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Screening for inhibitors of the hepatitis C virus internal ribosome entry site RNA

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Abstract

The highly conserved internal ribosome entry site (IRES) of hepatitis C virus (HCV) regulates translation of the viral RNA genome and is essential for the expression of HCV proteins in infected host cells. The structured subdomain IIa of the IRES element is the target site of recently discovered benzimidazole inhibitors that selectively block viral translation through capture of an extended conformation of an RNA internal loop. Here, we describe the development of a FRET-based screening assay for similarly acting HCV translation inhibitors. The assay relies on monitoring fluorescence changes that indicate rearrangement of the RNA target conformation upon ligand binding. Screening of a small pilot set of potential RNA binders identified a benzoxazole scaffold as a ligand that bound selectively to IIa IRES target and was confirmed as an inhibitor of *in vitro* viral translation. The screening approach outlined here provides an efficient method to discover HCV translation inhibitors that may provide leads for the development of novel antiviral therapies directed at the highly conserved IRES RNA.

1. Introduction

The diversity of complex structural folds that occur in non-coding functional RNAs in conjunction with the key roles they play in biological processes has spurred the interest in RNA as a potential drug target.^{1–3} The area of antiinfective therapy attests to the utility of RNA as a target since it is well established that many chemical classes of antibiotics selectively act on bacterial ribosomal RNA (rRNA).^{4, 5} While attractive RNA targets outside the bacterial ribosome have been identified, including regulatory domains in genomes of pathogenic viruses, drug discovery for these targets faces formidable challenges.⁶ A prime obstacle to drug discovery for RNA is the scarcity of synthetic small molecules that exhibit bias for binding to RNA as well as drug-like properties but which are not encumbered by the complexity of natural products.⁷ Further challenges arise from the difficulty to establish meaningful screening assays that report on functional consequences of ligand binding to an RNA target rather than returning binding affinities only. The triggering of a unique event, often related to conformational changes, in a specific target induced by ligand binding is tied to the achievement of selectivity over other cellular nucleic acids.⁸ Within the bacterial ribosome, the decoding site is a well-characterized RNA target of aminoglycoside antibiotics, which, upon binding, change the conformation of flexible adenine residues involved in mRNA decoding and thereby increase the error rate in protein synthesis.^{9, 10}

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Outside the ribosome, such conformational RNA targets for small molecule ligands had been largely elusive.

We have recently shown that a benzimidazole inhibitor of hepatitis C virus (HCV) blocks translation of the viral RNA by conformational induction at a target site in the 5' untranslated region (UTR) of the HCV genome.¹¹ The 5' UTR contains an internal ribosome entry site (IRES) which recruits host cell ribosomes directly at the start codon of the viral genome, independent of initiation factors and ribosomal scanning.^{12–14} The target site of the benzimidazole translation inhibitor is located in the IRES subdomain IIa which folds into a sharply bent motif that is required to accurately position the highly conserved hairpin loop IIb at the interface between the small (40S) and large (60S) ribosomal subunits (Fig. 1).^{15–17} Crystal structure analysis of the subdomain IIa revealed an L-shaped RNA fold that is stabilized by magnesium ions (Fig. 1b).¹⁸ We had previously suggested that specific ligand binding to the IIa RNA might affect the native conformation of the bent subdomain and prevent the correct positioning of the hairpin loop IIb at the ribosome, ultimately blocking viral translation. Subsequently we demonstrated that the benzimidazole translation inhibitor **2** of the HCV replicon acts by capture of an RNA conformation with a widened interhelical angle in the subdomain IIa.¹¹ Compound **2** emerged from optimization of the simple benzimidazole derivative **1** which was originally discovered at Isis Pharmaceuticals as a hit in a mass spectrometry-based affinity screen for IRES RNA-binding ligands (Fig. 1c).¹⁹

Crystal structure analysis of subdomain IIa RNA bound with the closely related ligand **3**²⁰ demonstrated that the benzimidazole inhibitors capture a dramatic conformational change in the RNA target, leading to an entirely straight conformation in the complex (Fig. 1d) and providing further support for a conformational mechanism of IRES inhibition.²¹ These structural data along with our previous discovery of another class of IRES inhibitors, 3,5-diamino piperidine (DAP) ligands such as compound **4**, which act by arresting the RNA fold in a bent state,²² support the notion of the subdomain IIa as a conformational switch that provides an attractive target for small molecule HCV inhibitors.

