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HIV-1 drug discovery: targeting folded RNA structures with branched peptides

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Human immunodeficiency virus type 1 (HIV-1) is an RNA virus that is prone to high rates of mutation. While the disease is managed with current antiretroviral therapies, drugs with a new mode of action are needed. A strategy towards this goal is aimed at targeting the native three-dimensional fold of conserved RNA structures. This perspective highlights medium-sized peptides and peptidomimetics used to target two conserved RNA structures of HIV-1. In particular, branched peptides have the capacity to bind in a multivalent fashion, utilizing a large surface area to achieve the necessary affinity and selectivity toward the target RNA.

Introduction

Human immunodeficiency virus (HIV) is a retrovirus that has been isolated and studied by scientists for over three decades.[1] It is responsible for the development of acquired immunodeficiency syndrome (AIDS), a condition that ultimately destroys the immune system of the persons infected, eventually leading to death. From its discovery in the early 1980s, it has taken the lives of approximately 25 million people, and over 33 million are currently infected.[1b,2] Its nature as a retrovirus, as well as its high rate of replication and mutation, has kept HIV/AIDS in the forefront as a continued worldwide epidemic that requires diligent efforts in discovering alternate methods of treatment.

Upon discovery of HIV, initial research focused on understanding the mechanism of infection (Fig. 1).[3] HIV infects CD4+ T cells, as well as other lymphatic cells, by fusing largely to the cells' receptors on the cell membrane through gp120 and gp41 proteins.[4] As HIV is a retrovirus, it must first reverse transcribe its RNA to DNA *via* reverse transcriptase, and once this is accomplished, the enzyme, integrase, incorporates the DNA into the host cell's genome.[5] The DNA is then transcribed back to RNA, and upon entry into the cytoplasm the genetic information is translated into proteins.[4a] HIV-1 protease cleaves the proteins and with use of export factors such as Crm-1, eIF-5A and Ran-GTP, the newly translated viral proteins are reencapsulated and released to repeat the viral cycle.[6]

Due to the rapid spread of HIV, many different anti-retroviral therapy (ART) drugs have been designed to combat CD4 cell infection and reduce viral loads. By the mid 1990s, nucleoside/nucleotide reverse transcriptase inhibitors were approved by the FDA for treatment of HIV, and a few years later non-nucleoside reverse transcriptase inhibitors were introduced. The intent of both inhibitors is to prevent HIV from replicating by preventing the viral RNA from being transcribed to DNA, one by terminating replication through insertion of a faulty nucleoside/nucleotide, and the other by binding HIV-1 reverse transcriptase, respectively.[7] In the early 2000s, protease and fusion inhibitors were introduced as new ART drug classes. Protease inhibitors function by blocking the action of HIV protease, preventing the cleavage and subsequent packaging of viral proteins,[7a,8] while fusion inhibitors prevent HIV from entering CD4 cells by mimicking a portion of the gp41 protein, inhibiting the formation of the helical bundle necessary to allow the fusion of HIV with the host cell membrane.[9] In 2007, integrase inhibitors were approved by the FDA; these prevent the viral DNA from becoming incorporated into the cell's genome by binding to the enzyme.[7a] CCR5 receptor antagonists were also approved as a drug class that binds to CCR5, a co-receptor on the surface of CD4 cells that interacts with HIV to allow for binding of the virus to the cell.[9]

The number and variety of drugs developed to treat HIV testify to the immense difficulty in treating the retrovirus. This is due to a number of factors, including genetic recombination from the two genomic copies in each viral capsid *via* reverse transcriptase,[10] the high mutation rate of reverse transcriptase, and fast replication of the virus.[3,11] Combinations of ART drugs taken together, known as Highly Active Anti-retroviral Therapy (HAART), are employed as a means of combating drug resistance,[3] but issues such as adverse side effects and maintaining strict dosing regimens remain problematic to treating HIV. Therefore, new drugs that target HIV-1 through new modes of action are needed as next generation therapeutics.

