-dependent

mechanism. Two different IRES elements lie within the HIV genome: one in the 5'UTR, which only operates in the G2/M phase of the cell cycle [42,43], and one embedded within the coding sequence (Figure 1(d)) [42,44]. Enormous efforts have gone into fathoming the induction mechanism(s) of these elements, but without much success. It is well known that the 5'UTR of HIV-1 RNA can adopt two different conformations, but a direct translational regulation mediated by conformational exchanges in the genomic RNA has been ruled out [45]. Furthermore, it seems likely that unknown ITAFs are differentially loaded by each conformer, providing a sophisticated and opportunistic viral strategy aimed at overcoming environmental difficulties.

It is tempting to propose that the induction of the IRES within the coding sequence is further modulated by long-distance RNA–RNA interactions with the 5'UTR, thus promoting alternative conformations that might regulate its functioning [46].

FUNCTIONAL DOMAINS LOCATED AT THE 3'UTR

FRDs are needed in the 3' end of viral genomes to promote the efficient binding of the polymerase and the regulation of the elongation step during RNA synthesis [7,12]. They are also important

partners in the preservation of viral genomic stability and the control of processes mediated by the 5' end [47]. The latter is mediated by the establishment of a dynamic crosstalk between both termini of the genomic RNA. The sequence and 3D structure of the 3' *cis*-acting domains are quite diverse, even among members of the same taxon (Figure 2), which must entail substantial differences in the mechanism of action if the same function is to be achieved. Such great complexity and variability preclude a detailed discussion of all the 3' RNA structures present in different RNA viruses; only those most extensively studied are therefore mentioned in the following.

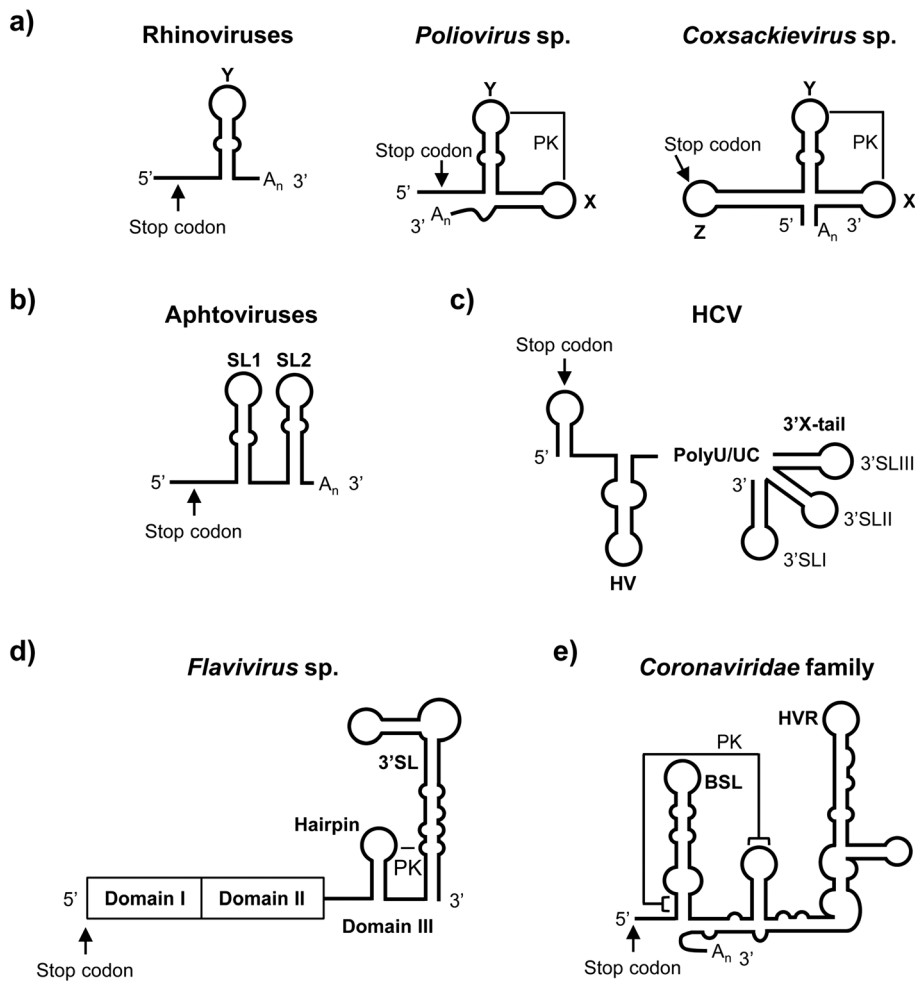


Figure 2. Diagram showing the structural diversity of the 3' untranslated regions (UTRs) of different viruses. (a) Several examples of 3' UTRs of enterovirus, (b) aphthoviruses, (c) hepatitis C virus (HCV), (d) *Flavivirus* sp. and (e) the *Coronaviridae* family. Proposed pseudoknot (PK) structures are indicated with a line. Structural motifs defining functional RNA elements are noted. Stop codon is marked by an arrow. BSL, bulged stem-loop; HV, highly variable region; HVR, hypervariable region; N, any nucleotide; R, purine

3'UTRs in the *Picornaviridae*

– *Enterovirus* sp.:

The genus *Enterovirus* includes human polioviruses, rhinoviruses and the human enteroviruses of groups B, C and D (which include different human coxsackieviruses). Their genomic RNA ends with the so-called *oriR* region, which is composed of a short, highly folded 3'UTR (75–100 nucleotides) capped by a polyA tail [48,49]. The *oriR* has been shown to play an important role in viral replication via its direct recruitment of viral RNA-dependent RNA polymerase and host cell factors such as PCBP, the La autoantigen and eEF1A [15]. A common architecture at the 3'UTR can be inferred for all the members of the same viral group (Figure 2(a)). Thus, rhinoviruses have a single stem-loop (named Y), polioviruses contain two stem-loops (X and Y) and coxsackieviruses have three consecutive stem-loops (X, Y and Z) [48]. The presence of two or more stem-loops defines a typical PK motif involving the apical loops of domains X and Y [50,51]. This PK may regulate dynamic spatial rearrangements in domains X and Y that fine-tune viral RNA synthesis [52,53]. Despite sequence diversity, enterovirus 3'UTR represents a good example of how RNA genome structure has diversified yet retains common essential functions.

– Aphthoviruses:

FMDV is the prototype of the aphthovirus group. Its genome contains an essential *oriR* region at the 3' end consisting of a well-defined 3'UTR of around 90 nucleotides, plus a genetically encoded polyA tract (Figure 2(b)). The 3'UTR adopts a quasi-globular conformation defined by two stem-loops, SL1 and SL2. It was recently shown that, whereas the deletion of SL2 completely interferes with virus viability, mutants defective for SL1 only show reduced replication capacity [54]. The *oriR* region in aphthoviruses is also involved in translational control by the establishment of molecular bridges with the IRES element at the 5' end of the viral genome [55].

cis-Acting elements within the 3' end of genomic *Flaviviridae* RNA

– *Hepacivirus* sp.:

The 3'UTR of the HCV genome is an evolutionarily conserved structural element of around 200

nucleotides with three different domains (Figure 2(c)): the HV, a polyU/UC tract of variable length and composition and the 3'X tail consisting of three stem-loops (3'SLI–3'SLIII) [56,57]. Both the polyU/UC tract and the 3'X tail contain elements important in replication and infectivity in cell culture and a chimpanzee model [58–64]. The 3'UTR in hepaciviruses operates by the recruitment of viral proteins, such as RNA-dependent RNA polymerase and helicase [65], as well as numerous host factors [66–70]. Moreover, the formation of high-order structures involving RNA motifs in the 3' end of the coding sequence provides additional regulation of 3'UTR folding and activity [71,72]. Together, the aforementioned elements constitute the complete functional scaffold required to achieve precise RNA replication.

– *Flavivirus* sp.:

This genus includes important human pathogens, such as DENV, YFV and JEV. Their positive RNA genome contains a 3'UTR ranging from 350 to 700 nucleotides in length. Despite this heterogeneity in size, common secondary structure elements are found at the very 3' end of the genomic RNA, defining the so-called domain III, which is indispensable for replication [73] (Figure 2(d)). Domain III is composed of a short hairpin followed by a long and stable stem-loop (3'SL). It has been shown that the formation of a PK motif involving the apical loop of the short hairpin and an internal loop of the 3'SL is critical for the recognition of viral and cellular proteins during the initiation of RNA synthesis [74].

