Review

RNA as a target for developing antivirals

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The base of knowledge concerning RNA structure and function has been expanding rapidly in recent years. Simultaneously, an increasing awareness of the pivotal role RNA plays in viral diseases has prompted many researchers to apply new technologies in high-throughput screening and molecular modeling to the design of antiviral drugs that target RNA. While the two RNA viruses with the greatest unmet medical need, HIV and HCV, have been most actively pursued, the approaches discussed in this review are relevant to all virus infections. Both traditional small-molecule and large-molecule therapeutics, such as antisense, ribozymes, and interfering dsRNAs, have been described, and several molecules are under development for commercialization. The purpose of this review is to summarize the current state of the art in this field and to postulate new directions in the future.

Keywords: antiviral, RNA, computer-modelling, high-throughput screening

Introduction

When compared to other biological macromolecules, RNA is not a well-represented therapeutic target. However, the large number of RNA-protein interactions that have been described and the extremely diverse structures and functions of RNA suggest that it may be an under-exploited target. RNA structure resembles DNA in that it is composed of nitrogenous bases linked to hydrophobic sugar structures. However, RNA also mimics proteins in that it folds into complex structures that can create unique hydrophilic binding sites suitable for small molecules. These attributes give RNA very attractive characteristics for use in therapeutic intervention.

The many important human diseases caused by RNA viruses, including human immunodeficiency virus (HIV), hepatitis C virus (HCV), and respiratory syncytial virus (RSV), highlight the importance of developing new compounds that can target viral RNAs. Clinical studies in which viral RNA is targeted for HIV or HCV therapeutics have now been reported for two approaches: antisense (Gunzburg et al., 1999; Furukawa et al., 1997; Witherell, 2001) and ribozymes (Macejak et al., 2001). In addition, significant preclinical effort has been directed toward finding small-molecule compounds that target these two viral RNAs. For example, high-throughput screens (HTS) based on cell-free biochemical reactions have been developed that streamline the search for small molecule–RNA interactions. This approach has led to the discovery of new inhibitors of HCV translation (Wang et al., 2000; McKnight & Heinz, unpublished results). More recently, there have also been attempts at rational design of small peptides that bind to viral RNA structures using techniques such as mass spectrometry (Stannex-Lowery et al., 1997).

Viral RNA genomes as a target

Viral RNAs utilize small, structured RNA elements to regulate and promote the processes that result in multiplication of their genomes. For a variety of viral systems, genetic studies have clearly demonstrated the absolute requirement for defined RNA elements in carrying out key steps in RNA synthesis, transcriptional regulation, and protein translation. Most RNA elements involved in viral replication function as basic stem-loop units, are highly conserved, and predominantly reside in the 5′ and 3′ non-coding regions of the viral genome (Pogue et al., 1994). Due to the conserved nature of most of these elements, there is an expectation that any change in nucleotide sequence would result in an inability for the element to function. Indeed, even single nucleotide mutations can result in inactive elements, probably through misfolding of RNA structure (Pogue et al., 1994). The ability for RNA viruses, which lack polymerases with proofreading function, to undergo mutation is notoriously high (Domingo et al., 2002). Thus, careful selection of an appropriately conserved RNA target is needed to reduce the occurrence of resistance mutation against a selected antiviral.
Most RNA targets contain combinations of non-Watson–Crick base pairs, bulges and internal loops within the stem–loop structure. A good example of this simple type of RNA structure is the 59-nt transactivation responsive (TAR) element that resides in the 5′-LTR of the HIV-1 viral genome, and which interacts with the TAR transactivator protein in order to regulate viral gene expression (Figure 1A) (Dingwall et al., 1990).