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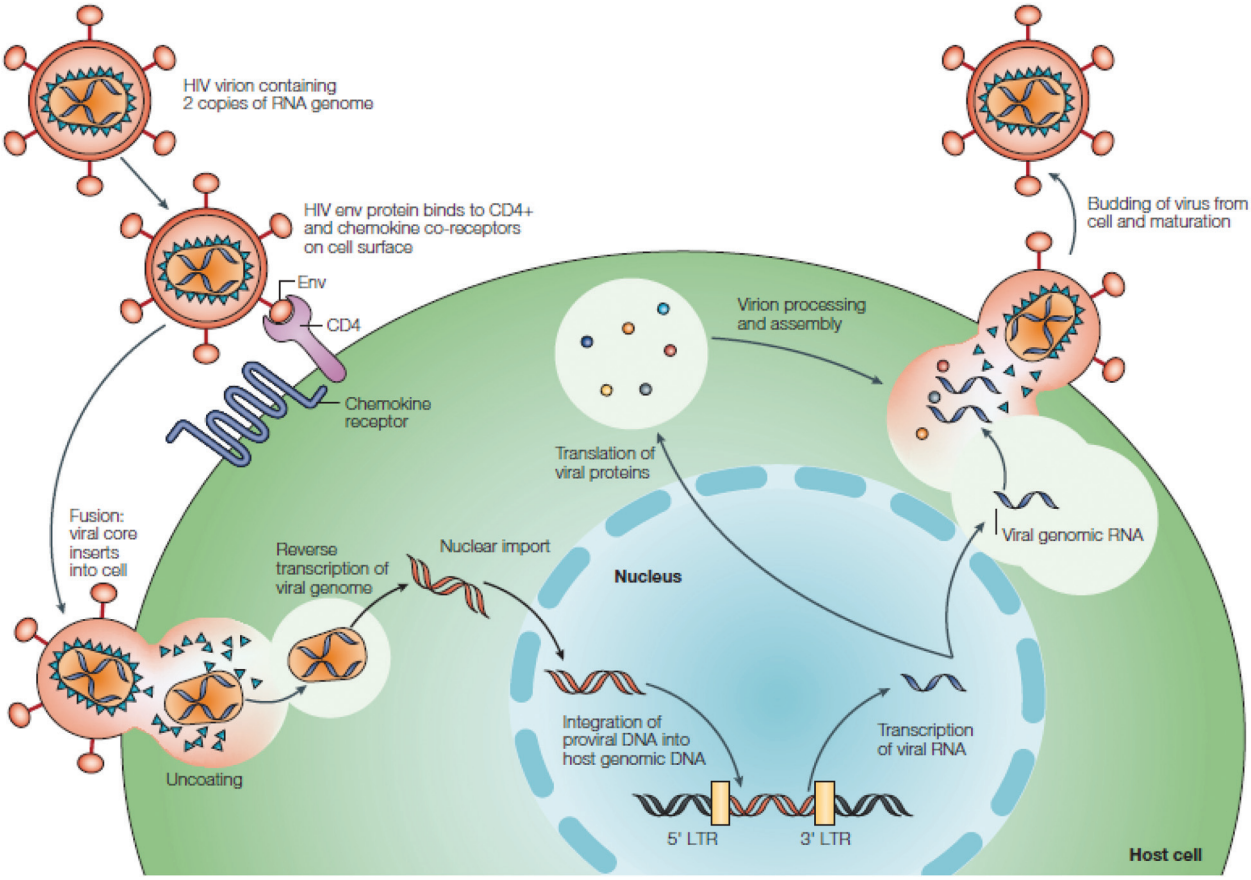


Fig. 1 HIV replication cycle. Reprinted from ref. 3 with permission from Nature.

Recognition of RNA as a therapeutic target

RNA serves many critical biological functions, from transfer of genetic information to regulating roles in the cell, such as transcription, translation, catalysis, as well as splicing.[12] A unique facet of RNA is its exquisite three-dimensional architecture derived from secondary structural elements such as hairpin loops, bulges, stems, turns, and pseudoknots, which minimize the energy of the structure. Also, the structure of RNA differs from DNA in that the major groove of A-form RNA is deeper and narrower than the B-form of DNA and the minor groove is shallower. This tertiary structure allows for binding interactions that could impart selectivity towards certain ligand constructs; thus, both the inherent functions and structure of RNA make it an ideal therapeutic target.[13] However, with the exception of antibiotics that work by binding to portions of rRNA,[14] and RNA gene suppression by antisense technology,[15] there has been limited success in developing small-molecule ligands that selectively target RNA.[16] This is due to several factors, including the large targetable surface area of most RNAs, the high binding affinity of endogenous ligands towards the RNA, permeability and stability of siRNA ligands, and conformational dynamics that make binding to as well as crystallizing a particular structure of RNA in solution difficult.[15,17]

In order to surmount these challenges, *in silico* studies have been employed to virtually screen ligands against various RNA motifs, and certain ligand-RNA dynamics have been studied using nuclear magnetic resonance and molecular dynamics studies.[16b,18] Another method is to perform a high-throughput screen using chemical libraries, in which a large number of diverse ligands can be screened against various RNAs with rapid turnover of results.[19] Several RNAs have been well-studied as therapeutic targets, including viral RNAs such as the HIV-1 dimerization initiation site (DIS)[20] and the HCV internal ribosome entry site (IRES),[21] as well as expanded nucleotide repeats r(CCUG) involved with the development of myotonic dystrophy type 2.[22] Herein, we focus on the review of medium-sized peptides and peptidomimetics used in targeting two conserved RNA structures of HIV-1: the transactivation response element (TAR) and rev response element (RRE) RNA, as well as the utilization of branched peptide scaffolds in therapeutics and our contribution in this field.

