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Inhibition of self-splicing group I intron RNA: high-throughput screening assays

Houng-Yau Mei*, Mei Cui, Scott T. Sutton, Hoa N. Truong, Fu-Zon Chung[1] and Anthony W. Czarnik

Bioorganic Chemistry Section, Department of Chemistry and [1]Molecular Biology, Department of Therapeutics, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, MI 48106, USA

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ABSTRACT

High-throughput screening assays have been developed to rapidly identify small molecule inhibitors targeting catalytic group I introns. Biochemical reactions catalyzed by a self-splicing group I intron derived from *Pneumocystis carinii* or from bacteriophage T4 have been investigated. *In vitro* biochemical assays amenable to high-throughput screening have been established. Small molecules that inhibit the functions of group I introns have been identified. These inhibitors should be useful in better understanding ribozyme catalysis or in therapeutic intervention of group I intron-containing microorganisms.

Group I introns have been found to catalyze their own splicing reactions in the absence of any proteins (1). An increasing number of activities including that of a ribonuclease, phosphotransferase, acid phosphatase, DNA and RNA restriction endonuclease, RNA ligase, RNA polymerase and aminoacyl esterase have been found for this catalytic RNA (ribozyme). Most interestingly, the same active site used in the self-splicing reactions (2) seems to be involved in all of these reactions. Since group I introns are found in biologically relevant genes of several microorganisms and are non-existent in human, it has been suggested that the self-splicing of group I introns serves as a therapeutic target (3). Arginine analogues (4) and metabolites such as streptomycin (5) and other aminoglycoside antibiotics (6) demonstrated inhibition of *in vitro* self-splicing of group I introns. There has been, however, no reports of low molecular weight organic inhibitors for RNA self-splicing reactions. We now report high-throughput screening assays using the intron systems in *Pneumocystis carinii* (7) or *SunY* (8) that successfully identifies small molecule inhibitors targeting catalytic group I introns.

A high-throughput *in vitro* self-splicing assay has been established using a 552 nt precursor RNA containing the 390 nt long group I intron and truncated 5′- and 3′-exons of *P.carinii* (shown in Fig. 1). To this 'cold' precursor RNA, α-^{32}P-labeled GTP (Amersham) is added as a cofactor to initiate the self-splicing reactions by attacking the 5′ terminus of the intron. The products of the first step reaction are the 5′-exon (113 nt) and a ^{32}P-labeled RNA fragment (439 nt, intron–3′ exon). This 5′-cleavage reaction is followed by the re-ligation of the 5′-exon

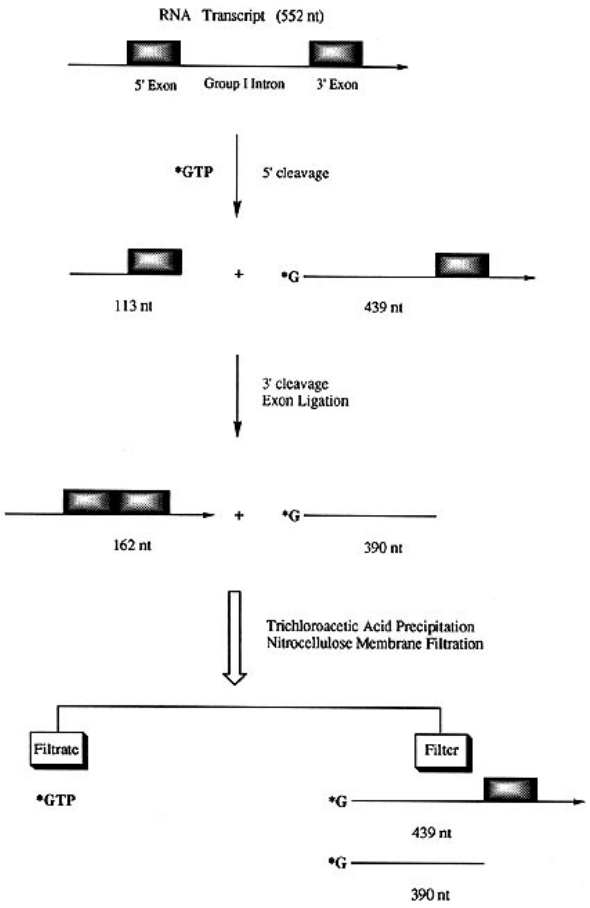

Figure 1. Schematic representation of the self-splicing reactions catalyzed by the group I introns derived from *P.carinii*. Also shown here is the separation protocol used for the high-throughput filtration assay.