A more complex HIV-1 RNA target is the Rev-responsive element (RRE) that functions primarily as an extended stem region that is joined to five additional hairpin loops and is present in the transmembrane domain of the env gene (Figure 2B). The Rev binds RRE and shifts protein synthesis so that the structural proteins are preferentially translated. Since the HIV-1 structural proteins are produced from incompletely spliced viral RNAs, the Rev protein acts to promote splicing and transport these RNAs from the nucleus to the cytoplasm (Emerman & Malim, 1998). The Rev protein binds with high affinity to a bubble structure located within the 351-nt RRE RNA in stem–loop II (Mann et al., 1994), thus making it an attractive target for antiviral intervention (BATTISTE et al., 1996; Peterson & Feigon, 1996). In addition, several groups have targeted the Tar–TAR RNA–protein interactions for the discovery and development of antiviral compounds (Yoo & Wilson, 1992; Bailly et al., 1996; Lim & Barton, 1997; Mei et al., 1997; HAMY et al., 1997) and Rev–RRE (Park et al., 1996; Wang et al., 1997; Xiao et al., 2001; Ratmeyer et al., 1996; ZAPP et al., 1997). These efforts will be discussed in more detail below.

The HCV internal ribosomal entry site (IRES) is another example of a complex RNA element that has recently become an accepted antiviral target (Figure 3C). The HCV genome consists of a positive-sense, single-stranded RNA genome that is translated as a polyprotein. The HCV IRES is a highly structured 345-nt region of RNA present at the 5′ terminus that is responsible for recruiting the cellular translation machinery to the RNA (Rjinbrand & Lemon, 2000). The HCV IRES sequence is highly conserved among all the many serotypes of the virus (Smith et al., 1997) and as a result has become a practical target for antiviral intervention. Numerous groups have created research platforms to search for inhibitors of the HCV IRES (Collier et al., 2002; Klinck et al., 2000; Lukavsky et al., 2000). As yet, only the antisense compound from ISIS (Witherell, 2001) and the ribozyme known as Hepazyme from Ribozyme Pharmaceuticals, Inc. (Maciejak et al., 2001) have been evaluated in clinical trials.

One other recently discovered viral RNA target that offers great potential as a unique approach to antiviral intervention is the picornavirus cis-acting replication element (cre, Figure 1D) (McKnight & Lemon, 1998; Gerbey et al., 2001). The cre is an approximately 33-nt long hairpin that resides not within the terminal 5′ and 3′ non-coding regions, but within coding sequence in the middle of the 7-kb viral genome. The structure and sequence of the cre are absolutely required for initiation of RNA synthesis (Paul et al., 2000; Yang et al., 2002), and since it also resides in and functions as coding sequence, the possibility for drug-resistant mutations is very low. The 5′-terminal cloverleaf RNA structure that also participates in regulating RNA synthesis of the picornavirus genome (Andino et al., 1993) is another reasonable target for antivirals specific for the picornaviruses. The advantage of designing compounds that would interfere with the function of the picornavirus cloverleaf or cre structures is apparent, and reports of their discovery are expected in the near future.

**Targeting viral RNA as functional genomic sub-domains: the power of genetics**

A crucial aspect of targeting viral RNAs for antivirals is the ability to identify individual, functional targets within the large genome. A component for any viral RNA inhibitor discovery platform is the confirmation that small RNA motifs remain biologically functional even when isolated from surrounding structures. The identification of individual RNA sub-domains such as Tar and the HCV IRES, which can function separately from their respective genomes, has greatly aided in the search for antivirals targeted to these RNAs. For example, studies that showed Tar could specifically interact with TAR directly led to suggestions that peptides or other small molecules could be designed to inhibit this important RNA–protein interaction (Tan et al., 1997).

Antiviral research efforts centred on the HCV IRES have relied heavily on surrogate systems where the IRES is fused to reporter or viral sequences for readout of translation efficiency (Wang et al., 2000; Maciejak et al., 2001; Lee et al., 2000). Toward this end, most drug-hunting efforts for the HCV IRES have relied on an IRES-firefly reporter gene construct that readily produces enzyme activity in *in vivo* or *in silico* translation assays. This HTS approach to targeting the ribosomal ‘machinery’ involved in protein synthesis opens new opportunities for novel therapeutic mechanisms. However, the surrogate approach to studying a particular RNA target outside of an actively replicating viral genome does have its limitations, as the opportunity to monitor the development of resistant mutations is not available. In contrast, it is possible to use surrogates in reverse genetics studies on mutations purposely engineered into the HCV IRES (Zhao & Wimmer, 2001).
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**Figure 1.** Secondary structure of RNA antiviral targets