HIV-1 TAR RNA as a therapeutic target

HIV-1 TAR RNA has been widely investigated due to its critical role in HIV-1 replication. TAR RNA is a highly conserved 59 base pair sequence located at the 5′ end of transcribed HIV-1 RNA. The secondary structure reveals a double-stranded

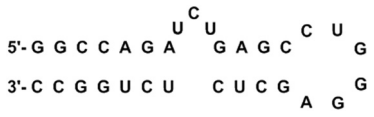


Fig. 2 Secondary structure of TAR RNA.

stem that contains a hexanucleotide loop as well as a three nucleotide bulge UCU (Fig. 2), through which the arginine rich motif (ARM) of the transcriptional activator protein Tat binds; this leads to further binding of cofactors cyclin T1-ckd1 and cyclin-dependent kinase 9 (CDK9), and the resulting complex promotes efficient transcription elongation of the RNA from the long terminal repeat.[23] Without the formation of a Tat–TAR complex, the rate of viral transcription is minimal, leading to the production of fragmented transcripts.[24] Therefore, disrupting the Tat–TAR interaction is a utilized strategy to interrupt viral replication, and has been pursued through the use of a wide variety of ligands such as intercalators,[25] aminoglycosides,[26] small molecules,[27] siRNA,[28] and nucleic acids.[29]

Peptides and peptidomimetics have also been used as medium-sized molecules to disrupt Tat–TAR interactions. A Tat-derived linear analog, RKKRRQRRK, was shown to compete with Tat for TAR, inhibiting the virus at the post-transcriptional level.[30] Concomitantly in the 1990s, Hamy and co-workers employed the use of peptoids and D-amino acids in a combinatorial library to generate inhibitors containing unique secondary structures. One of the first peptidomimetics developed was a hybrid peptoid/peptide **CGP64222**, which was shown to inhibit formation of the Tat–TAR complex at nanomolar concentrations by inducing a conformational change of the RNA upon binding (Fig. 3).[31] This compound was also shown to block viral entry through interaction with a CXC-chemokine receptor 4 co-receptor, making **CGP64222** a dual-acting HIV-1 inhibitor.[32] In 2000, Friedler and co-workers synthesized cyclic peptides with an arginine-rich scaffold to generate ARM mimetics, with one compound **Tat11** shown to inhibit nuclear import and disrupt Tat-RNA binding.[33] Cyclic peptides were also investigated by inducing a β-hairpin turn through use of a D-Pro-L-Pro motif.[34] Several of these cyclic peptides bound TAR in the low nanomolar regime, with one compound **L50** (cyclic L-ProRVRTRGKRRI-D-Pro) displaying a

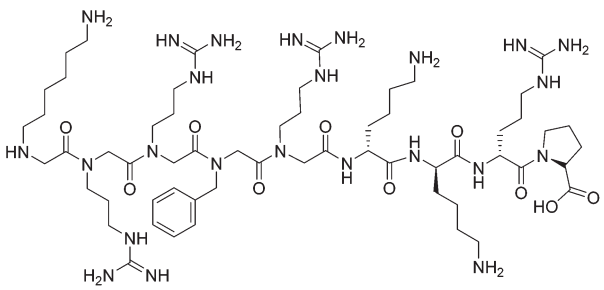


Fig. 3 Peptoid/peptide hybrid CGP64222.

K_d of 1 nM and an IC_{50} of 250 nM, inhibiting both reverse transcription and Tat-dependent transcription.[35]

HIV-1 RRE RNA as a therapeutic target

The continued struggle to combat HIV has led to great interest in also examining the rev response element (RRE) RNA as a potential drug target. RRE is a highly conserved region in the HIV-1 genome, consisting of approximately 351 nucleotides in the *env* gene.[36] RRE interacts with a Rev protein, also encoded in the *env* region, to allow for transport of singly spliced and unspliced mRNAs from the nucleus into the cytoplasm with complexation of nuclear export factors such as Ran-GTP, eLF-5A and Crm-1.[37] Unspliced mRNAs are required for translation of *gag* and *pol* genes in order to both encode structural proteins for packaging as well as to serve as the genome for new viruses.[37,38] Thus, disruption of the Rev–RRE interaction would serve to inhibit the replication of HIV-1, making RRE a suitable drug target. RRE contains a high affinity binding site, stem IIB, where a Rev dimer initially binds, generating cooperative binding that extends to stem IA (Fig. 4). Frankel and co-workers proposed models showing that the hydrophobic portion of Rev bound with itself to form a V-shaped dimer, while the ARM segment interacted with the RRE RNA.[39] Recently, this model was expanded upon with the report of the RRE–Rev complex in solution, revealing an "A" shape structure of RRE, with Rev dimers cooperatively binding on the "leg" portions that correspond to stems IIB and IA (Fig. 5).[40]

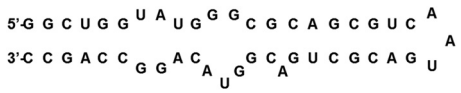


Fig. 4 Secondary structure of RRE IIB.