Here, we describe the development of a screening method for compounds that upon binding trigger a specific conformational change in the IRES subdomain IIa RNA. An assay was established that relies on fluorescence resonance energy transfer (FRET) between cyanine dye labels attached to the 5' termini of the RNA target (Fig. 2). Screening of a small pilot library of synthetic compounds in a functional binding assay identified ligands based on their ability to induce a widened interhelical angle in the subdomain IIa which was detected by FRET quenching. Hit molecules discovered in the FRET assay were tested for target selectivity and biological activity as inhibitors of IRES-driven *in vitro* translation.

2. Results and discussion

2.1. FRET assay for ligands affecting the conformation of the IRES subdomain IIa

The cyanine dye labeled RNA construct for the FRET assay was designed using geometrical constraints derived from the three-dimensional structure of the subdomain IIa (Fig. 2a) which we had determined earlier by X-ray crystallography.¹⁸ In the folded L-shaped state of the RNA, the 5'-attached dye labels are at a distance that allows FRET to occur with high efficiency. FRET intensity will be reduced when ligand binding induces a conformation with a widened RNA interhelical angle which translates into an increased distance between the dye labels (Fig. 2b). As was expected from the conformational mechanism of action of the translation inhibitor **3**,¹¹ titration to the IIa RNA caused a dose-dependent decrease of the FRET signal (Fig. 2c). Due to the diminishing FRET efficiency at growing distances of the cyanine dyes, emission from the excited Cy3 donor increased accordingly. This observation along with the persistence of residual FRET signal at high ligand concentrations

demonstrated that FRET reduction was not due to optical interference by the ligand or target precipitation through nonspecific binding and aggregation, which are two complications likely to be expected when assaying chemically diverse sets of small molecules.^{23, 24} For the screening of compounds at a single, high concentration (200 μ M), we proposed two necessary conditions to select useful hit compounds: firstly, observation of a statistically significant ($>3\sigma$) decrease of the FRET signal relative to a blank (DMSO) control, and secondly, persistence of residual fluorescence throughout the titration to exclude RNA target precipitation.

2.2. Screening for ligands affecting the conformation of the IRES subdomain IIa

An exploratory set of 97 synthetic small molecules comprised of commercially available as well as proprietary compounds was tested in the FRET assay in a 96-well format. Preferred scaffolds included benzimidazoles, benzoxazoles, indoles, isatin hydrazones and diazepanes. Selection criteria for the biased library and experimental details of the screening procedure are outlined in Methods. The benzimidazole HCV translation inhibitor **3**, which had recently been co-crystallized with the subdomain IIa RNA target, was included in the screen as a positive control. In the primary screen, each compound was tested for its impact on the FRET signal at a single concentration of 200 μ M in triplicate (Fig. 3). Compounds that showed reduction of the FRET fluorescence at $>3\sigma$ relative to solvent (DMSO) control were further evaluated. Standard deviation of the assay was determined by averaging over a larger number of physical replicates in which the FRET signal was measured in the absence of compound.

Of the tested exploratory set, eight compounds in three scaffold classes significantly decreased the FRET signal at 200 μ M concentration. One class of 2-amino-benzimidazoles was excluded from further investigations as the low residual FRET signal indicated nonspecific target interaction and precipitation for at least two of these compounds (**5**, **6**). Titration of the remaining five molecules revealed also isatin hydrazones (**7**, **8**, **12**) as likely general RNA precipitants at higher concentrations while the 2-amino-benzoxazole **9** showed a dose-dependent FRET reduction similar to the known benzimidazole inhibitors **2** and **3** albeit with lower potency (Fig. 4a). Binding of the compound **9** was not affected by the presence of excess competitor tRNA, demonstrating target selectivity of the benzoxazole comparable to benzimidazole inhibitors such as **2** and **3** whose selective interaction with the subdomain IIa RNA we had established in previous studies.^{11, 21}

2.3. Testing RNA binders for translation inhibition

To investigate functional consequences of ligand binding to the subdomain IIa target, we tested candidate compounds in an *in vitro* translation assay using a bicistronic firefly reporter construct that carried a Renilla luciferase reporter driven by an HCV IRES and, as a control, a cap dependent firefly luciferase (Fig. 4b).²⁵ The benzoxazole **9** selectively inhibited IRES-initiated translation (30% inhibition at 100 mM compound concentration) while not affecting cap dependent translation (Fig. 4c). In contrast, nonspecific RNA binding compounds identified as fluorescence quenching hits in the binding screen showed indiscriminate inhibition of both cap and IRES driven translation consistent with RNA precipitation observed in the FRET assay.