3' terminal domains in coronaviruses

The genome of the *Coronaviridae* family encodes for five to seven overlapping, capped and polyadenylated subgenomic mRNAs [75]. The 3'UTR contains two highly conserved structural motifs: an essential bulged stem-loop (BSL) immediately followed by a hairpin (Figure 2(e)) [76,77]. Under certain conditions, this folding may be displaced towards the formation of a PK motif between the base of the first domain and the apical loop of the hairpin [78]. This leads to a conformational equilibrium that must be preserved for RNA replication to occur. At the 3' position of the PK, an HVR is found. It is highly divergent in terms of both primary and secondary structures, with the exception of the 5'-GGAAGAGG-3' motif. This octanucleotide is situated 70–80 nucleotides from the 3' end of all

coronavirus genomes, thus being considered a characteristic signature of these pathogenic agents [75]. Such stringent conservation implies a critical yet unknown function in the coronavirus biology [79].

There is a long list of viral and cellular proteins that also bind the 3'UTR of different coronavirus and affect replication [75]. These include viral RNA polymerase, non-structural virus proteins, nuclear factors and proteins involved in the translation process. Thus, once again, RNA–RNA and RNA–protein interactions provide a complex regulatory network that controls viral infection.

CIS-ACTING RNA ELEMENTS WITHIN THE CODING SEQUENCE

Recent work has shown a great variety of *cis*-acting RNA motifs embedded throughout the coding sequence [80–86]. These operate in a coordinated fashion with other functional regions and with viral and host proteins, providing complex control systems for replication.

Some of the best-characterised CREs are found in the picornavirus genome [87–89]. All of them contain one or more short hairpins responsible for efficient priming during RNA synthesis [90]. Two adenine residues placed in an apical loop of one of these hairpins function as a template for the uridylylation of the essential viral protein VPg, involved in picornaviral replication [90]. This mechanism has been shown to contribute to the overcoming of the rate-limiting effects of low nucleotide concentrations [91], thus providing a robust system for the initiation of replication.

A CRE was identified within the 3' end of the coding sequence of the HCV genome [81]. It is a highly conserved structure composed of three stem-loops (5BSL3.1–5BSL3.3) (Figure 3(a)) [92], which were shown to be required for viral replication [64,93]. The establishment of long-range RNA–RNA interactions between 5BSL3.2 and the 3'SLII within the 3'UTR [71,94–96], and with the SL9110 hairpin motif in the coding sequence [72,95,96], is critical for the proper functioning of this region during viral RNA synthesis (Figure 3(a)) [71,72]. Importantly, the existence of an additional, direct, long-range RNA–RNA interaction involving the subdomain III_d of the IRES and the essential stem-loop 5BSL3.2 of the CRE region has important consequences for the regulation of the HCV IRES function (Figure 3(a)) [96–98].

5'–3' COMMUNICATION IN VIRAL GENOMES

End-to-end communication in viral genomes is directly responsible for promoting and regulating different steps of the infectious cycle. Via the establishment of long-distance contacts, differentially evolved functional regions at both ends of viral genomes can act in a coordinated fashion to generate molecular switch control systems between different steps of the viral cycle. Such crosstalk seems to provide important benefits, such as an increase in the local concentration of essential proteins and cofactors and additional protection against the action of exonucleases. Genome circularisation provides new proof of the great versatility shown by compact viral RNA genomes.

The formation of the circular topology in picornaviruses is dependent on direct RNA contacts between the IRES and the 3'UTR (Figure 3(b)) [55]. Furthermore, the RNA binding capacity of different factors, such as eIF4G to the IRES region or PABP to the 3' polyA tail, ensures the stability of the closed-loop conformation.

In hepacivirus genomes, circularisation was initially proposed to be mediated by protein factors alone [47], which would simultaneously bind to both ends of the viral genome. More recently, a long-range contact between the IRES and the CRE was described to induce significant conformational changes in precise domains of the IRES (Figure 3(a)) [97,99], resulting in the regulation of the IRES function [98]. Undoubtedly, these results do not exclude the participation of viral and cellular proteins in the stabilisation of circular topology, nor the performance of important functional roles [100–103].

In other viruses, such as *Flavivirus* sp., RNA genome circularisation does not require the presence of proteins but the use of canonical and complementary sequences (cyclisation sequence, upstream AUG region and downstream AUG region) at either end of the viral genome [104–106] (Figure 3(c)). These motifs achieve the association of the genomic termini by direct base pairing. The closed-loop topology is essential for viral propagation and could be very advantageous for the regulation of viral translation and RNA synthesis, as well for the control of the switch between different steps of the viral cycle.

VIRAL RNA STRUCTURES AS NOVEL TARGETS FOR THERAPEUTIC AGENTS

Complex viral evolutionary dynamics are a great obstacle in the development of efficient therapeutic