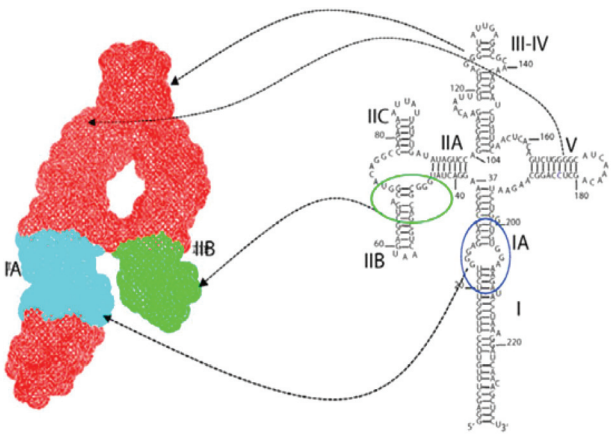


Fig. 5 Right: secondary structure of full-length RRE RNA with domain locations; left: SAXS topological structure, with the high affinity binding site IIB in green and oligomerization site between IIB and IA (cyan) legs. Reprinted from ref. 40 with permission from Elsevier.

Similar ligands have been designed to disrupt Rev–RRE binding, including aminoglycosides,[41] small molecules,[42] peptide nucleic acids,[43] nucleobase amino acids,[44] metallopeptide complexes,[45] and bifunctional compounds such as aminoglycoside-acridine[41b,46] and aminoglycoside–PNA conjugates.[47] Also, developments have been made to covalently link drug-RNA targets; for example, *hv* was utilized to covalently photocrosslink a diazinine-containing Met analogue with RRE RNA.[48] However, there has been a greater focus in the use of α-helical peptidomimetics to target RRE RNA. Using a highly specific RRE-binding peptide R_6QR_7, Kaplin and co-workers induced alpha helicity in linear peptide analogs through macrolactam constraints generated from the amide bond formation at Lys and Asp residues, generating a hit compound Ac-RRR**E**RQR**K**RRR-OH with a K_d of 45 nM and a 26-fold selectivity for RRE.[49] Yu and co-workers also pursued the development of peptidomimetics by first designing *N*-methylated peptides modeled from calmodulin (Table 1, **1**). A compound containing two *N*-methylated lysines (K*), LKK*LLKLLK*KLLKLKG, had a K_d of 9.1 nM and showed a five-fold selectivity for RRE against a mixture of tRNAs as well as TAR RNA.[50] Further modifications of calmodulin included the introduction of acridine (K**) at the epsilon amine of lysine as an intercalator, and mono- and bis-acridinylated peptides were examined as RRE binders. It was found that the position of the acridinyl lysine affected the binding affinity of the peptides, with mono acridinylated peptides **3**, **4**, and **5** demonstrating better binding affinities towards RNA but with little selectivity. Sequences containing two acridinyl lysines (**6–8**) showed improvement of binding affinity, with **6** and **8** displaying increased selectivity for RRE and TAR over $tRNA^{mix}$ by almost 10 and 20-fold, respectively. The addition of another acridinyl lysine **8** slightly raised binding affinity but selectivity was lost. Through an alanine scan using **1** as a lead peptide, sequences **10** and **11** were found to improve binding affinities towards RRE, with each showing selectivity against TAR RNA.[21a] Yu and co-workers also developed constrained peptides from **1** in which the 5th and 12th Leu residues were replaced with Cys and then cross-linked by various maleimido derivatives.[21b] Unfortunately, none of the peptides showed increased binding affinity towards RRE in relation to **1**, with K_ds ranging from 46 to 90 nM. K_ds in the picomolar range were achieved through the covalent cross-linking of the peptides through intermolecular disulfide bonds; however, these peptides did not display selectivity for RRE or TAR RNA.

Branched peptides as medicinal scaffolds

Branched peptides have been used as a scaffold for a variety of applications, including vaccine development, metal chelation, tumor targeting, as well as transfection agents.[51] Their prominence in drug therapy stems from their modulation of biological activity through multivalent binding, as well as improved resistance to proteolysis *in vivo* compared to linear counterparts.[51,52] This resistance is thought to occur in part due to the deep channel in the active site of metallopeptidases such as neurolysin; only small peptides have access to this channel, allowing for more bulky peptides to escape rapid proteolysis.[53] The concept of utilizing branched peptides surfaced in 1988; Tam developed the multiple antigen peptide (MAP) system where multiple lysines served as a core matrix on which peptide antigens could be attached *via* a triglyceryl linker.[54] This MAP design has also been exploited as a drug delivery system, with conjugation of polyhedral boron and dihydroxyboryl-phenylalanine to branched poly-lysine-alanine systems for boron neutron capture therapy,[55] and the use of branched cell permeable peptides (CPPs) or branched histidine-lysine peptides to improve the efficiency in internalization and gene delivery through transduction or transfection of cells.[56] Recently, histidine-rich branched peptides $(GH)_2K$ and $(HH)_2K$ have also been utilized as a potential therapeutic for Alzheimer's Disease through the chelation of Cu^{2+} and Zn^{2+} ions, which