(A) The 59-nt HIV TAR element that is encoded immediately 3′ to the transcription start site in the LTR promoter (Muesing et al., 1987). (B) The 351-nt RRE RNA (Mann et al., 1994). (C) The 341-nt HCV 5′ UTR that contains the IRES structure (Honda et al., 1999). The individual subdomains are labelled and the AUG initiator codon is indicated in grey. (D) The cre elements that are involved in controlling picornavirus RNA replication are shown and include those for rhinovirus type 14 (Yang et al., 2002) (left) and poliovirus (Paul et al., 2000) (right).
Proof of concept: antibiotics, antisense, ribozymes and RNAi

In contrast to many early-stage efforts in antiviral discovery, developing viral RNA as an antiviral target has few major theoretical obstacles to overcome. Proof of concept has already been provided by the success of several classes of antibiotics that have been shown to work by binding to RNA or RNA–protein complexes. Of these, the aminoglycosides seem to be the most intriguing as they target binding sites on the RNA that appear to be difficult for bacterial cells to change by mutation (Amyes, 1998). In addition, several types of large molecules that target RNA have been used successfully in both preclinical and clinical settings as therapeutic agents against viral infections (Table 1).

Aminoglycosides as antivirals

Aminoglycosides offer a number of valuable lessons in the design of RNA-based antivirals. Since they have general RNA binding properties, aminoglycosides have been used as models or scaffolds for developing antiviral platforms (Litovchick et al., 2001; Hermann & Westhof, 1999; Hendrix et al., 1997; Wang et al., 1998). Neomycin was found to be a potent inhibitor of Tat binding to TAR, and footprinting experiments showed that neomycin binds in the bulge region of TAR (Wang et al., 1998). Thus, a potential for a structure–activity relationship (SAR) study exists with neomycin-like compounds that inhibit Tat–TAR interactions.

The ability to target the critically important Rev–RRE interaction was demonstrated by Zapp and colleagues (Zapp et al., 1997). A panel of aminoglycosides was found to inhibit Rev–RRE complex and block virus production in tissue culture. This effort led to a SAR analysis of similar neomycin B compounds created by combinatorial chemistry in an effort to refine inhibitors of the RRE and Rev...
Table 1. Antiviral compounds in commercial development

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<th>Product/Approach</th>
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<td>Small molecules identified by structural modelling</td>
<td>HCV IRES</td>
<td>Ribotargets</td>
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<td>Enzo Biochem</td>
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<td>Phase I</td>
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<td>Next-generation ribozymes</td>
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<td>Ribozyme Pharma Inc.</td>
<td>Preclinical</td>
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<td>2′-5′ oligoadenylate-linked antisense</td>
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Antisense GEM 92 (oral delivery) HIV

Next-generation ribozymes HCV IRES Ribozyme Pharma Inc. Preclinical

Antisense HGTV43 expressed from Stealth Vectors HIV Enzo Biochem Phase I

Small molecules identified by structural modelling Various viruses IBIS Preclinical

Antisense #14803 HCV IRES ISIS Phase II

Small molecules identified by mass spectroscopy and modelling Various viruses IBIS Preclinical

Product/approach Viral target Company Status

Antiviral compounds in commercial development*

protein interaction (Zapp et al., 1997). Hendrix and co-workers (Hendrix et al., 1997) studied a related set of aminoglycosides using surface plasma resonance (SPR). A hypothesis was established by Hendrix et al. that implicating the sugars linked to neomycin core structures as scaffolds that present hydrogen moieties in a proper spatial aspect that promotes RNA binding; additional work using neomycin B as a scaffold subsequently confirmed this idea (Alper et al., 1998; Wong et al., 1998). Neomycins and other aminoglycosides have also been shown to be potent inhibitors of the viral RNA catalytic activity of human hepatitis delta virus ribozyme (Rogers et al., 1996), as well as efficient binders of the HIV RNA packaging signal (McFike et al., 2002).