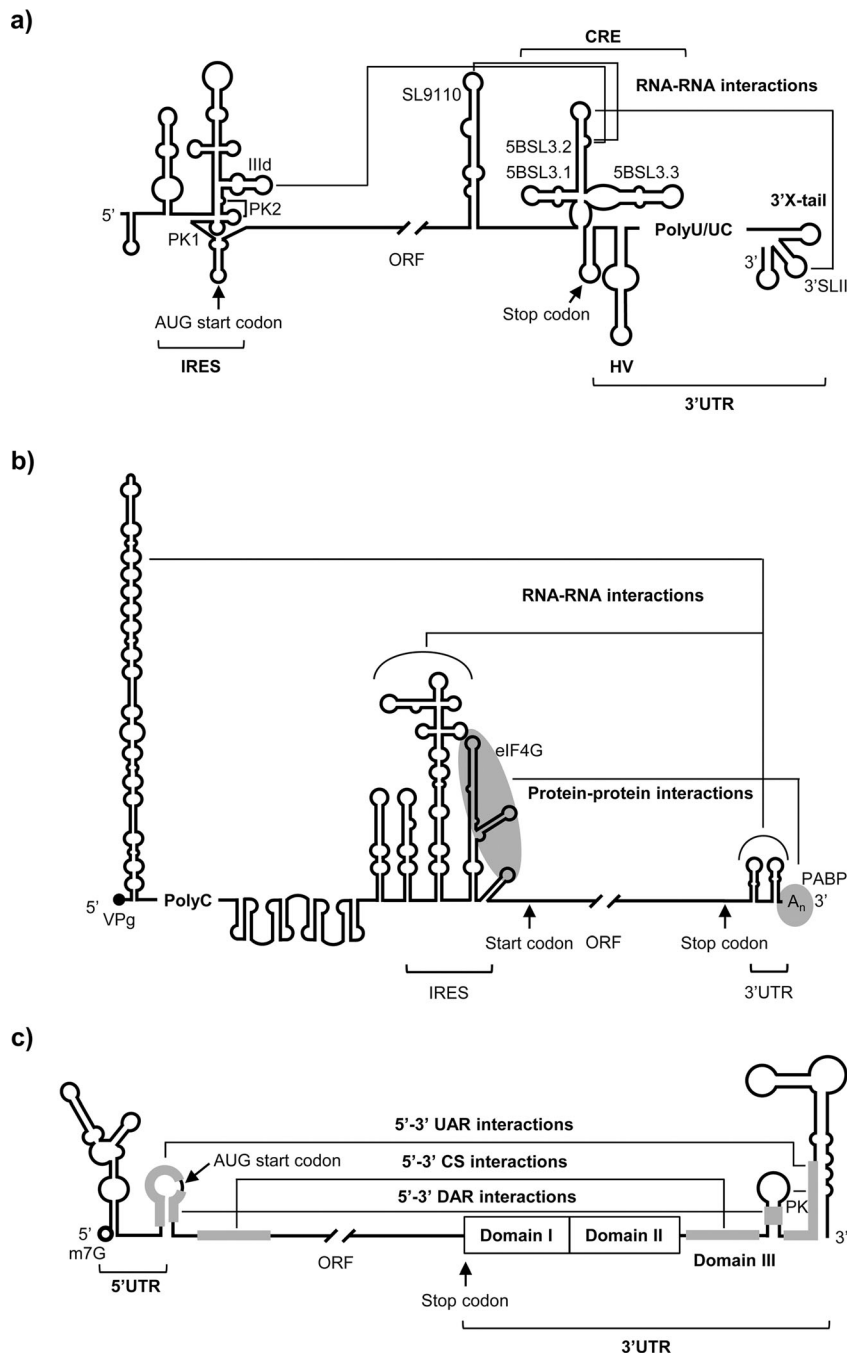


Figure 3. Circularisation mechanisms in different viral genomic RNA molecules. Detailed diagrams of the secondary structure proposed for the 5' and 3' ends, as well as for the described *cis*-acting replicating elements (CREs), are shown. (a) Hepatitis C virus. The region required for internal ribosome entry site (IRES) activity is noted. The 3' end of the viral genomic RNA is organised into two structural elements: the CRE region and the 3' untranslated region (UTR). (b) Foot and mouth disease virus RNA genome with the VPg viral protein bound to its 5' end. The IRES region is indicated. Binding sites for eIF4G and polyA binding protein (PABP) are shadowed. The circular form is stabilised by protein-protein (eIF4G and PABP, among others) and RNA-RNA interactions. (c) *Flavivirus* genome is capped at its 5' end (indicated by m7G) to ensure viral protein synthesis. The locations of the complementary sequences, cyclisation sequence (CS), upstream AUG region (UAR) and downstream AUG region (DAR), are marked by grey solid lines. The black solid lines indicate long-distance interactions (direct RNA-RNA and protein-protein contacts). Arrows denote the position of the start and stop translation codons. Pseudoknots (PK) are noted as described in Figure 1

tools. The constant appearance of sequence mutants resistant to current treatments has prompted the search for alternative targets. Structurally conserved regions involved in the progression of the viral cycle are excellent candidates. The combination of different compounds targeting multiple viral regions and processes might contribute greatly to reducing the appearance of resistant variants. The following section summarises the main advances recently made in the development of molecules directed against different functional domains of viral RNA genomes.

Nucleic-acid-based drugs

Nucleic acids, particularly RNA, are excellent candidates for the development of antiviral agents targeting functional regions of viral genomes. The success of these strategies relies on sequence complementarity and/or structural recognition between the inhibitor and the target. Therefore, highly conserved genomic domains are the most promising regions for targeting new nucleic-acid-based antiviral compounds. Importantly, a number of aspects need to be taken into account in pursuing this goal [107], such as the cellular uptake of the inhibitor, target and inhibitor colocalisation, the active inhibitor concentration inside the cell, the intracellular inhibitor stability and the access and binding to the target region. In recent years, great progress has been made in the field of nucleic acid treatment, and some of the aforementioned problems have been now successfully solved. This has led to the design of new generations of antiviral RNA and DNA drugs that are currently being extensively developed [108].

– Antisense oligonucleotides:

Antisense oligonucleotides are short nucleic acid molecules whose sequence is complementary to an existing motif in a target RNA. The molecular mechanisms of action of antisense oligonucleotides are diverse (Figure 4(a)) [109]. They act by hiding essential sites in the viral genomic RNA, such as those required for protein synthesis, replication and packaging, or if DNA based, by inducing target degradation mediated by RNase H [108].

Antisense oligonucleotides can be administered either directly, by subcutaneous injection or as pharmacological compositions. In this context, great advances in the combinatorial chemistry have allowed for the development of oligonucleotides with

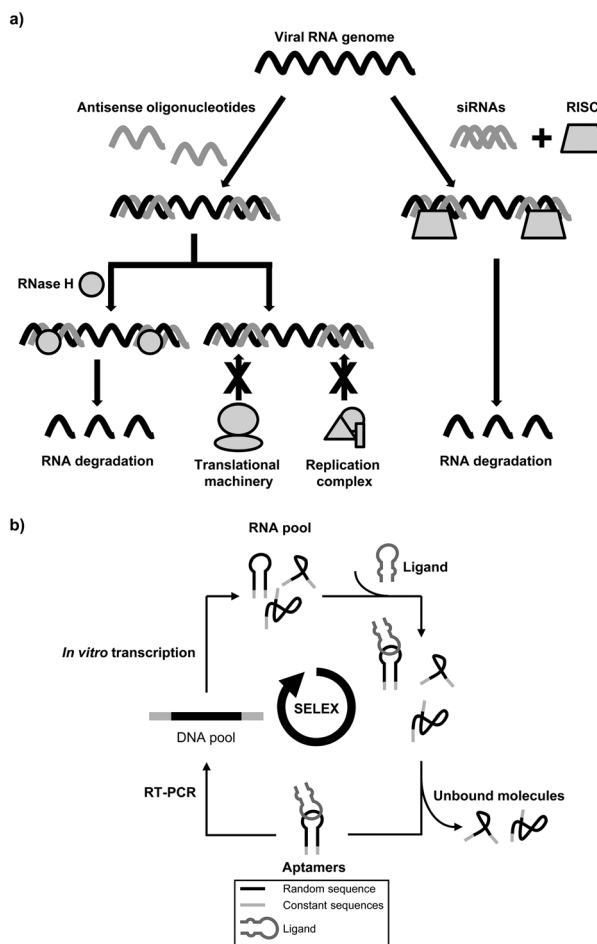


Figure 4. Nucleic-acid-based antiviral compounds. (a) Mechanism of action for antisense oligonucleotides and siRNAs. RNase H mediates the cleavage of the heteroduplex antisense deoxyoligonucleotide : target RNA. For non-DNA oligomers, inhibition is achieved by directing the oligonucleotides against functional regions of the RNA, such as those involved in the recruitment of the translational or replicative machinery. Small interfering RNAs (siRNAs) bind to the macromolecular complex RNA-induced silencing complex (RISC), which selects the guide strand that recognises the target sequence in the RNA of interest. This promotes the RNA degradation by specific endonucleases. (b) Diagram of a systematic evolution of ligands by exponential enrichment process to identify RNA aptamers targeting a specific RNA domain. Suppression of transcription and reverse transcription allows for the isolation of active DNA aptamers. RT-PCR, reverse-transcription polymerase chain reaction

chemical substitutions at precise positions [108]. These modifications confer important features, such as significant half-life increase in serum and higher thermal stability of the duplex [110]. This strategy was followed by McHutchinson *et al.* for the design of an anti-HCV compound [111], but with low success in phase I clinical trials.

A different administration strategy is based on the intracellular production of unmodified antisense oligonucleotides. It requires gene therapy protocols to deliver the construct inside the cell. Its main advantage is that chimeric constructs encompassing different antisense sequences (or even different nucleic-acid-based inhibitors) can be produced, thus targeting multiple sites simultaneously. The use of this strategy may greatly contribute in diminishing the appearance of resistant viral variants. This method has been successfully used for the inhibition of the HIV-1 replication [112–114].

– *Small interfering RNAs (siRNAs)*:

The cellular RNAi pathway can be triggered by synthetic double-stranded siRNA molecules. These compounds are usually 21 nucleotides long, with a two-nucleotide 3' overhang. The siRNAs are loaded into the RNA-induced silencing complex, where the sense or guide strand is selected to target the complementary sequence within the genomic viral RNA for further degradation (Figure 4(a)) [115].

To date, numerous studies have been conducted using siRNAs as antiviral drugs [115]. Importantly, previous advances made for the therapeutic use of antisense oligonucleotides have served as the starting point for the development of this new strategy. Thus, the incorporation of stabilising chemical modifications in the passenger (antisense) strand may greatly increase the potency of the siRNA without toxicity [116].

The application of gene therapy strategies to promote a long-term decrease of viral loads has been also studied for the siRNA technology. One of the main drawbacks of this alternative is the appearance of escape mutants [117]. To overcome this problem, several strategies have been developed from the generation of constructs combining multiple siRNAs targeting different regions of the viral genomes [118].

The great potential of siRNA-based therapeutics has prompted the development of novel internalisation strategies that should allow for a better-sustained antiviral response. Important contributions have been made in the field for the HCV infection. For example, the use of nanosomes (lipidic nanoparticles) achieves the encapsulation of multiple siRNAs with a 100% delivery yield in a liver tumour xenotransplant mouse model of HCV [119]. Alternatively, multilayered polyelectrolyte films have been also shown to be an efficient way of nucleic acid intracellular

delivery, providing dose-dependent, specific and long-term inhibition of HCV replication in hepatocyte-derived cells [120].

