Branched peptide boronic acids (BPBAs): a novel mode of binding towards RNA†

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We report branched peptide boronic acids (BPBAs) that bind to RRE IIB from an on-bead high-throughput screening of a 3.3.4-library (46 656 compounds). We demonstrate that boronic acids are tunable moieties that afford a novel binding mode towards RNA. 

The highly structured HIV-1 Rev response element (RRE), which is a span of ~240-nucleotides located in the env gene of all singly spliced and unspliced HIV-1 transcripts, is an example of an extremely well-conserved sequence of RNA across different HIV-1 isolates and plays an essential role in RNA replication by interaction with the Rev protein. It has been demonstrated that proviral colonies without the rev gene have no replicative abilities, and in the absence of rev protein, the stability of unspliced mRNA is decreased. While the details of the RRE–Rev export pathway is being investigated, some steps have been identified. It is known that of the singly spliced transcripts and multiply spliced transcripts, only multiply spliced transcripts can be exported to the cytoplasm and translated to their corresponding proteins including Rev. Once Rev is expressed, it is imported into the nucleus where it binds cooperatively to RRE. In particular, the stem-loop IIB of RRE (RRE IIB) has been recognized as the high affinity site where Rev initially binds. The resulting Rev–RRE ribonucleoprotein complex binds the host Crm1 and is then shuttled out of the nucleus through the nuclear pore after the larger complex binds to Ran-GTP. Since this cooperative binding allows for the export of full-length and singly spliced transcripts, the Rev–RRE export pathway has become a high profile drug target for its critical role in proliferation of HIV-1.

In continuation of our effort toward developing molecules that target the tertiary structure of RNA, we focused our attention on RRE and envisioned utilizing RNA–ligand interactions that are outside the typical canonical mode of binding. We previously demonstrated that branching in peptide ligands provides strong multivalent interactions with another HIV-1 related RNA, the transactivation response element (TAR). These branched peptides (BPs) displayed no cytotoxicity, provided excellent cell permeability, and bound to TAR in the submicromolar regime. Herein, we report the discovery and biophysical characterization of branched peptide boronic acids (BPBAs) as medium-sized ligands that bind to the tertiary structure of HIV-1 RRE IIB. Our investigations suggest that the boronic acid moiety plays a pivotal role in increasing binding affinity.

We embarked on a strategy to improve the selectivity and binding affinity to the RNA target through the incorporation of unnatural amino acid side chains featuring the boronic acid functional group. Boronic acids have been used in various applications including biomolecules. For example, boronic acids anchored to a cellulose polymer support was first used to separate and purify RNA. Peptides displaying boronic acid moieties have been demonstrated to form reversible covalent bonds with alizarin and glucose, in addition to being utilized as potent protease inhibitors. Furthermore, boron-containing compounds are well tolerated in vivo as is evident from the FDA approval of the first boron-containing drug, Bortezomib (Velcade, Fig. 1A). Another boron-containing small molecule, Tavaborole (AN2690), is currently in phase III clinical trials for treatment of onychomycosis, and its mode of action involves trapping...
the 2'- and 3'-oxygen atoms of the terminal adenosine in leucyl-tRNA synthetase as a boronate adduct (Fig. 1A). Since peptideyl boronic acids have not been investigated to target RNA, we hypothesized that we can capitalize on the empty p-orbital of boron by forming a reversible covalent bond to the Lewis bases in RNA. In particular, the 2'-hydroxyl group in RNA is well-suited as an electron donor towards boron. As a result, the strategy could promote selectivity for RNA over DNA and also boost affinity to the RNA target due to the formation of a reversible covalent bond (Fig. 1B). The boronic acid moiety as an unnatural side chain in peptides increases the complexity and diversity of peptide libraries. Further, the boronic acid functional group is an excellent candidate for probing RNA–ligand binding interactions that are atypical of canonical mode of binding.

A BPBA peptide library was synthesized on Tentagel beads by split and pool synthesis. The library was prepared such that there were three variable amino acid positions at both the N- and C-termini (A1–A3 and A4–A6, respectively) and one variable position in the library to examine whether RRE IIB had a preference in the side chain length or boron Lewis acidity. Hence, the 3.3.4 branched peptide boronic acid library was composed of six possible side chains (Fig. 2A). Each of the six possible side chains was chosen for its potential to interact with the RRE IIB target RNA. The specific amino acids for each type of binding interaction were chosen by random assignment in order to preclude bias in the library design. In position A1, for example, we selected amino acids with functional groups that can interact with the RNA through hydrophobic interaction (Val), electrostatic attraction (Lys), hydrogen bonding (Ser), pi-stacking (Trp), and reversible covalent bonding between boron (KBB) and a Lewis base presented by the RNA target (Fig. 2B). We chose to incorporate two boron-containing side chains at each variable position in the library to examine whether RRE IIB had a preference in the side chain length or boron Lewis acidity. Hence, the 3.3.4 branched peptide boronic acid library was composed of 46 656 possible amino acid sequences linked to the bead by a photocleavable linker [3-amino-3-(2-nitrophenyl) propionic acid, ANP]. Tyr was included at position A7 as a convenient spectroscopic handle in quantifying peptide concentrations.

The BPBA library was subjected to on-bead high throughput screening against DY547 labeled HIV-1 RRE IIB RNA. During the screening process, the beads were first pretreated with an excess of both bovine serum albumin and competitor tRNA to minimize non-specific binding. Specific binding of the target RNA to peptide resulted in increased fluorescence of the bead, which was monitored by fluorescence microscopy. Eleven beads were selected as possible hits. These peptides were photocleaved via UV irradiation and then sequenced by MALDI MS-MS analysis. Compound hits were resynthesized for further biophysical characterizations.