While most existing aminoglycosides do not provide sufficient specificity for development as useful antivirals, SAR and modelling studies involving systematic replacement of sugars with different scaffolds and functional groups using aminoglycosides as a backbone may prove to be an important complement for the field of small-molecule viral RNA recognition.

The extreme specificity of antisense offers real therapeutic promise for virus infections

Antisense molecules are designed to be transported into the cell where they bind to complementary RNA sequences, forming a nucleic acid duplex. Cellular enzymes then degrade the RNA section of the duplex, which releases the antisense molecule for further binding to other complementary RNAs (for a review of the antisense approach, see the references by Agrawal & Zhao, 1998, or Branch, 1998). The antisense method of targeting RNA offers specificity and precise control over the point of attack. That is, one can selectively target antisense sequences that are unique to the viral gene whose expression is to be inhibited. This is a key feature for diseases like viral infections that usurp normal cell functions in their disease process. In general, it has been difficult to devise small-molecule therapeutic strategies against these diseases without also hampering normal cell functioning. Therefore, an antisense therapeutic should theoretically have fewer side effects than many other antiviral agents. Another advantage to an antisense method is that it interferes at the source of the disease by blocking the formation of unwanted proteins rather than stopping these proteins from functioning. Since viral proteins tend to be multifunctional, this approach more effectively shuts down any effect of the protein at the viral, cellular or organ level.

Unfortunately, antisense methods are susceptible to numerous pharmacological problems related to nonspecific immune responses, sensitivity to RNases and low cellular uptake (Orr, 2001; Hanecak et al., 1996). Moreover, chemical modifications of the antisense molecule typically used to alleviate these problems can introduce new toxicities (Brown-Drivers et al., 1999). Finally, it is possible that many viral RNA sequences will not be accessible to antisense agents due to their highly structured nature or tight association with proteins and cellular membranes (Sharp, 2001).

In a traditional approach, ISIS Pharmaceuticals and Hybridon, Inc. have developed phosphorothioate oligonucleotide antisense molecules for direct administration therapies. The phosphorothioate chemistry, in which sulfur atoms replace non-bridging oxygens on the molecule, provides a margin of protection from breakdown by nucleases. Phosphorothioate oligonucleotides have been shown to exert their antisense effects by hybridizing to any mRNA, resulting in activation of RNase H, which then cleaves the target RNA strand (Conklee, 2000). This type of mechanism allows the antisense approach to be applied to viral infections where RNAs function only as intermediates during replication. As such, DNA viruses such as papillomavirus and herpesvirus have also been studied extensively as targets for antisense therapy (Lewis et al., 2000; Anderson et al., 1996). ISIS 2922, which is an oligonucleotide complementary to a segment of the IE2 mRNA that encodes a protein critical for replication of cytomegalovirus, became the world’s first antisense compound to be approved for commercial use in the USA (Orr, 2001; Azad et al., 1993).
the clinic (Hancek et al., 1996; Brown-Driver et al., 1999, Zhang et al., 1999; Lima et al., 1997). This compound is directed against the IRES component of the virus genome and serves to block translation of its RNA.

Intracellular immunity that would result in efficient inhibition of HIV-1 genes would be an important step toward clinical gene therapy for the HIV-1 disease, AIDS (Baltimore, 1988). One unique example is work reported by Enzo Biochem, a group investigating and developing anti-sense as a potential gene therapy approach against AIDS (Gunzburg et al., 1999). The company's novel vector delivery technology (known as Stealth Vector), which is configured to express independent antisense RNA sequences directed against two functional HIV-1 RNA elements ( TAR and RRE), is showing promise and is in clinical trials (Gunzburg et al., 1999). Stealth Vector localizes primarily in the cell nucleus, where it is most effective in inhibiting HIV-1 growth by blocking RNA production and processing. Importantly, the vector is also designed to be invisible to the immune system. However, the therapy is not straightforward, as it must be applied to immune cells removed from the patient and altered ex vivo by the introduction of antisense-producing genes. Following re-implantation in the patient, altered cells are expected to propagate HIV-resistant immune cells via the expression of antisense sequences. In contrast to currently used antivirals targeted at viral reverse transcriptase or protease, this vector-based antisense approach is designed not to require repeated applications and should completely overcome the stability and cost of production issues of direct administration of antisense molecules.