– *Aptamers*:

Aptamers are nucleic acids that bind to a specific ligand with high affinity. They are isolated by SELEX, an *in vitro* selection strategy [121,122]. This process consists of iterative series of the synthesis, binding, positive selection and amplification of a randomised oligonucleotide pool to yield a population enriched in those molecules that can bind to a desired target molecule (Figure 4(b)). This strategy has identified RNA and DNA molecules of potential therapeutic use in a great variety of diseases, including many viral infections [123]. In addition, aptamers offer useful information about the 3D architecture of the target molecule. They are therefore a valuable alternative to complementary oligonucleotides (antisense and siRNAs). An aptamer's mode of action is dependent on its folding and mediated by direct loop–loop interactions (Figure 4(b)), which block the function of the RNA target domain, either by interfering with the recruitment of essential factors or by affecting the folding of the surrounding area and thus impeding RNA–RNA interactions, or both. This may contribute to the diminished chance of appearance of resistant viral mutants.

Among the preferred viral targets, the most extensively investigated has been the 5'UTR, including that of the genomic RNA of HIV [124–129] and HCV [130–133]. Other functional RNA regions have been used as targets for aptamers, such as the 3'UTR [130] and the CRE region of HCV [134]. These inhibitors have returned promising results both in cell culture and *in vitro* and have been used to produce antiviral compounds efficient in the nanomolar range [135,136]. Furthermore, this research has served as a starting point for the development of novel strategies combining two or more inhibitor RNA molecules [137–142].

The emergence of SELEX methodologies has provided novel therapeutic and diagnostic agents with improved pharmacokinetic and pharmacodynamic properties and low immunogenicity. These compounds are already being investigated by several companies for possible clinical use (reviewed in [143]). The conjugation of the 5' end of the aptamer sequence with stabilising agents such as polyethylene glycol has been shown to be very effective in pharmacological formulations [144]. The additional

incorporation of ligands for specific cell surface receptors helps in the efficient and precise delivery of agents to target cell types or tissues. Given the great progress being made in this field, aptamers may be in widespread use as antiviral agents in the future.

Antibiotics against RNA functional domains

The use of antibiotics for targeting RNA structural elements was firstly described for the aminoglycoside paromomycin. This compound recognises the aminoacyl A site of the bacterial ribosome by its interaction with an enlarged deep groove defined by two bulging and one unpaired adenine residues [145,146]. This 3D conformation was shown to be strongly similar to that acquired by the HIV-1 dimerisation initiation site (DIS) kissing-loop complex [147]. Further modelling and crystal structure resolution provided a strong basis for the development of modified, high-affinity antivirals derived from aminoglycosides [148] and a more accurate view of the folding of the HIV DIS region [149]. These results will greatly prompt the design of small, improved ligands able to interfere with the folding of the essential DIS domain.

Branched peptides targeting the *trans*-activation response element of HIV

Branched peptides are a common therapeutic tool used in the development of synthetic peptide vaccines and drug delivery vehicles [150]. Their usefulness as efficient ligands of RNA molecules is associated with the so-called multivalent targets, that is, FRDs with multiple interacting sites. Additionally, amino acids may carry a radical of specific molecular architecture that increases the target selectivity. These molecules have been isolated from random peptide libraries to generate efficient, non-toxic and cell-permeable antiviral compounds that target the HIV *trans*-activation response element [151].

Benzimidazole derivatives

The benzimidazole nucleus is the main constituent of many therapeutic drugs, all of them based on the combination of heterocyclic compounds to yield isosters of naturally occurring nucleotides. The chemical composition of these isosters makes benzimidazole derivatives excellent candidates for interaction with natural biopolymers. One of these derivatives, ISIS-11, has attracted much attention

in recent years as a potential anti-HCV molecule. This compound specifically interacts with the basal portion of the IRES domain II, the so-called domain IIa [152,153]. During the recruitment of the 40S ribosomal particle, domain II adopts an L shape that directs the apical hairpin loop IIb towards the 40S subunit E site, thus inducing a conformational shift that promotes the positioning of the start codon close to the active site. In the presence of ISIS-11, domain IIa acquires the shape of an open helix that alters the proper folding of domain II, thus impeding the correct positioning of the viral RNA in the catalytic binding cleft. These findings confirm the important principle of interfering with RNA folding to achieve an inhibitory effect.

CONCLUDING REMARKS

The compactness of RNA viral genomes provides important advantages in terms of propagation efficiency. Such compactness, however, requires the intervention of a supracoding system (i.e. information in the genome beyond that of the nucleotide sequence) to direct the folding required; without such a supracoding system, no such compactness would exist, and essential functions of the genome in transcription, replication and encapsidation associated with this folding would be lost. Structurally conserved functional RNA elements interact with protein factors and other RNA domains to direct and regulate these functions as well as switch between different steps of the viral cycle. Interfering with the functioning of these structural domains offers a potential means of treating viral infections. The great advances made in the field of chemical synthesis and nucleic acid production have prompted novel initiatives involving small molecules that can alter the 3D conformation of viral genomic RNA domains. This might be used to complement classical antiviral therapies, such as the use of neutralising antibodies or interferon treatment. The combination of clinical strategies is currently under extensive investigation with the aim of improving therapeutic responses and long-term outcomes while minimising toxicity and secondary effects.

CONFLICT OF INTEREST

The authors have no competing interest.

ACKNOWLEDGEMENTS

Work at our laboratory is supported by grants BFU2012-31213 from the Spanish Ministry of

Economy and Competitiveness and CVI-7430 from the *Junta de Andalucía* and by FEDER funds from the EU.