*To whom correspondence should be addressed. Tel: +1 313 996 3031; Fax: +1 313 996 5229; Email: meih@aa.wl.com

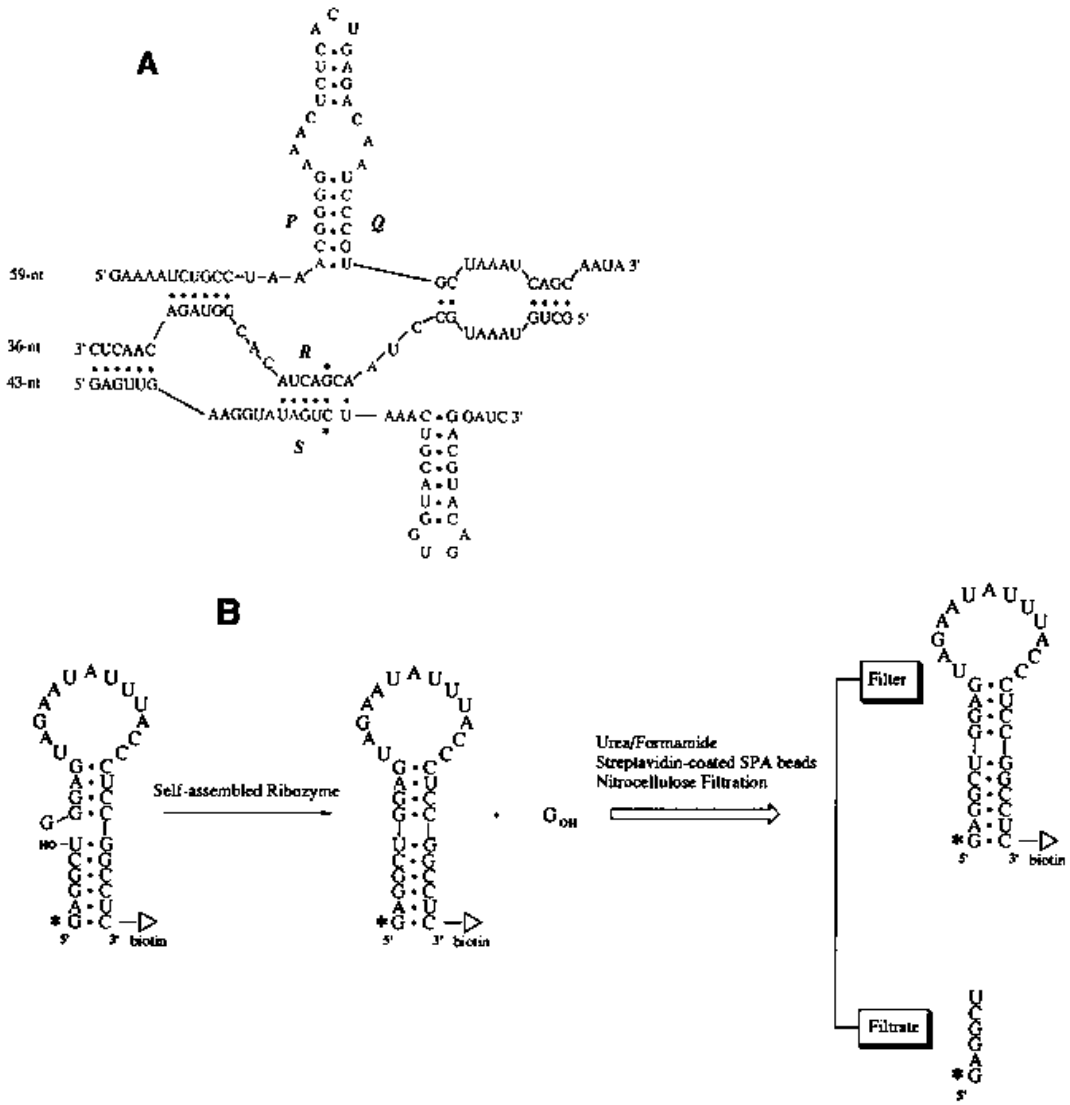

Figure 2. (**A**) Primary sequence and predicted secondary structure of the self-assembled ribozyme system. The catalytic domains conserved in group I introns are represented by ***P***, ***Q***, ***R*** and ***S***. The G/C base pair responsible for binding guanosine cofactor is highlighted by asterisks. (**B**) RNA ligation reaction catalyzed by the self-assembled ribozyme. An asterisk at the 5′-end of the 6 nt RNA may indicate radioisotopes such as ^{32}P and ^{33}P while an arrow at the 3′-end of either 28 or 33 nt RNA represents a biotin tag.

and the 3′-exon to produce a ligated exon (162 nt) and a 5′-^{32}P-labeled intron RNA (390 nt). Free [^{32}P]GTP and the longer ^{32}P-labeled RNA products can be readily separated through acid precipitation and filtration. Only longer RNA fragments are acid precipitable and remain on the membrane after filtration. The efficiency of the self-splicing reactions in the absence or presence of inhibitors can be followed by measuring the amount of the radioactivity retained on the filter membrane. If the first step (5′-cleavage) is inhibited by small molecules, incorporation of [^{32}P]GTP to the precursor RNA will be inhibited rendering a decreased amount of radioactivity on the membrane.