In another novel approach to the antisense technology, Ridgeway Biosystems, Inc. has developed an antisense technology that involves the administration of 2′,5′-oligoadenylate covalently linked to antisense (2′5′A-antisense) and that results in the selective cleavage of targeted oligoadenylate covalently linked to antisense (2′-5′A-anti-sense) RNA (Capodici et al., 2002). A 2-5A-antisense approach has also been successfully applied to HIV-1 (Player et al., 1998). However, it should be noted that not all of the antisense molecules functioned in reducing HIV-1 replication, confirming the hypothesis that certain regions of a viral RNA genome, including those believed to interact with cellular proteins, may not be accessible to antisense molecules (Sharp, 2001).

Antisense oligonucleotides with catalytic activity

Ribophyme Pharmaceuticals, Inc. (RPI) has also devised a novel antisense technology for developing antivirals. Based on the catalytic activity of synthetically designed antisense molecules, ribozymes are designed as human therapeutics to recognize, bind and digest viral RNA sequence (Usman & Blatt, 2000). In addition, RPI has shown that proprietary chemical modifications allow ribozymes to be stable and active in human serum for several days (Usman et al., 1996). RPI has been actively developing a ribozyme for treating HCV patients known as Heptazyme (Lee et al., 2000); a second-generation molecule is currently undergoing clinical testing.

Double-stranded RNA molecules that can initiate gene silencing

A final proof of concept that viral RNAs can be efficiently targeted for therapeutic intervention is a new approach called RNA interference (RNAi). RNAi was first described in lower eukaryotes when it was observed that the response to double-stranded RNA (dsRNA) resulted in potent sequence-specific gene silencing at the post-transcriptional level. The RNAi pathway has since been recognized as a conserved biological mechanism that spans across almost the entire animal and plant kingdoms. RNAi is a two-step process. In the first step, the dsRNA that triggers the silencing response is cleaved by cellular RNase-III-like nucleases into small interfering RNAs (siRNAs) that are 21–23 nt in length (Sharp, 2001). In the second step, the siRNAs are incorporated into a nuclease-containing complex, known as RNA-induced silencing complex (RISC), which digests mRNAs that are homologous to the siRNA (Sharp, 2001).

The end result is suppression of gene expression.

Several groups have begun to apply RNAi to viral RNA targets. Griffin et al. showed that siRNAs can be used to protect human tissue culture cells from poliovirus infection (Griffin et al., 2002). However, the virus was quickly able to mutate to overcome the interference, suggesting that this approach may require targeting of conserved viral sequences that are not amenable to mutation. Many groups have successfully inhibited HIV in cell culture using RNAi (Capodici et al., 2002; Coburn & Cullen, 2002; Novina et al., 2002; Jacque et al., 2002). One of these studies even demonstrated that when siRNAs are expressed within cultured cells, the cells become refractory to subsequent infection by HIV (Jacque et al., 2002). This suggests that the use of RNAi as a therapeutic might have an additional prophylactic application. Other laboratories have gone on to show inhibition of HIV when RNAs is targeted against host cellular RNAs that are required for the virus infection (Novina et al., 2002; Martinez et al., 2002). Finally, recent studies have shown that RNAs can inhibit HCV RNA replicon
Using RNA structure to target antivirals by computer modelling

Biologically, RNA has functional characteristics that fall between DNA and protein. Messenger RNAs are like DNA in that they serve as carriers of genetic information. Other RNAs, such as transfer and ribosomal RNAs, behave much like proteins in a structural-like fashion. RNA molecules depend on both structure and sequence for function and, as demonstrated by ribozymes, can even have enzymatic activities. However, even though the need for RNAs to adopt structure has long been recognized, current technologies have been much better suited to the analysis of protein structure than of RNA. This fact is easily realized by comparing the over 8000 coordinates that have been deposited in the Protein Data Bank, whereas the number for RNA structures is only on the order of 100.