Table 1 Dissociation constants and discrimination ratios for dimethyl- and acridinyl-lysine peptides against RRE and TAR RNA

Peptide	Sequence (position(s) of K*, K**)[a,b]	α-Helicity[c] (%)	K_d *vs.* RRE [nM]	K_d *vs.* TAR[d] [nM]	K_d *vs.* $tRNA^{mix}$ [nM]
1	LKKLLKLLKKLLKLKG	26/57	22 (2.5)	62 (2.8)	55 (2.5)
2	LKK*LLKLLK*KLLKLKG	8/52	9.1	53 (5.8)	42 (4.6)
3	LK**KLLKLLKKLLKLKG	14/52	3.2 (4.4)	2.5 (5.6)	14
4	LKKLLKLLK**KLLKLKG	36/62	4.3 (3.0)	1.3 (10)	13
5	LKKLLKLLKK**LLKLKG	25/63	6.8 (2.1)	1.5 (9.3)	14
6	LK**KLLKLLK**KLLKLKG	35/57	0.61 (8.0)	0.55 (8.9)	4.9
7	LK**KLLKLLKK**LLKLKG	15/48	0.72 (8.5)	0.64 (9.5)	6.1
8	LKKLLKLLK**K**LLKLKG	12/54	0.92 (7.5)	0.37 (18)	6.9
9	LK**KLLKLLK**K**LLKLKG	15/49	0.25 (0.92)	0.20 (1.1)	0.23
10	WKKLLKLLKKLLKLAG	48/65	2.5 ± 0.3	2.0 ± 0.3	nd
11	LKKLLKWLKKLLKLAG	19/71	1.2 ± 0.07	21 ± 1	nd

[a] K* = N^{ε},N^{ε}-dimethyl Lys. [b] K** = N^{ε}-acridinyl-Lys. [c] In 10 mM H_3PO_4/50% 2,2,2-trifluoroethanol (TFE) in 10 mM H_3PO_4, pH 7.4. [d] Discrimination ratios (K_d against other RNA/K_d against RRE) are given in parenthesis.

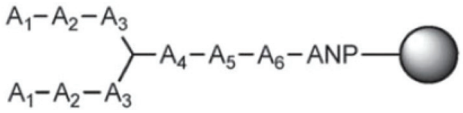
have been shown to encourage the formation of beta-amyloid (Aβ) plaques.[57]

Branched peptide libraries targeting folded RNA structures

As discussed earlier, the genetic diversity and high rate of mutation of HIV-1 have made attempts to eradicate the virus unsuccessful, so the conserved properties of both TAR and RRE RNA make them suitable targets to inhibit the virus through a new mode of action. In developing an RNA binder, several features are desired: the inhibitor (i) must be cell permeable to reach the target in the nucleus, (ii) must be selective against a variety of RNAs including tRNAs, and (iii) must have the potency required to disrupt RNA:cognate protein binding. Synthesis of the binder should be efficient, and the reaction should be one in which structural diversity can be readily introduced. Due to the structural complexity of RNA, the binder may benefit from multivalent interactions that could aid in both selectivity and binding affinity towards accessible pockets of the RNA. Considering these factors, medium-sized (~1000 to 2500 Da) branched peptides were proposed as a scaffold for targeting RNA.[58] Amino acids have a wide variety of functional groups that can interact with RNA *via* non-canonical modes of binding, such as with electrostatic interactions (Lys, Arg), π–π interactions (Phe, Trp), hydrophobic interactions (Val, Leu) and hydrogen bonding (Ser, Tyr). Also, imposed architecture and function of other reactive groups due to the wide availability and synthesis of unnatural amino acids can be achieved. First developed by Merrifield in 1963,[59] the synthesis of peptides using solid phase is straightforward and large numbers of sequences can be generated using a split and pool method to generate libraries of peptides in an expedient manner, with subsequent on-bead screening against the desired RNA target.[60] Branching the peptide allows the potential for increased surface area interaction with the RNA, and the structural diversity present in a combinatorial library should afford sequences that are biased toward specific tertiary structures amidst an ensemble of RNA conformations. Further, branching in peptides typically results in improved metabolic stability making them amenable to therapeutic development.

First-generation 3.3.3 branched peptide library targeting HIV-1 TAR RNA

Our first-generation branched peptide library was developed to target HIV-1 TAR RNA.[61] A combinatorial library of 4096 peptides linked to TentaGel resins *via* a photocleavable linker was synthesized (Fig. 6). The goal was to interrogate all possible binding modes that maximize RNA binding; hence, monomers with the capability to interact *via* electrostatic, π–π, and hydrogen bonding were utilized. Equally important is the ability to distinguish non-selective binders from false positives. For these, we opted to block promiscuous binders with BSA and competitor RNA. After high throughput screening with fluorescently labelled RNA, branched peptide hits containing an Arg–Arg motif were identified and characterized. In this proof-of-principle assay, the presence of positively charged moieties was not a surprise due to the negatively charged phosphates on RNA as well as the fact that the native ligand Tat interacts with TAR *via* an ARM (*vide supra*). Interestingly, large scale synthesis of FITC-labelled peptides for fluorescence polarization and dot blot assays resulted in an acid-mediated deletion of the N-terminal residue. Luckily, use of an aminohexanoic acid (Ahx) spacer between the N-terminus and FITC group eliminated this autocleavage and improved overall yields.[62]