REFERENCES

- Schuster P. RNA based evolutionary optimization. *Orig Life Evol Biosph* 1993; **23**: 373–391.
- Eigen M. Error catastrophe and antiviral strategy. *Proceedings of the National Academy of Sciences of the USA* 2002; **99**: 13374–13376. DOI: 10.1073/pnas.212514799
- Wagner A, Stadler PF. Viral RNA and evolved mutational robustness. *Journal of Exposure Zoological* 1999; **285**: 119–127. DOI: 10.1002/(SICI)1097-010X(19990815)285:2<119::AID-JEZA>3.0.CO;2-D
- Contreras AM, Hiasa Y, He W, et al. Viral RNA mutations are region specific and increased by ribavirin in a full-length hepatitis C virus replication system. *Journal of Virology* 2002; **76**: 8505–8517.
- Churkin A, Cohen M, Shemer-Avni Y, et al. Bioinformatic analysis of the neutrality of RNA secondary structure elements across genotypes reveals evidence for direct evolution of genetic robustness in HCV. *Journal of Bioinformatics and Computational Biology* 2010; **8**: 1013–1026. DOI: S0219720010005087
- Wan Y, Kertesz M, Spitale RC, et al. Understanding the transcriptome through RNA structure. *Nature Reviews Genetics* 2011; **12**: 641–655. DOI: 10.1038/nrg3049
- Liu Y, Wimmer E, Paul AV. cis-Acting RNA elements in human and animal plus-strand RNA viruses. *Biochemical et Biophysical Acta* 2009; **1789**: 495–517. DOI: 10.1016/j.bbagr.2009.09.007
- Appel N, Schaller T, Penin F, et al. From structure to function: new insights into hepatitis C virus RNA replication. *Journal Biological Chemistry* 2006; **281**: 9833–9836. DOI: R500026200
- Fernández-Miragall O, López de Quinto S, Martínez-Salas E. Relevance of RNA structure for the activity of picornavirus IRES elements. *Virus Research* 2009; **139**: 172–182. DOI: 10.1016/j.virusres.2008.07.009
- Roberts L, Holcik M. RNA structure: new messages in translation, replication and disease. Workshop on the role of RNA structures in the translation of viral and cellular RNAs. *EMBO Reports* 2009; **10**: 449–453. DOI: 10.1038/embor.2009.56
- Reineke LC, Lloyd RE. Animal virus schemes for translation dominance. *Current Opinion in Virology* 2011; **1**: 363–372. DOI: 10.1016/j.coviro.2011.10.009.
- Schlesinger S, Makino S, Linial ML. cis-Acting genomic elements and trans-acting proteins involved in the assembly of RNA viruses. *Seminars in Virology* 1994; **5**: 39–49.
- Thurner C, Witwer C, Hofacker IL, et al. Conserved RNA secondary structures in Flaviviridae genomes. *Journal of General Virology* 2004; **85**: 1113–1124.
- Nakashima N, Uchiumi T. Functional analysis of structural motifs in dicistroviruses. *Virus Research* 2009; **139**: 137–147. DOI: 10.1016/j.virusres.2008.06.006
- Zoll J, Heus HA, van Kuppeveld FJ, et al. The structure-function relationship of the enterovirus 3'-UTR. *Virus Research* 2009; **139**: 209–216. DOI: 10.1016/j.virusres.2008.07.014
- Filbin ME, Kieft JS. Toward a structural understanding of IRES RNA function. *Current Opinion in Structural Biology* 2009; **19**: 267–276. DOI: 10.1016/j.sbi.2009.03.005
- Jan E, Sarnow P. Factorless ribosome assembly on the internal ribosome entry site of cricket paralysis virus. *Journal of Molecular Biology* 2002; **324**: 889–902. DOI: 10.1016/s0022-2836(02)01099-9
- Wilson JE, Pestova TV, Hellen CU, et al. Initiation of protein synthesis from the A site of the ribosome. *Cell* 2000; **102**: 511–520. DOI: 10.1016/S0092-8674(00)00055-6
- Kanamori Y, Nakashima N. A tertiary structure model of the internal ribosome entry site (IRES) for methionine-independent initiation of translation. *RNA* 2001; **7**: 266–274.
- Costantino D, Kieft JS. A preformed compact ribosome-binding domain in the cricket paralysis-like virus IRES RNAs. *RNA* 2005; **11**: 332–343. DOI: 10.1261/rna.7184705
- Pfingsten JS, Costantino DA, Kieft JS. Structural basis for ribosome recruitment and manipulation by a viral IRES RNA. *Science* 2006; **314**: 1450–1454. DOI: 10.1126/science.1133281
- Yamamoto H, Nakashima N, Ikeda Y, et al. Binding mode of the first aminoacyl-tRNA in translation initiation mediated by Plautia stali intestine virus internal ribosome entry site. *Journal of Biological Chemistry* 2007; **282**: 7770–7776. DOI: 10.1074/jbc.M610887200
- Costantino DA, Pfingsten JS, Rambo RP, et al. tRNA-mRNA mimicry drives translation initiation from a viral IRES. *Nature Structural and Molecular Biology* 2008; **15**: 57–64. DOI: 10.1038/nsmb1351
- Reynolds JE, Kaminski A, Kettinen HJ, et al. Unique features of internal initiation of hepatitis C virus RNA translation. *EMBO Journal* 1995; **14**: 6010–6020.
- Wang TH, Rijnbrand RC, Lemon SM. Core protein-coding sequence, but not core protein, modulates the efficiency of cap-independent translation directed by the internal ribosome entry site of hepatitis C virus. *Journal of Virology* 2000; **74**: 11,347–11,358.
- Kieft JS, Zhou K, Jubin R, et al. The hepatitis C virus internal ribosome entry site adopts an ion-dependent tertiary fold. *Journal of Molecular Biology* 1999; **292**: 513–529.
- Honda M, Brown EA, Lemon SM. Stability of a stem-loop involving the initiator AUG controls the efficiency of internal initiation of translation on hepatitis C virus RNA. *RNA* 1996; **2**: 955–968.
- Berry KE, Waghray S, Doudna JA, Waghray S, Doudna JA. The HCV IRES pseudoknot positions the initiation codon on the 40S ribosomal subunit. *RNA* 2010; **16**: 1559–1569. DOI: 10.1261/ma.2197210
- Spahn CM, Kieft JS, Grassucci RA, et al. Hepatitis C virus IRES RNA-induced changes in the conformation of the 40s ribosomal subunit. *Science* 2001; **291**: 1959–1962.
- Fraser CS, Doudna JA. Structural and mechanistic insights into hepatitis C viral translation initiation. *Nature Reviews Microbiology* 2007; **5**: 29–38.
- Terenin IM, Dmitriev SE, Andreev DE, et al. Eukaryotic translation initiation machinery can operate in a bacterial-like mode without eIF2. *Nature Structural and Molecular Biology* 2008; **15**: 836–841.

32. Fernández-Miragall O, Martínez-Salas E. Structural organization of a viral IRES depends on the integrity of the GNRA motif. *RNA* 2003; **9**: 1333–1344.
33. Fernández-Miragall O, Martínez-Salas E. *In vivo* footprint of a picornavirus internal ribosome entry site reveals differences in accessibility to specific RNA structural elements. *Journal of General Virology* 2007; **88**: 3053–3062.
34. Fernández N, Fernández-Miragall O, Ramajo J, *et al.* Structural basis for the biological relevance of the invariant apical stem in IRES-mediated translation. *Nucleic Acids Research* 2011; **39**: 8572–8585. DOI: 10.1093/nar/gkr560
35. Fernández N, García-Sacristán A, Ramajo J, *et al.* Structural analysis provides insights into the modular organization of picornavirus IRES. *Virology* 2011; **409**: 251–261. DOI: S0042-6822(10)00649-5
36. López de Quinto S, Martínez-Salas E. Interaction of the eIF4G initiation factor with the aphthovirus IRES is essential for internal translation initiation *in vivo*. *RNA* 2000; **6**: 1380–1392.
37. López de Quinto S, Lafuente E, Martínez-Salas E. IRES interaction with translation initiation factors: functional characterization of novel RNA contacts with eIF3, eIF4B, and eIF4GII. *RNA* 2001; **7**: 1213–1226.
38. Jang SK, Pestova TV, Hellen CU, *et al.* Cap-independent translation of picornavirus RNAs: structure and function of the internal ribosomal entry site. *Enzyme* 1990; **44**: 292–309.
39. Pilipenko EV, Pestova TV, Kolupaeva VG, *et al.* A cell cycle-dependent protein serves as a template-specific translation initiation factor. *Genes & Development* 2000; **14**: 2028–2045.
40. Kolupaeva VG, Lomakin IB, Pestova TV, *et al.* Eukaryotic initiation factors 4G and 4A mediate conformational changes downstream of the initiation codon of the encephalomyocarditis virus internal ribosomal entry site. *Molecular and Cellular Biology* 2003; **23**: 687–698.
41. Song Y, Tzima E, Ochs K, *et al.* Evidence for an RNA chaperone function of polypyrimidine tract-binding protein in picornavirus translation. *RNA* 2005; **11**: 1809–1824. DOI: 10.1261/rna.7430405
42. Brasey A, Lopez-Lastra M, Ohlmann T, *et al.* The leader of human immunodeficiency virus type 1 genomic RNA harbors an internal ribosome entry segment that is active during the G2/M phase of the cell cycle. *Journal of Virology* 2003; **77**: 3939–3949.
43. Camerini V, Decimo D, Balvay L, *et al.* A dormant internal ribosome entry site controls translation of feline immunodeficiency virus. *Journal of Virology* 2008; **82**: 3574–3583. DOI: 10.1128/JVI.02038-07
44. Buck CB, Shen X, Egan MA, *et al.* The human immunodeficiency virus type 1 gag gene encodes an internal ribosome entry site. *Journal of Virology* 2001; **75**: 181–191. DOI: 10.1128/JVI.75.1.181-191.2001
45. Abbink TE, Ooms M, Haasnoot PC, *et al.* The HIV-1 leader RNA conformational switch regulates RNA dimerization but does not regulate mRNA translation. *Biochemistry* 2005; **44**: 9058–9066. DOI: 10.1021/bi0520588
46. Abbink TE, Berkhout B. A novel long distance base-pairing interaction in human immunodeficiency virus type 1 RNA occludes the Gag start codon. *Journal of Biological Chemistry* 2003; **278**: 11,601–11,611. DOI: 10.1074/jbc.M210291200
47. Edgil D, Harris E. End-to-end communication in the modulation of translation by mammalian RNA viruses. *Virus Research* 2006; **119**: 43–51.
48. Pilipenko EV, Maslova SV, Sinyakov AN, *et al.* Towards identification of cis-acting elements involved in the replication of enterovirus and rhinovirus RNAs: a proposal for the existence of tRNA-like terminal structures. *Nucleic Acids Research* 1992; **20**: 1739–1745.
49. Wimmer E, Hellen CU, Cao X. Genetics of poliovirus. *Annual Review of Genetics* 1993; **27**: 353–436. DOI: 10.1146/annurev.ge.27.120193.002033
50. Jacobson SJ, Konings DA, Sarnow P. Biochemical and genetic evidence for a pseudoknot structure at the 3' terminus of the poliovirus RNA genome and its role in viral RNA amplification. *Journal of Virology* 1993; **67**: 2961–2971.
51. Wang J, Bakkens JM, Galama JM, *et al.* Structural requirements of the higher order RNA kissing element in the enteroviral 3'UTR. *Nucleic Acids Research* 1999; **27**: 485–490. DOI: 10.1093/nar/27.2.485
52. Melchers WJ, Bakkens JM, Bruins Slot HJ, *et al.* Cross-talk between orientation-dependent recognition determinants of a complex control RNA element, the enterovirus oriR. *RNA* 2000; **6**: 976–987.
53. van Ooij MJ, Glaudemans DH, Heus HA, *et al.* Structural and functional integrity of the coxsackievirus B3 oriR: spacing between coaxial RNA helices. *Journal of General Virology* 2006; **87**: 689–695. DOI: 10.1099/vir.0.81558-0
54. Rodriguez Pulido M, Sobrino F, Borrego B, *et al.* Attenuated foot-and-mouth disease virus RNA carrying a deletion in the 3' noncoding region can elicit immunity in swine. *Journal of Virology* 2009; **83**: 3475–3485. DOI: 10.1128/JVI.01836-08
55. Serrano P, Pulido MR, Saiz M, *et al.* The 3' end of the foot-and-mouth disease virus genome establishes two distinct long-range RNA-RNA interactions with the 5' end region. *Journal of General Virology* 2006; **87**: 3013–3022.
56. Tanaka T, Kato N, Cho MJ, *et al.* Structure of the 3' terminus of the hepatitis C virus genome. *Journal of Virology* 1996; **70**: 3307–3312.
57. Blight KJ, Rice CM. Secondary structure determination of the conserved 98-base sequence at the 3' terminus of hepatitis C virus genome RNA. *Journal of Virology* 1997; **71**: 7345–7352.
58. Kolykhalov AA, Feinstone SM, Rice CM. Rice CM. Identification of a highly conserved sequence element at the 3' terminus of hepatitis C virus genome RNA. *Journal of Virology* 1996; **70**: 3363–3371.
59. Yanagi M, St Claire M, Emerson SU, *et al.* *In vivo* analysis of the 3' untranslated region of the hepatitis C virus after *in vitro* mutagenesis of an infectious cDNA clone. *Proceedings of the National Academy of Sciences of the United States of America* 1999; **96**: 2291–2295.
60. Kolykhalov AA, Mihalik K, Feinstone SM, *et al.* Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3' nontranslated region are essential for virus replication *in vivo*. *Journal of Virology* 2000; **74**: 2046–2051.
61. Friebe P, Bartenschlager R. Genetic analysis of sequences in the 3' nontranslated region of hepatitis C virus that are important for RNA replication. *Journal of Virology* 2002; **76**: 5326–5338.