The potential for small-molecule recognition based on molecular shape is enhanced by the flexibility of RNA. Binding of molecules to specific RNA targets can be predicted by global conformation and the distribution of charged, aromatic and hydrogen-bonding groups placed in a relatively rigid scaffold. Validated procedures for using computer programs to dock small molecules to RNA structures are now available (Chen et al., 1997; Leclerc & Cedergren, 1998). This approach relies heavily on 3-dimensional models of RNA targets that can be based on X-ray, NMR or even phylogenetic data. In analysing these data for virtual screening, one of the first steps is to propose regions that may be amenable to small-molecule binding. This can be done manually or by using a computer program that identifies potential binding pockets on molecules such as RNA. Potential binding sites are referred to as pockets, cavities, cups, bowls and so on, all formed by tertiary structure that goes beyond the typical major and minor groove regions commonly found in helical RNA. There may also be experimental information regarding interaction between RNA and proteins or small molecules that can be exploited. Once interesting sites are identified, the docking procedure is typically a three-step process. The shape and electronic character of the docking site is distinguished first. This is followed by placing test ligands in the site and sampling hundreds to thousands of orientations and conformations (poses). Lastly, an estimate of the binding affinity (score) is calculated for the best fitting poses. The docking approach can be used to select top-scoring ligands for testing in high- to medium-throughput screens.

Fortunately, the number of potential viral RNA targets in which 3-dimensional structures exist is rapidly growing and new technologies have been established that increase NMR data analysis tremendously, thus speeding up solution structure determination (Lukavsky & Puglisi, 2001). Among the medically relevant viruses, several NMR structures are available: the HIV TAR and packaging signals (Amarasinghe et al., 2001; Amarasinghe et al., 2000; Zeffman et al., 2000; Nifoni et al., 2000; Lind et al., 2002), the HBV packaging signal (Fiodelli et al., 2002) and various sub-domains of the HCV IRES (Lukavsky et al., 2000; Collier et al., 2002; Klink et al., 2000; Jabin et al., 2000). In Figure 2, four sub-domains of the HCV IRES are shown that could be used in virtual screens as described above.

Finally, X-ray crystallographic determinations of crucial RNA subunits representing the HIV Rev and Tar elements (Ippolito & Steitz, 2000; Ippolito & Steitz, 1998), as well as the stem–loop IIIabc structure of the HCV IRES have been reported (Kief et al., 2002). The ability to utilize NMR and X-ray crystallography data on viral RNA structures through computer-aided in silico screening is now becoming routine. Several published reports describe screening efforts directed towards targeting 3-dimensional viral RNA structures with small-molecule binders (Hermann and Westhof, 1999; Leclerc & Cedergren, 1998; Wilson et al., 1996; Fildler et al., 2000; Du et al., 2002). While no therapeutic application has yet been reported, the drug-hunting community is anxiously awaiting the first true antiviral discovery to result from these efforts.

High-throughput screening possibilities

A mass spectrometry approach

A drug discovery approach using an HTS system based on mass spectrometry has been proposed (Hofstraad et al., 1999). This scheme can rapidly determine the exact chemical composition and mass of compounds that bind to an RNA target as well as the binding constants (Kd) of compounds for the RNA target (Sannes-Lowery et al., 2000). With this system, the authors claim that thousands of compounds can be screened for binding capacity within several weeks time. Once strong binders are determined it is envisioned that an SAR can be devised around these, and coupled with a biological assay, a complete RNA targeting platform is formed that theoretically can quickly result in replication in cell culture (Kapadia et al., 2003; Rundall et al., 2003; Seo et al., 2003). However, the utility of RNAs on stable HCV replica cell lines is still a relatively unexplored area. Moreover, the application of RNAs in a clinical setting has not been demonstrated for any virus infection. While the development of RNAs as an antiviral is in its infancy and may suffer from delivery or cost of production issues, it clearly has some advantages over antisense. The double-stranded molecules are very stable and special chemistry is not required to render them resistant to nuclease digestion (Sharp, 2001). Thus, we anticipate that RNAs will prove to be a valuable new approach for developing viral therapeutics that target RNA.
An in vivo reporter-based approach