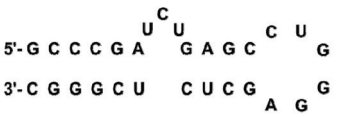
A_1	A_2	A_3	A_4	A_5	A_6
Asp	Arg	Tyr	His	Tyr	Phe
Arg	Asn	Leu	Val	Arg	Leu
Tyr	His	Trp	Asn	Ala	Asp
His	Val	Ala	Trp	Gln	Ser

Fig. 6 3.3.3 Branched peptide library.

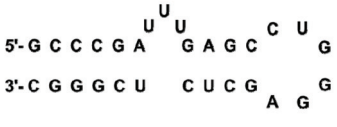
Results indicated binding affinities towards TAR RNA in the low micromolar range, with the best binder **FL4** [$(RRW)_2$*HAL] having a K_d of 600 nM, comparable to native Tat–TAR binding (K_d = 780 nM). It was also discovered that the lack of arginine groups at the N-terminus for several hits led to poor aqueous solubility, thus suggesting the need for basic residues within the peptides. To probe the effect of branching on RNA binding affinity, a linear version of **T4-1** (RRWGHAL) was synthesized and revealed a 125-fold decrease in binding affinity, demonstrating the key role of branching in the peptide. As electrostatic interactions can, in principle, account for a substantial amount of binding energy, multiple basic residues that arise from the screening assay may generate false positives. To our delight, several peptides containing Arg–Arg motifs at the N-terminus as well as peptides containing a larger number of positive charges relative to **FL4** also had lower binding affinities. This suggested that the decrease in binding affinity seen for **T4-1** was not simply due to the loss of electrostatic interactions, thus supporting the role of branching design and sequence in increasing the binding affinity of **FL4** towards the RNA.

The selectivity of branched peptides towards the native TAR structure was also probed using competition assays. In the

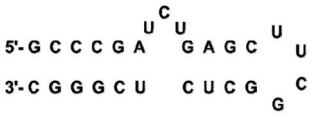
presence of excess competitor tRNA, the binding affinity of **FL4** to ^{32}P-labeled TAR RNA was shifted, indicating partial selectivity of the compound towards TAR. Further titrations against several mutant versions of TAR including point mutation TAR 24C>U, bulgeless TAR, and tetraloop TAR, showed a decrease in binding affinity for the tetraloop and bulgeless mutants, indicating that **FL4** interacted with the bulge and apical loop moieties on TAR RNA (Fig. 7). Hill analyses showed noncooperative binding for both the native structure TAR and TAR 24C>U, supporting the multivalent interaction of **FL4** towards the RNA. In the case of the point mutation TAR 24C>U, the binding affinity was similar to the native structure. This indicated that the compound may not interact specifically with the C24 nucleobase; however, since this mutation should not alter the native structure of TAR this result was not completely unexpected.[63]



TAR



TAR 24C>U



Tetraloop TAR

Bulgeless TAR

TAR RNA Variants	K_d (μM)
wt TAR	0.6 ± 0.1
TAR 24C>U	0.8 ± 0.2
Tetraloop TAR	5.5 ± 2.0
Bulgeless TAR	6.6 ± 1.3

Fig. 7 Structures of TAR RNA variants and dissociation constants for FL4.

Lastly, the cellular uptake and cytotoxicity of the branched peptides were examined. We predicted these compounds to be cell permeable due to their medium molecular weight ($500 < x < 1500$ Da) as well as the presence of arginine moieties in the peptides.[56c,64] It was demonstrated that medium-sized branched peptides were internalized into the cytoplasm and nucleus of HeLa cells. MTT assays also revealed relative cell viabilities of greater than 70% for all compounds at a concentration of 1 μM.

With proof-of-principle studies in hand—*i.e.*, the demonstration of the pivotal role of branching in peptides, cell permeability as a result of their 'medium' molecular weight, and good binding affinity, an improved peptide library was envisioned along with the possibility of targeting other structured RNAs.