62. Yi M, Lemon SM. Structure-function analysis of the 3' stem-loop of hepatitis C virus genomic RNA and its role in viral RNA replication. *RNA* 2003; **9**: 331–345.
63. Yi M, Lemon SM. 3' nontranslated RNA signals required for replication of hepatitis C virus RNA. *Journal of Virology* 2003; **77**: 3557–3568.
64. You S, Rice CM. 3' RNA elements in hepatitis C virus replication: kissing partners and long poly(U). *Journal of Virology* 2008; **82**: 184–195. DOI: JVI.01796-07[pil] 10.1128/JVI.01796-07
65. Banerjee R, Dasgupta A. Specific interaction of hepatitis C virus protease/helicase NS3 with the 3'-terminal sequences of viral positive- and negative-strand RNA. *Journal of Virology* 2001; **75**: 1708–1721.
66. Ito T, Lai MM. Determination of the secondary structure of and cellular protein binding to the 3'-untranslated region of the hepatitis C virus RNA genome. *Journal of Virology* 1997; **71**: 8698–8706.
67. Luo G. Cellular proteins bind to the poly (U) tract of the 3' untranslated region of hepatitis C virus RNA genome. *Virology* 1999; **256**: 105–118.
68. Spangberg K, Goobar-Larsson L, Wahren-Herlenius M, *et al.* The La protein from human liver cells interacts specifically with the U-rich region in the hepatitis C virus 3' untranslated region. *Journal of Human Virology* 1999; **2**: 296–307.
69. Wood J, Frederickson RM, Fields S, *et al.* Hepatitis C virus 3'X region interacts with human ribosomal proteins. *Journal of Virology* 2001; **75**: 1348–1358.
70. Chang KS, Luo G. The polypyrimidine tract-binding protein (PTB) is required for efficient replication of hepatitis C virus (HCV) RNA. *Virus Research* 2006; **115**: 1–8.
71. Friebe P, Boudet J, Simorre JP, *et al.* Kissing-loop interaction in the 3' end of the hepatitis C virus genome essential for RNA replication. *Journal of Virology* 2005; **79**: 380–392.
72. Diviney S, Tuplin A, Struthers M, *et al.* A hepatitis C virus *cis*-acting replication element forms a long-range RNA-RNA interaction with upstream RNA sequences in NS5B. *Journal of Virology* 2008; **82**: 9008–9022.
73. Brinton MA, Fernandez AV, Dispoto JH. The 3'-nucleotides of flavivirus genomic RNA form a conserved secondary structure. *Virology* 1986; **153**: 113–121.
74. Davis WG, Blackwell JL, Shi PY, *et al.* Interaction between the cellular protein eEF1A and the 3'-terminal stem-loop of West Nile virus genomic RNA facilitates viral minus-strand RNA synthesis. *Journal of Virology* 2007; **81**: 10,172–10,187. DOI: 10.1128/JVI.00531-07
75. Sola I, Mateos-Gomez PA, Almazan F, *et al.* RNA-RNA and RNA-protein interactions in coronavirus replication and transcription. *RNA Biology* 2011; **8**: 237–248. DOI: 10.4161/rna.8.2.14991
76. Hsue B, Masters PS. A bulged stem-loop structure in the 3' untranslated region of the genome of the coronavirus mouse hepatitis virus is essential for replication. *Journal of Virology* 1997; **71**: 7567–7578.
77. Hsue B, Hartshorne T, Masters PS. Masters PS. Characterization of an essential RNA secondary structure in the 3' untranslated region of the murine coronavirus genome. *Journal of Virology* 2000; **74**: 6911–6921.
78. Williams GD, Chang RY, Brian DA. A phylogenetically conserved hairpin-type 3' untranslated region pseudoknot functions in coronavirus RNA replication. *Journal of Virology* 1999; **73**: 8349–8355.
79. Goebel SJ, Miller TB, Bennett CJ, *et al.* A hypervariable region within the 3' *cis*-acting element of the murine coronavirus genome is nonessential for RNA synthesis but affects pathogenesis. *Journal of Virology* 2007; **81**: 1274–1287. DOI: 10.1128/JVI.00803-06
80. Cuceanu NM, Tuplin A, Simmonds P. Evolutionarily conserved RNA secondary structures in coding and non-coding sequences at the 3' end of the hepatitis G virus/GB-virus C genome. *Journal of General Virology* 2001; **82**: 713–722.
81. Tuplin A, Wood J, Evans DJ, *et al.* Thermodynamic and phylogenetic prediction of RNA secondary structures in the coding region of hepatitis C virus. *RNA* 2002; **8**: 824–841.
82. Simmonds P, Karakasiliotis I, Bailey D, *et al.* Bioinformatic and functional analysis of RNA secondary structure elements among different genera of human and animal caliciviruses. *Nucleic Acids Research* 2008; **36**: 2530–2546. DOI: 10.1093/nar/gkn096
83. Wang Q, Barr I, Guo F, *et al.* Evidence of a novel RNA secondary structure in the coding region of HIV-1 pol gene. *RNA* 2008; **14**: 2478–2488. DOI: 10.1261/rna.1252608
84. Victoria M, Colina R, Miagostovich MP, *et al.* Phylogenetic prediction of *cis*-acting elements: a CRE-like sequence in Norovirus genome?. *BMC Research Notes* 2009; **2**: 176. DOI: 10.1186/1756-0500-2-176
85. Moss WN, Priore SF, Turner DH. Identification of potential conserved RNA secondary structure throughout influenza A coding regions. *RNA* 2011; **17**: 991–1011. DOI: 10.1261/rna.2619511
86. Liu ZY, Li XE, Jiang T, *et al.* Novel *cis*-acting element within the capsid-coding region enhances flavivirus viral-RNA replication by regulating genome cyclization. *Journal of Virology* 2013; **87**: 6804–6818. DOI: 10.1128/JVI.00243-13
87. McKnight KL, Lemon SM. The rhinovirus type 14 genome contains an internally located RNA structure that is required for viral replication. *RNA* 1998; **4**: 1569–1584.
88. Lobert PE, Escriou N, Ruelle J, *et al.* A coding RNA sequence acts as a replication signal in cardiocoronaviruses. *Proceedings of the National Academy of Sciences of the United States of America* 1999; **96**: 11,560–11,565.
89. Goodfellow I, Chaudhry Y, Richardson A, *et al.* Identification of a *cis*-acting replication element within the poliovirus coding region. *Journal of Virology* 2000; **74**: 4590–4600.
90. Paul AV, Rieder E, Kim DW, *et al.* Identification of an RNA hairpin in poliovirus RNA that serves as the primary template in the *in vitro* uridylylation of VPg. *Journal of Virology* 2000; **74**: 10,359–10,370.
91. Steil BP, Barton DJ. Poliovirus *cis*-acting replication element-dependent VPg uridylylation lowers the K_m of the initiating nucleoside triphosphate for viral RNA replication. *Journal of Virology* 2008; **82**: 9400–9408. DOI: 10.1128/JVI.00427-08
92. You S, Stump DD, Branch AD, *et al.* A *cis*-acting replication element in the sequence encoding the NS5B RNA-dependent RNA polymerase is required for hepatitis C virus RNA replication. *Journal of Virology* 2004; **78**: 1352–1366.