Another assay was established based on a self-assembled ribozyme (Fig. 2A) composed of three RNA fragments (59, 43 and 36 nt). This ribozyme contains the conserved catalytic core derived from self-splicing *SunY* group I intron and catalyzes the ligation of a 6 nt RNA fragment to a 28 nt RNA (shown in Fig. 2B). The assembled ribozyme catalyzes the nucleophilic attack of the 3′-OH from the 6 nt to the 3′-phosphodiester linkage following the 5′-guanine residue of the 28 nt. The products include a free guanosine and a ligated 33 nt RNA product. This ligation reaction represents a mimicry of either the reversal of the first step or the second step reaction occurring in the group I intron RNA self-splicing process. To facilitate the separation of the 33 nt product from the 6 nt RNA, a protocol using biotin-streptavidin conjugation (9) is incorporated into this assay (Fig. 2B). Each 6 nt RNA is 5′-labeled with a radioactive isotope and the 28 nt RNA is chemically synthesized with a biotin at the 3′-end. When the ligation reaction occurs, the 33 nt product should be 5′-isotope-labeled and 3′-biotin-tagged. Upon conjugation with streptavidin, the product can be readily separated from the 6 nt RNA through filtration. The non-radioactive 28 nt substrate RNA in this assay does not interfere with the detection of the product formation.

Both assays are amenable to high-throughput screening. A typical example of the results obtained from the *P.carinii* screen

Results(% Reaction)												
	1	2	3	4	5	6	7	8	9	10	11	12
A	110	111	100	92	90	75	82	100	87	90	98	4
B	108	113	96	104	82	72	82	88	91	89	56	-3
C	105	99	89	69	78	70	73	70	64	81	59	-2
D	100	88	81	67	70	67	71	57	58	76	82	1
E	104	85	61	66	64	60	57	54	60	67	66	-1
F	93	93	59	66	57	56	11	57	63	68	71	-3
G	93	101	85	117	29	69	65	72	75	132	88	4
H	87	82	75	72	63	70	66	64	58	71	70	0

Figure 3. High-throughput screening data of a 96 well microtiter plate from the nitrocellulose filter assay carried out on the precursor RNA of*P.carinii*. In each well, precursor RNA (50 nM) was mixed first with inhibitors (20 µM) and the self-splicing reaction was initiated with the addition of [α-^{32}P]GTP (0.6 µCi). The self-splicing buffer contains 50 mM Tris–HCl, pH 7.5, 100 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 5 mM spermidine and 5% glycerol. The reaction mixture was incubated at 50°C for 3 h. Addition of 11% trichloroacetic acid (TCA) stopped the splicing reaction and precipitated the RNA products. The TCA/reaction mixture was incubated and transferred to a nitrocellulose filter plate (Millipore, MHAB). The plate was filtered, washed and dried. The retained radioactivities were determined using scintillation counting (Wallac Microbeta Counter). All solution handling was automated using a robotic workstation (Beckman, Biomek 1000). A statistical analysis of the inhibition data was obtained from the high-throughput filtration assay. Out of 1440 samples tested, most (~85%) exhibit little or no inhibition. The active ones (≥50% inhibition) represent ~5% of the collection.

is shown in Figure 3. The percentages shown in Figure 3 represent the extent of the self-splicing reactions. Column 1 represents eight repeats of the self-splicing reactions in the absence of any inhibitors while column 12 represents eight repeats of solution containing [^{32}P]GTP only. Eighty different samples (columns 2–11) of potential inhibitors were tested per plate. The difference between the mean values of raw data from columns 1 and 12 serves as the common denominator in calculating the inhibitory effect. The percentage in each well was obtained by subtracting its raw value with the mean value of column 12 and then dividing this value by the common denominator. In this example, samples in wells F7 and G5 demonstrated >70% inhibitory effect and were selected for follow-up inhibition studies. The eight repeats in column 1 (or column 12) suggest that there may be up to 20% error associated with this filter assay. This should not affect the usage of this filter method as a primary screening assay if the high-throughput screen is to quickly identify significant positive or negative effects in a large collection of compounds. Results from biotin-streptavidin assay for self-assembled ribozyme screen resemble but are not identical to that of Figure 3.

Results from the high-throughput filtration assay were verified using a gel electrophoresis method. The reaction yields were determined and a good correlation between the filtration and the gel electrophoresis assays was found. For example, at 20 µM concentration, samples in wells F7 and G5 (Fig. 3) demonstrated >70% inhibition in both filtration and gel electrophoresis assays. Viomycin, streptomycin and pentamidine, previously reported as group I intron inhibitors at high micromolar concentrations (10), were also tested in both assays as controls. Under similar conditions, viomycin, streptomycin or pentamidine exhibited 20–50% inhibition.

In summary, reproducible, sensitive and high-throughput assays have been established for targeting self-splicing group I introns. The reported high-throughput filtration assay is meant to rapidly screen mass numbers of compounds. Further studies are required to understand the mechanism of action of individual inhibitor. Although unproven yet, inhibitors acting on this specific mechanism could be of clinical utility in treating infections caused by microorganisms whose life cycle is regulated by the catalytic function of group I introns. If such agents are shown to be clinically useful, the *in vitro* assays described here might be more generally used to screen agents targeting a variety of RNA-catalyzed reactions.

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