Second-generation 3.3.4 branched peptide library targeting HIV-1 RRE RNA

Due to increased interest in another conserved structure of HIV-1 RNA, we focused on targeting RRE RNA.[65] In this library, two unnatural amino acids containing boronic acid moieties were incorporated: a phenylalanine derivative, F_{BPA} and a benzoyl lysine derivative, K_{BBA} (Fig. 8). Installation of boronic acids in biomolecules present a unique mode of interaction due to their ability to form reversible covalent bonds with Lewis bases. Further, we envisioned the boronic acid moiety as a potential surrogate for positive charge. Peptides containing boronic acids have been shown to form reversible covalent bonds with sugars such as alizarin and glucose, and have been employed to inhibit various proteases.[66] In fact, in 2003 a peptidyl proteasome inhibitor, Bortezomib (Velcade), was approved by the FDA to treat multiple myeloma,[67] and another boron-containing drug, Tavaborole (AN2690), recently received FDA approval in 2014 for treatment of onychomycosis.[68] Both of these drugs work by binding hydroxyl groups to form reversible boronate adducts: Bortezomib *via* the Thr-OH in the 26S proteasome and Tavaborole *via* the 2′ and 3′-oxygen atoms of the terminal adenosine in leucyl-tRNA synthetase. Inspired by this work, the use of boronic acid to form reversible covalent bonds with the 2′-OH of RNA was employed.

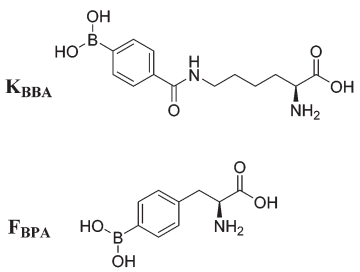


Fig. 8 Structures of boronic acid monomers K_{BBA} and F_{BPA}.

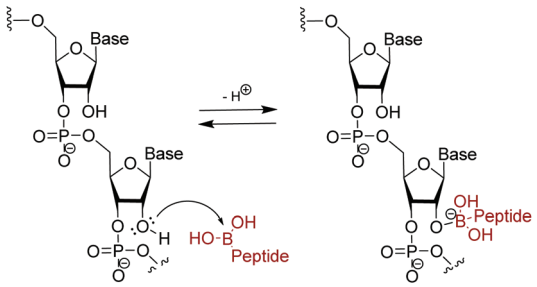
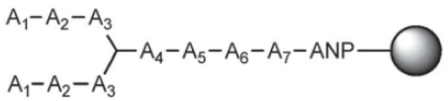


Fig. 9 Suggested reversible covalent bond formation between boronic acid on branched peptide and 2′-OH on RNA.

In principle, a likely increase in selectivity for the RNA target over DNA as well as improvement of binding affinity *via* a non-canonical mode of binding is envisioned (Fig. 9).

In this second generation of branched peptides, the library was expanded to 46 656 possible sequences, and a tyrosine was included at position A7 as a spectroscopic handle for quantification of the peptides (Fig. 10). Once again, amino acids were selected at each position to engage different interactions with the RNA as described above, and K_{BBA}/F_{BPA} was chosen at each variable position to explore the binding potential of boronic acid both with a shorter or longer alkyl tether. Of the eleven hits sequenced using MALDI MS/MS,[69] **BPBA1-BPBA3** had dissociation constants in the low micromolar range and seven contained one or both of the boronic acid moieties. These boronic acids were preferred at the A1–A3 N-terminus region of the sequences, while the longer-chain boronic acid K_{BBA} was preferred over F_{BPA}. Lysine was a preferred residue in the majority of positions. However, the number of lysines present had no correlation to an increase or decrease in binding affinity suggesting again that while electrostatic interactions were important for peptide:RNA interactions, the binding affinity was not solely dependent on this type of interaction. Also, it was shown that the hydrophobic side chain residues were least preferred, indicating these interactions were not beneficial for binding interactions with the RNA.



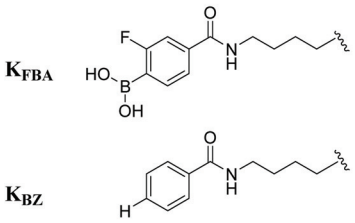
A_1	A_2	A_3	A_4	A_5	A_6	A_7
Trp	Tyr	Phe	His	Tyr	Trp	Tyr
Ser	Asn	Thr	Gln	Thr	Ser	Tyr
Val	Pro	Ala	Pro	Leu	Ala	Tyr
Lys	Lys	Lys	Lys	Lys	Lys	Tyr
K_{BBA}	K_{BBA}	K_{BBA}	K_{BBA}	K_{BBA}	K_{BBA}	Tyr
F_{BPA}	F_{BPA}	F_{BPA}	F_{BPA}	F_{BPA}	F_{BPA}	Tyr

Fig. 10 3.3.4 Branched peptide boronic acid (BPBA) library.

The role of boronic acid in binding was explored using **BPBA1** and **BPBA3**.[65a] When the boronic acid moiety was removed from K_{BBA} to afford **BPBA1.1**, a six-fold decrease of binding affinity towards RRE IIB was observed (Fig. 11). In contrast, when an electron withdrawing fluorine atom was installed *ortho* to the boronic acid group (**BPBA1.2**), the binding affinity improved to 0.8 μM. This result supports the hypothesis that increasing the Lewis acidity of boron can lead to better complexation with the RNA. Further, when multiple boronic acid residues were removed in **BPBA 3.1**, binding was undetectable, indicating that these boronic acids play a pivotal role in recognizing and binding RRE IIB. These results support the use of boronic acid as a unique mode of binding for peptide:RNA interactions, and show the capacity for tuning the Lewis acidity of these boronic acids to increase their affinity towards RNA.