93. Lee H, Shin H, Wimmer E, *et al.* *cis*-Acting RNA signals in the NS5B C-terminal coding sequence of the hepatitis C virus genome. *Journal of Virology* 2004; **78**: 10,865–10,877.
94. Tuplin A, Struthers M, Simmonds P, *et al.* A twist in the tail: SHAPE mapping of long-range interactions and structural rearrangements of RNA elements involved in HCV replication. *Nucleic Acids Research* 2012; **40**: 6908–6921. DOI: 10.1093/nar/gks370
95. Palau W, Masante C, Ventura M, *et al.* Direct evidence for RNA-RNA interactions at the 3' end of the Hepatitis C virus genome using surface plasmon resonance. *RNA* 2013; **19**: 982–991. DOI: 10.1261/rna.037606.112
96. Shetty S, Stefanovic S, Mihailescu MR. Hepatitis C virus RNA: molecular switches mediated by long-range RNA-RNA interactions? *Nucleic Acids Research* 2013; **41**: 2526–2540. DOI: 10.1093/nar/gks1318
97. Romero-López C, Berzal-Herranz A. A long-range RNA-RNA interaction between the 5' and 3' ends of the HCV genome. *RNA* 2009; **15**: 1740–1752.
98. Romero-López C, Berzal-Herranz A. The functional RNA domain 5BSL3.2 within the NS5B coding sequence influences hepatitis C virus IRES-mediated translation. *Cellular and Molecular Life Sciences* 2012; **69**: 103–113. DOI: 10.1007/s00018-011-0729-z
99. Romero-López C, Barroso-delJesus A, García-Sacristán A, *et al.* The folding of the hepatitis C virus internal ribosome entry site depends on the 3'-end of the viral genome. *Nucleic Acids Research* 2012; **40**: 11,697–11,713. DOI: 10.1093/nar/gks927
100. McCaffrey AP, Ohashi K, Meuse L, *et al.* Determinants of hepatitis C translational initiation in vitro, in cultured cells and mice. *Molecular Therapy* 2002; **5**: 676–684.
101. Bradrick SS, Walters RW, Gromeier M. The hepatitis C virus 3'-untranslated region or a poly(A) tract promote efficient translation subsequent to the initiation phase. *Nucleic Acids Research* 2006; **34**: 1293–1303.
102. Song Y, Friebe P, Tzima E, *et al.* The hepatitis C virus RNA 3'-untranslated region strongly enhances translation directed by the internal ribosome entry site. *Journal of Virology* 2006; **80**: 11,579–11,588.
103. Lourenco S, Costa F, Debarges B, *et al.* Hepatitis C virus internal ribosome entry site-mediated translation is stimulated by *cis*-acting RNA elements and trans-acting viral factors. *FEBS Journal* 2008; **275**: 4179–4197.
104. Hahn CS, Hahn YS, Rice CM, *et al.* Conserved elements in the 3' untranslated region of flavivirus RNAs and potential cyclization sequences. *Journal of Molecular Biology* 1987; **198**: 33–41. DOI: 10.1016/0022-2836(87)90455-4
105. Alvarez DE, Lodeiro MF, Luduena SJ, *et al.* Long-range RNA-RNA interactions circularize the dengue virus genome. *Journal of Virology* 2005; **79**: 6631–6643. DOI: 10.1128/JVI.79.11.6631-6643.2005
106. Friebe P, Harris E. Interplay of RNA elements in the dengue virus 5' and 3' ends required for viral RNA replication. *Journal of Virology* 2010; **84**: 6103–6118. DOI: 10.1128/JVI.02042-09
107. Rossi JJ. Controlled, targeted, intracellular expression of ribozymes: progress and problems. *Trends in Biotechnology* 1995; **13**: 301–306.
108. Haasnoot J, Berkhout B. Nucleic acids-based therapeutics in the battle against pathogenic viruses. *Handbook of Experimental Pharmacology* 2009; **243**–263. DOI: 10.1007/978-3-540-79086-0_9
109. Crooke ST. Molecular mechanisms of action of antisense drugs. *Biochimica et Biophysica Acta* 1999; **1489**: 31–44.
110. Darfeuille F, Arzumanov A, Gait MJ, *et al.* 2'-O-methyl-RNA hairpins generate loop-loop complexes and selectively inhibit HIV-1 Tat-mediated transcription. *Biochemistry* 2002; **41**: 12,186–12,192. DOI: 10.1021/bi025974d
111. McHutchison JG, Patel K, Pockros P, *et al.* A phase I trial of an antisense inhibitor of hepatitis C virus (ISIS 14803), administered to chronic hepatitis C patients. *Journal of Hepatology* 2006; **44**: 88–96.
112. Puerta-Fernández E, Barroso-delJesus A, Romero-López C, *et al.* Inhibition of HIV-1 replication by RNA targeted against the LTR region. *AIDS* 2005; **19**: 863–870.
113. Gu S, Ji J, Kim JD, *et al.* Inhibition of infectious human immunodeficiency virus type 1 virions via lentiviral vector encoded short antisense RNAs. *Oligonucleotides* 2006; **16**: 287–295. DOI: 10.1089/oli.2006.16.287
114. Levine BL, Humeau LM, Boyer J, *et al.* Gene transfer in humans using a conditionally replicating lentiviral vector. *Proceedings of the National Academy of Sciences of the United States of America* 2006; **103**: 17,372–17,377. DOI: 10.1073/pnas.0608138103
115. Blake SJ, Bokhari FF, McMillan NA. RNA interference for viral infections. *Current Drug Targets* 2012; **13**: 1411–1420. DOI: CDT-EPUB-20120528-25
116. Elmen J, Thonberg H, Ljungberg K, *et al.* Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. *Nucleic Acids Research* 2005; **33**: 439–447. DOI: 10.1093/nar/gki193
117. Boden D, Pusch O, Lee F, *et al.* Human immunodeficiency virus type 1 escape from RNA interference. *Journal of Virology* 2003; **77**: 11,531–11,535.
118. Schopman NC, ter Brake O, Berkhout B. Anticipating and blocking HIV-1 escape by second generation antiviral shRNAs. *Retrovirology* 2010; **7**: 52. DOI: 10.1186/1742-4690-7-52
119. Chandra PK, Kundu AK, Hazari S, *et al.* Inhibition of hepatitis C virus replication by intracellular delivery of multiple siRNAs by nanosomes. *Molecular Therapy* 2012; **20**: 1724–1736. DOI: 10.1038/mt.2012.107
120. Dimitrova M, Affolter C, Meyer F, *et al.* Sustained delivery of siRNAs targeting viral infection by cell-degradable multilayered polyelectrolyte films. *Proceedings of the National Academy of Sciences of the United States of America* 2008; **105**: 16,320–16,325. DOI: 10.1073/pnas.0800156105
121. Ellington AD, Szostak JW. *In vitro* selection of RNA molecules that bind specific ligands. *Nature* 1990; **346**: 818–822.
122. Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* 1990; **249**: 505–510.
123. Marton S, Reyes-Darias JA, Sánchez-Luque FJ, *et al.* *In vitro* and *ex vivo* selection procedures for identifying potentially therapeutic DNA and RNA molecules. *Molecules* 2010; **15**: 4610–4638.
124. Ducongé F, Toulmé JJ. *In vitro* selection identifies key determinants for loop-loop interactions: RNA aptamers selective for the TAR RNA element of HIV-1. *RNA* 1999; **5**: 1605–1614.