The activity of the benzoxazole **9** as a ligand of the subdomain IIa and an IRES inhibitor is readily explained by the structural similarity of this compound to previously discovered benzimidazole inhibitors such as **3** (Fig. 5). The heterocycle edge that engages in key hydrogen bonding interactions of the amino-imidazole in **3** with the RNA target (G110) is conserved in the amino-oxazole of **9** as well as the benzene ring which stacks between nucleobases G52 and A53.²¹ While the attachment site of the N,N-dimethylaminopropyl

group is moved to the exocyclic amine in **9**, flexibility of the alkyl linker permits a similar projection vector as compared to that in benzimidazoles **1–3** which facilitated interaction with A109.

3. Conclusion

The aim of this study was to establish a FRET based screening assay for the identification of ligands that bind to the HCV IRES subdomain IIa and capture conformational changes in the RNA target which lead to translation inhibition. We performed screening of a small pilot set containing compounds biased for RNA binding. A benzoxazole derivative (**9**) was discovered that inhibited HCV IRES-driven translation likely through binding to the subdomain IIa target. While the potency of the hit compound was inferior to established benzimidazole IRES inhibitors, the oxazole scaffold will lend superior drug-like properties to derivatives of **9** due to reduced basicity compared to imidazole as well as a lower hydrophilicity. Future studies will explore structure activity relationships around the amino-benzoxazole in hit compound **9**.

4. Experimental section

4.1. RNA constructs

Cyanine dye labeled oligoribonucleotides were obtained from chemical synthesis and purified by HPLC (Integrated DNA Technologies, Coralville, IA). RNA constructs were annealed from single strands by heating to 65°C for 5 minutes followed by snap cooling in 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, pH 7.0, containing 2mM MgCl₂.

4.2. FRET assay implementation

The FRET assay was performed on a Spectra Max Gemini monochromator plate reader (Molecular Devices, Sunnyvale, CA) at 23°C in black Costar 96-well plates (Corning, Lowell, MA). The RNA construct shown in Fig. 2a, was used at 100nM concentration in 10mM HEPES buffer, pH 7.0, 2mM MgCl₂, in a final volume of 120μL per well. Cy3 label was excited at 520nm and fluorescence was read as Cy5 emission at 670nm. A cutoff filter at 550nm was applied. FRET signal was recorded from the Cy5 emission intensity while exciting the Cy3 label and corrected by subtraction of the signal from direct excitation and crosstalk, both of which were derived from measurements of RNA single-labeled with Cy3 or Cy5 only (see Supplementary Methods). Optimization of ionic conditions suggested that robust folding of the IIa RNA was achieved in the presence of 2mM MgCl₂ (Supplementary Fig. 1), which was subsequently used for all FRET experiments. Testing of the impact of DMSO as a co-solvent for compound screening showed that addition of up to 25vol% of the organic solvent affected the FRET signal by less than 20% (Supplementary Fig. 2).

4.3. FRET screening of compounds

Primary screening for ligands that induce FRET changes in the cyanine dye labeled IIa RNA was performed at 200μM compound concentration in the presence of 5vol% DMSO. RNA stock at 1μM in buffer (12μL) was diluted in the 96-well plate with buffer (102μL) and mixed with compound stock in DMSO (6μL) to a final volume of 120μL per well at room temperature. For the (negative) RNA control, only DMSO was added to 5vol%. For the (positive) RNA control, the benzimidazole inhibitor **3** was used. Each condition was performed in triplicate. For background correction of optical interference, control wells were set up containing compound in buffer/DMSO but no RNA. For each compound concentration tested (200μM in the primary screen; range in dose response titrations) a compound only control well was included at the same concentration. For tRNA competition

experiments total tRNA from wheat germ (Sigma, St. Louis, MO) was added to the desired concentration (2x and 10x excess over Ila target RNA concentration). Plates were sealed and mixed in the plate reader for 10 sec followed by incubation at 23°C for 60 min. Incubation temperature and time were chosen to ensure that any ligand induced conformational change in the RNA target had reached equilibrium (Supplementary Fig. 3). After removal of the plate seal, fluorescence data were recorded at 23°C and processed as described in the Supplementary Methods.