Wang et al. have devised a cell-based HTS that utilizes a dual reporter system consisting of cellular capped chloramphenical acetyl transferase gene followed by firefly luciferase that is under translational control of the HCV IRES (Wang et al., 2000). A screen of 132,000 compounds resulted in the discovery of several phenazine and phenazine-like molecules that specifically inhibited IRES translation activity. A subsequent SAR established that the phenazine core and polar substitutions on the molecule are crucial for potent activity, indicating again that there is an importance for unique hydrogen-bonding arrangements in designing small molecules that are specific for RNA. However, there is no indication that any of these molecules are being developed further.

An in vitro reporter-based approach

Figure 3. An in vitro reporter-based approach to screening HCV IRES inhibitors

Shown are schematic diagrams of the two RNAs constructed for HTS in the in vitro assay that is described in the text. (A) HCV IRES-firefly luciferase RNA. (B) Capped firefly luciferase control mRNA. Note the β-globin leader sequence that precedes the luciferase coding sequence in order to provide appropriate ribosomal scanning from the CAP complex.

We and others have utilized the generation of synthetic RNAs that contain a 5′ terminal HCV IRES fused to the firefly luciferase coding sequence. A polyadenylate tract that is present at the 3′ end of the molecule enhances stability of the message (Figure 3A). Based on work with poliovirus in vitro translation systems (Barton et al., 1996), one can use a modified in vitro translation protocol using HeLa cell lysates such that HCV IRES-mediated translation can be readily monitored in the presence of compound. In our HTS, we synthesized a control capped mRNA to effectively triage the compounds (Figure 3B). Compounds that inhibited both capped and IRES-mediated translation were eliminated, thus enriching for selectivity against the IRES. We also eliminated compounds that demonstrated a non-specific inhibition of the firefly luciferase enzyme.

Optimizing small-molecule libraries for HTS

One area of concern with HTS approaches is the diversity of the compound library used in the screen. While several small molecules that interact with RNA have been described, most of these do so by intercalation or other non-specific interactions that would lead to cellular toxicity (Hermann & Westhof, 1998; Wilson & Li, 2000). Utilizing a biased library is one approach to thwarting the non-specificity seen with most organic small-molecule libraries. For example, one can start with a panel of known compounds that interact specifically with RNA and chemically expand on these to form a diverse platform library. Many specific RNA-interacting small molecules have been described that could be used in this approach (for a review see references Wilson et al., 1996; Hermann & Westhof, 1998; Gallego & Varani, 2001; Wilson & Li, 2000; Opalinska & Gewirtz, 2002). In addition, given the success of antibiotics that bind RNA, the inclusion of natural products should also be considered in designing compound libraries biased towards RNA interaction. In addition, the concept of incorporating counter-screens against non-specific targets is critical for culling out the many nuisance compounds that non-specifically target RNA.

Borrowing from HTS results

Based on the findings from a study in which RNA aptamers were selected from a random library in an HTS, cyanocobalamin (vitamin B12) was reported to have one of the strongest binding affinities yet reported for a small molecule to RNA (Kd=88 nM) (Loesch & Suwak, 1994). Since cobalamins, the class of compounds to which vitamin B12 belongs, are found most abundantly in the human liver, Lott and colleagues speculated that B12 may bind to the HCV IRES and serve as a natural inhibitor of viral translation (Lott et al., 2001). These authors then demonstrated that cyanocobalamin inhibited the HCV IRES-dependent translation of a reporter gene in vitro in a dose-
and colleagues has identified aptamers targeted against TAR sub-domain within the RRE, they also contained novel aptamers for the Rev protein exhibited some similarity to a naturally occurring guanosine–guanosine pair, bound Rev proteins to the IRES RNA, but instead bound to the IRES-80S ribosome complex, stalling the initiation of translation only at this point (Takyar et al., 2002). Nevertheless, this finding points to the value of exploring molecules known to have RNA-binding activity as potential starting points for antivirals.