Biophysical characterizations of branched peptide **BPBA1** revealed several observations. First, branching in peptides is critical to binding with RRE RNA. For example, removal of the WKK N-terminus linked either at the (i) α- or (ii) ε-nitrogen of the lysine branch or (iii) removal of the C-terminus branch showed a marked decrease in binding affinity. Second, electrostatic interactions play an important contribution to binding affinity, but the location within the branched peptide is far more critical. Indeed, linear peptides where the ε-nitrogen N-terminal branch was translocated either to N- or C-terminus revealed K_d values that were 5-fold weaker than parent **BPBA1**. Further, a sequence scrambled branched peptide had a >75 fold increase in K_d. These results indicated that both branching and sequence were important in contributing towards the binding affinity of **BPBA1** towards RRE IIB.

Investigations on the selectivity of **BPBA1** towards other variants of RRE IIB RNA demonstrated significant improvement when compared to the first generation library. In summary, all variants (stem and loop deletions/mutations) showed an increase in K_d values compared to the native structure, and a variant in which all loops and bulges were deleted resulted in a ~50-fold decrease in binding affinity (K_d of 91.7 μM), indicating that optimal binding of **BPBA1** was achieved with the native tertiary structure of RRE IIB wild type RNA. Competition studies with $tRNA^{mix}$ and a DNA analogue of RRE IIB RNA also showed over 30 fold preference for the target RNA. These studies highlight the importance of three dimensional architecture and functional group exposure such as hydroxyl groups to binding affinity and selectivity. Ribonuclease protection assays revealed specific nucleotide contacts of **BPBA1** with RRE IIB RNA (Fig. 12). For example, RNase VI showed protection along the upper stem portion, where the native protein Rev binds,[37a,70] whereas RNase A showed protection in the internal loops of RRE IIB, specifically U7 and U36. These data indicate that **BPBA1** spans a large portion of the RRE IIB RNA, with multiple contact points along the RNA.



Peptide	Sequence	K_d (μM)
BPBA1	$(WKK)_2$*K_{BBA}YWY	1.4 ± 0.4
BPBA1.1	$(WKK)_2$*K_{BZ}YWY	8.2 ± 2.3
BPBA1.2	$(WKK)_2$*K_{FBA}YWY	0.8 ± 0.2
BPBA2	$(K_{BBA}KF_{BPA})_2$*K_{BBA}KKY	3.3 ± 1.2
BPBA3	$(F_{BPA}YF_{BPA})_2$*NKSY	8.7 ± 2.3
BPBA3.1	$(FYF)_2$*NKSY	NB

Fig. 11 Left: structures of modified boronic acid monomers K_{BZ} and K_{FBA}; right: dissociation constants of the highest affinity binders from the 3.3.4 BPBA library (BPBA1-3) and their analogues (1.1, 1.2, and 3.1). NB = no binding.

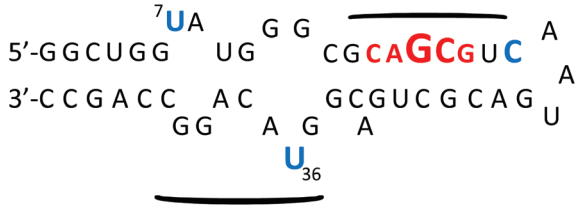


Fig. 12 Summary of RNase protection of RRE IIB in the presence of increasing concentrations of BPBA1. Font size reflects level of protection from RNase A (blue) and RNase VI (red). Curved lines indicate regions of peptide:RNA contact.

Conclusions

Drug discovery using RNA as a therapeutic target remains a challenging, herculean task. Our approach in using branched peptides provides an alternative strategy to sequence selective recognition of RNA; that is, conserved, highly structured RNA can be targeted based on the three-dimensional arrangement of nucleic acid bases. Indeed, HIV-1 TAR and RRE RNAs fall into this category and have been a focus of many studies by researchers. An attractive facet of this chemical biology approach is that the RNA:peptide interaction can be aided using virtually any exotic amino acid functional group; in our case, we focused on boronic acids in order to capitalize on their Lewis acidic properties. Thus far, we have demonstrated that selective tuning of this Lewis acidity can increase or decrease binding affinity to RRE RNA. To effectively inhibit RNA:protein interactions where the binding constants are in the low nanomolar range, RNA inhibitors need to be tight, selective binders. Bias towards a specific RNA target can be generated by conformational restriction of the peptide that induces a preorganized scaffold and protrudes key functional groups to interact favourably with RNA. In our investigations, branched peptide boronic acids possess K_ds near the 1 μM range and improvements are clearly needed. The ability of medium molecular weight branched peptides to utilize a large surface area for binding, and thus create additional opportunities of selective engagement with the RNA, suggests a viable strategy towards this goal and perhaps the targeting of other RNA structures as well.

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