125. Darfeuille F, Cazenave C, Gryaznov S, *et al.* RNA and N3'->P5' kissing aptamers targeted to the trans-activation responsive (TAR) RNA of the human immunodeficiency virus-1. *Nucleosides, Nucleotides & Nucleic Acids* 2001; **20**: 441–449.
126. Darfeuille F, Arzumanov A, Gryaznov S, *et al.* Loop-loop interaction of HIV-1 TAR RNA with N3'->P5' deoxyphosphoramidate aptamers inhibits *in vitro* Tat-mediated transcription. *Proceedings of the National Academy of Sciences of the United States of America* 2002; **99**: 9709–9714.
127. Beaurain F, Di Primo C, Toulmé JJ, *et al.* Molecular dynamics reveals the stabilizing role of loop closing residues in kissing interactions: comparison between TAR-TAR* and TAR-aptamer. *Nucleic Acids Research* 2003; **31**: 4275–4284.
128. Boucard D, Toulmé JJ, Di Primo C. Bimodal loop-loop interactions increase the affinity of RNA aptamers for HIV-1 RNA structures. *Biochemistry* 2006; **45**: 1518–1524.
129. Lebars J, Richard T, Di Primo C, *et al.* NMR structure of a kissing complex formed between the TAR RNA element of HIV-1 and a LNA-modified aptamer. *Nucleic Acids Research* 2007; **35**: 6103–6114.
130. Aldaz-Carroll L, Tallet B, Dausse E, *et al.* Apical loop-internal loop interactions: a new RNA-RNA recognition motif identified through *in vitro* selection against RNA hairpins of the hepatitis C virus mRNA. *Biochemistry* 2002; **41**: 5883–5893.
131. Kikuchi K, Umehara T, Fukuda K, *et al.* RNA aptamers targeted to domain II of hepatitis C virus IRES that bind to its apical loop region. *Journal of biochemistry (Tokyo)* 2003; **133**: 263–270.
132. Tallet-López B, Aldaz-Carroll L, Chabas S, *et al.* Antisense oligonucleotides targeted to the domain III_d of the hepatitis C virus IRES compete with 40S ribosomal subunit binding and prevent *in vitro* translation. *Nucleic Acids Research* 2003; **31**: 734–742.
133. Kikuchi K, Umehara T, Fukuda K, *et al.* A hepatitis C virus (HCV) internal ribosome entry site (IRES) domain III-IV-targeted aptamer inhibits translation by binding to an apical loop of domain III_d. *Nucleic Acids Research* 2005; **33**: 683–692.
134. Marton S, Romero-López C, Berzal-Herranz A. RNA aptamer-mediated interference of HCV replication by targeting the CRE-5BSL3.2 domain. *Journal of Viral Hepatitis* 2013; **20**: 103–112. DOI: 10.1111/j.1365-2893.2012.01629.x
135. Romero-López C, Sanchez-Luque FJ, Berzal-Herranz A. Targets and tools: recent advances in the development of anti-HCV nucleic acids. *Infectious Disorders Drug Targets* 2006; **6**: 121–145.
136. James W. Aptamers in the virologists' toolkit. *Journal of General Virology* 2007; **88**: 351–364. DOI: 88/2/351
137. Romero-López C, Barroso-delJesus A, Puerta-Fernández E, *et al.* Interfering with hepatitis C virus IRES activity using RNA molecules identified by a novel *in vitro* selection method. *Biological Chemistry* 2005; **386**: 183–190.
138. Romero-López C, Díaz-González R, Berzal-Herranz A. Inhibition of hepatitis C virus internal ribosome entry site-mediated translation by an RNA targeting the conserved III_f domain. *Cellular and Molecular Life Sciences* 2007; **64**: 2994–3006.
139. Zhou J, Li H, Li S, *et al.* Novel dual inhibitory function aptamer-siRNA delivery system for HIV-1 therapy. *Molecular Therapy* 2008; **16**: 1481–1489. DOI: 10.1038/mt.200892
140. Kikuchi K, Umehara T, Nishikawa F, *et al.* Increased inhibitory ability of conjugated RNA aptamers against the HCV IRES. *Biochemical and Biophysical Research Communications* 2009; **386**: 118–123. DOI: S0006-291X(09)01118-8
141. Romero-López C, Díaz-González R, Barroso-delJesus A, *et al.* Inhibition of HCV replication and IRES-dependent translation by an RNA molecule. *Journal of General Virology* 2009; **90**: 1659–1669.
142. Romero-López C, Berzal-Herranz B, Gomez J, *et al.* An engineered inhibitor RNA that efficiently interferes with hepatitis C virus translation and replication. *Antiviral Research* 2012; **94**: 131–138. DOI: 10.1016/j.antiviral.2012.02.015
143. Wang RE, Wu H, Niu Y, *et al.* Improving the stability of aptamers by chemical modification. *Current Medicinal Chemistry* 2011; **18**: 4126–4138. DOI: BSP/CMC/E-Pub/2011/ 310
144. Healy JM, Lewis SD, Kurz M, *et al.* Pharmacokinetics and biodistribution of novel aptamer compositions. *Pharmaceutical Research* 2004; **21**: 2234–2246.
145. Carter AP, Clemons WM, Brodersen DE, *et al.* Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. *Nature* 2000; **407**: 340–348. DOI: 10.1038/35030019.
146. Vicens Q, Westhof E. Crystal structure of paromomycin docked into the eubacterial ribosomal decoding A site. *Structure* 2001; **9**: 647–658. DOI: S0969212601006293
147. Ennifar E, Paillart JC, Marquet R, *et al.* HIV-1 RNA dimerization initiation site is structurally similar to the ribosomal A site and binds aminoglycoside antibiotics. *Journal of Biological Chemistry* 2003; **278**: 2723–2730. DOI: 10.1074/jbc.M205726200
148. Ennifar E, Paillart JC, Bodlenner A, *et al.* Targeting the dimerization initiation site of HIV-1 RNA with aminoglycosides: from crystal to cell. *Nucleic Acids Research* 2006; **34**: 2328–2339. DOI: 10.1093/nar/gkl317
149. Sakuragi J, Ode H, Sakuragi S, *et al.* A proposal for a new HIV-1 DLS structural model. *Nucleic Acids Research* 2012; **40**: 5012–5022. DOI: 10.1093/nar/gks156
150. Pini A, Falciani C, Bracci L. Branched peptides as therapeutics. *Current Protein and Peptide Science* 2008; **9**: 468–477.
151. Bryson DI, Zhang W, McLendon PM, *et al.* Toward targeting RNA structure: branched peptides as cell-permeable ligands to TAR RNA. *ACS Chemical Biology* 2012; **7**: 210–217. DOI: 10.1021/cb200181v
152. Parsons J, Castaldi MP, Dutta S, *et al.* Conformational inhibition of the hepatitis C virus internal ribosome entry site RNA. *Nature Chemical Biology* 2009; **5**: 823–825. DOI: 10.1038/nchembio.217
153. Dibrov SM, Ding K, Brunn ND, *et al.* Structure of a hepatitis C virus RNA domain in complex with a translation inhibitor reveals a binding mode reminiscent of riboswitches. *Proceedings of the National Academy of Sciences of the United States of America* 2012; **109**: 5223–5228. DOI: 10.1073/pnas.1118699109