**SELEX and aptamers**

Several studies have used in vitro systematic evolution of ligands by exponential enrichment (SELEX) to isolate minimal, high-affinity RNA ligands (aptamers) to a viral protein that might serve as decoys and inhibit activity. One analysis revealed that although some of the tightest binding aptamers for the Rev protein exhibited some similarity to a sub-domain within the RRE, they also contained novel sequence and structural motifs (Giver et al., 1993). These authors designed and synthesized a short (37 nt) RNA molecule embodying the best binding sequence, and found that this RNA ligand exhibited three- to fivefold tighter binding than the full-length wild-type RRE element. RNA ligands that contained non-Watson–Crick base pairings, and which could be modelled as isosteric to an important naturally occurring guanosine–guanosine pair, bound Rev up to 160-fold tighter than elements that contained canonical Watson–Crick pairings or non-isosteric mismatches. These results support the hypothesis that Rev recognizes structural features associated with a non-Watson–Crick base pair.

In a similar studies, the SELEX procedure was used to isolate a number of TAR RNA variants from a pool of 1013 RNA sequences (Marozzi et al., 1998). Compared to the primary and presumptive secondary structures of the wild-type TAR, the selected RNA variants showed an almost complete sequence conservation of the Tat-binding domain. Marozzi and colleagues further showed that the TAR variants had lost the ability to bind cell factor(s) in vitro and thus should represent useful decoys for the inhibition of HIV-1 replication. Additional work by Darfeuille and colleagues has identified aptamers targeted against TAR that inhibit Tat-based transcription (Darfeuille et al., 2002a; Darfeuille et al., 2002b). However, an application of these molecules that would demonstrate any antiviral activity has not been forthcoming.

A group at Novartis used a novel compound library consisting of oligomers containing both peptide and peptide residues (peptoids are isomers of peptides in which all the side chains are carried by the backbone nitrogen, making them more flexible than peptides) in a SELEX-based approach for finding TAR inhibitors (Hamby et al., 1997). Of interest to therapeutic use is the fact that peptoids have the advantage of being resistant to enzymatic degradation. The combinatorial chemistry approach resulted in the discovery of a single compound with high affinity for TAR RNA, CGP64222 (only 12 nM was needed to inhibit formation of the Tat–TAR RNA complex by 50%). CGP64222 also exhibited antiviral activity in HIV-infected cell cultures (Hamby et al., 1997).

Finally, Urvil et al. used SELEX to isolate high-affinity RNA aptamers that bind to the HCV NS3 protease domain (Urvil et al., 1997). Starting from an RNA pool that had a random sequence core of 12–18 nt, aptamers that bind specifically to the NS3 protein were selected. A single aptamer, 10G-1, was found that bound to the NS3 protein with a binding constant of 650 nM and inhibited the proteolytic activity in vitro. By phosphate-modification–interference, the phosphate residues critical for the binding of 10G-1 to NS3 were found to lie within the selected regions of the aptamer; binding involved electrostatic contacts with these phosphates. The authors are continuing studies that use the NS3-binding region in 10G-1 as a basis for designing more potential inhibitors of the NS3 protein.

**Concluding remarks**

The next stage of targeting RNA for antiviral compounds must not only focus on the hunt for new antivirals, but also on how to develop discovery platforms that give rise to lead compounds with high affinity and specificity. The rules for establishing the characteristics of a good inhibitor of RNA function are slowly being documented. FDA-approved therapeutics such as antibiotics and antisense already exist that target bacteria or host RNAs involved in cancer (Druker et al., 1996), and an antisense against a cellular protein kinase involved in non-small cell lung tumour has reached phase III clinical trials (Yien et al., 1999). As such, whether an antiviral targeted to RNA is based on a small molecule or a large molecule such as antisense or RNAi, the promise of establishing commercially available antivirals based on the viral RNA target now seems very close to reality. However, this promise can only be realized after issues such as compound specificity and bioavailability are overcome.

**References**


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