The sequence and dissociation constant of the hit BPBAs are shown in Table 1. Using standard dot blot assay techniques, 32P-labeled RRE IIB was titrated with increasing concentration of BPBAs (see ESI for details).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>$K_d$ (mM)</th>
<th>MW (g mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPBA1</td>
<td>(WKK)$_2$ * KBBAYWY</td>
<td>1.4 ± 0.4</td>
<td>1817.99</td>
</tr>
<tr>
<td>BPBA2</td>
<td>(KBB,FK)$_2$ * KBBAYKY</td>
<td>3.3 ± 1.2</td>
<td>2031.10</td>
</tr>
<tr>
<td>BPBA3</td>
<td>(F$<em>{val}$,YP$</em>{val}$)$_2$ * NKSY</td>
<td>8.7 ± 2.3</td>
<td>1727.78</td>
</tr>
<tr>
<td>BPBA4</td>
<td>(KKB)$<em>2$ * F$</em>{val}$TSY</td>
<td>26.8 ± 4.4</td>
<td>1751.98</td>
</tr>
<tr>
<td>BPBA5</td>
<td>(KBB,FK)$_2$ * KKKWY</td>
<td>27.2 ± 6.9</td>
<td>1853.04</td>
</tr>
<tr>
<td>BPBA6</td>
<td>(WYK)$_2$ * PTWY</td>
<td>28.5 ± 4.4</td>
<td>1646.34</td>
</tr>
<tr>
<td>BPBA7</td>
<td>(KBB,FK)$_2$ * KLKY</td>
<td>58.4 ± 4</td>
<td>1742.09</td>
</tr>
<tr>
<td>BPBA8</td>
<td>(KBB,FK)$_2$ * HKKY</td>
<td>86.5 ± 10</td>
<td>1836.04</td>
</tr>
</tbody>
</table>

$^a$ * = lysine branching unit. Each value is an average of at least three experiments.

$^b$ 32P-labeled RRE IIB was titrated with increasing concentration of BPBAs (see ESI for details).

Three peptides (BPBA1, BPBA2 and BPBA3) had low micromolar binding affinities (1.4, 3.3 and 8.7 μM, respectively) and were investigated further. To further confirm that the dissociation constants determined from the dot blot assay were reliable, electrophoretic mobility shift assay (EMSA) was performed with BPBA1, BPBA2 and BPBA3. The dissociation constants determined through EMSA (0.3 ± 0.1, 0.6 ± 0.2 and 4.6 ± 2.4 μM, respectively) were comparable to the low micromolar results obtained via dot blot assay and were consistent in rank order of affinity (ESI). The slight discrepancy with the $K_d$ values may be a result of nonspecific interactions between the peptide and nitrocellulose. Nevertheless, these sequences contained boronic acid residues with boron containing amino acids present in positions A1, A4 and A6. Interestingly, no boron containing amino acids were found at positions A2 or A3 in any hit sequence nor was there any correlation between the number of boronic acid moieties and the resulting binding affinities. Further, it was observed that the longer, more Lewis acidic residue of the Rev protein binds RRE IIB through the polyarginine nuclear localization signal (NLS) located in its N-terminal region. The positively charged Lys side chains in our hits may function similarly to the Arg residues of the Rev NLS and likely provide the necessary...
When the increased Lewis acidity and is expected to facilitate complexation. To binding to RRE IIB RNA, we designed control peptide variants of binding between these BPBAs and RRE IIB.

The change was accompanied by an approximate 6-fold increase in the observed $K_d$ value (1.4 vs. 8.2 μM, Fig. 3). Although the precise role of the boronic acid moiety in binding is currently unknown, the decrease in affinity suggests that the boronic acid functional group interacts with RRE. The single boronic acid moiety in BPBA1 contributes a modest fraction of binding affinity with RRE IIB. In this case, electrostatic attraction accounts for the majority of interaction with the RNA provided by the high density of Lys residues. In order to investigate the hypothesis that the Lewis acidity of boron can increase binding affinity, we synthesized a fluorinated analog, BPBA1.2, wherein fluorine was placed ortho to the boronic acid at position A4. The electron withdrawing ability of fluorine results in increased Lewis acidity and is expected to facilitate complexation. To our delight, when the $K_d$ was determined by dot blot assay, the binding affinity improved to 0.8 ± 0.1 μM, suggesting an induction of stronger interaction with RRE through the boronic acid moiety. This result supports the notion that the binding affinity of BPBAs can be tuned by manipulating the Lewis acidity of the boronic acid and is consistent with the observation that more electrophilic boronic acids are more acidic as they can form stable Lewis acid–base complexes.17

We also investigated the effect of the number of boronic acid moieties in hit peptide BPBA3, which contains four boron atoms. When a boronic acid-free analog of BPBA3 was synthesized [BPBA3.1], nearly all binding was abolished (Fig. 3). The drastic loss of binding with BPBA3.1 is likely due to the high density of boron containing side chains because the major source of binding interaction, presumably Lewis acid–base complexation, is lost. These results indicate that boronic acid side chains can be utilized in peptides to boost binding affinity with a highly structured RNA target. Further, such interaction represents an additional and unique mode of binding that increases the repertoire of RNA binding motifs.

In conclusion, we generated a BPBA library that was designed to interrogate the effect of boronic acids when screened against the tertiary structure of an RNA target: RRE IIB. High throughput screening and biophysical characterization of hit compounds resulted in peptides with binding affinities in the low micromolar range, wherein the presence of boronic acid groups introduced a novel, alternative mode of interaction. Structure-activity relationship studies demonstrated that the binding affinity of BPBAs can be tuned by changing the electronic property of the boron center. This result highlights that boronic acid moieties can impact binding towards RNA, and this strategy is applicable to other systems since boronic acids can be readily engineered into peptides.

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Notes and references