4.4. Selection of compounds for screening

The set of 97 compounds submitted to screening in the FRET assay was comprised of molecules designed and synthesized in our laboratory as well as compounds purchased from ChemBridge and ChemDiv (both, San Diego, CA). Commercial compounds had the following characteristics: polar surface area < 120 Å², 0–3 rotatable bonds, 4–6 H-bond acceptors, 4–6 H-bond donors. Proprietary compounds synthesized in our laboratory were designed following our guidelines for molecules biased for binding to RNA targets.^{2, 26–28} All compounds had MW < 380D and cLogP in the range of –0.5–3.5.

4.5. Testing of compounds for inhibition of viral *in vitro* translation

The *in vitro* transcription-translation assay (IVT) was conducted using the T_NT Quick coupled reticulocyte lysate system (Promega, Madison, WI) and a bicistronic luciferase reporter (Fig. 4b) as previously described.²⁵ Compound stock solution in DMSO was added to the assay mixture to a final concentration of 10μM or 100μM, respectively, and adjusted to a final DMSO concentration of 1vol%. Detection of firefly and Renilla luciferase levels was done using the Dual-Glo Luciferase Assay System (Promega) according to manufactures recommendations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The subdomain IIa RNA target in the HCV IRES. (a) Secondary structure of the IRES element in the HCV 5' UTR (nucleotides 1–341 of HCV genotype 1b). The boxed region indicates the subdomain IIa whose sequence is shown. (b) Crystal structure of the subdomain IIa RNA.¹⁸ Mg^{2+} ions are shown as green spheres. (c) HCV translation inhibitors that bind at the subdomain IIa RNA.^{11, 19, 20, 22} (d) Crystal structure of the subdomain IIa RNA in complex with the benzimidazole inhibitor 3.²¹

Figure 2.

Concept of a FRET-based assay for ligands that induce a conformational change in the HCV IRES subdomain IIa target. (a) The cyanine dye-labeled RNA construct used for the FRET assay. Nucleotides that deviate from the HCV genotype 1b sequence are in outlined font. (b) Calculation of the dependence of the Cy3/Cy5-FRET efficiency on the interhelical angle assuming the RNA geometry derived from the crystal structure of subdomain IIa. Error bars indicate the range of FRET signal for geometries with the dye labels oriented maximally towards or away from each other, depending on the flexibility of the 3-carbon linker. (c) FRET (●) and Cy3 (○) fluorescence signal for a titration of cyanine dye-labeled subdomain IIa RNA with benzimidazole **3** in the presence of 2 mM Mg^{2+} .

Figure 3.

Result of a FRET binding screen of 97 compounds from a biased library of synthetic compounds against the IRES subdomain IIa RNA. The FRET intensity in the presence of 200 μ M compound was normalized relative to the signal of free RNA. Compounds were assayed in duplicate. Standard deviation of the FRET signal in the absence of compound (DMSO control) was calculated from 12 physical replicates, each performed in duplicate. Compounds that gave FRET reduction greater than 3 standard deviations relative to free RNA were designated as hits. Structures of hit compounds (5–12) are shown. The known benzimidazole inhibitor **3** was included as a control.

Figure 4.

Dose-dependent activity of the benzoxazole derivative **9**. (a) Subdomain IIa RNA binding in the FRET assay. (b) Bicistronic reporter construct used for compound testing in a coupled *in vitro* transcription-translation assay (IVT). A cap-driven firefly reporter gene is followed by *Renilla* luciferase reporter under the control of the internal ribosome entry site (IRES) from hepatitis C virus (HCV).²⁵ (c) Translation inhibition by compound **9** which at a concentration of 100 μM selectively inhibits the IRES-dependent process while not affecting cap-driven translation.

Figure 5. Overlay of the benzimidazole inhibitor **(3)** crystal structure (yellow) and an energy minimized conformation of the benzoxazole compound **9** (grey) identified in the FRET screen. Indicated are interactions of **3** with the subdomain IIa RNA as observed in the crystal structure of the complex (Fig. 1d).²¹