Bidirectional effectors of a group I intron ribozyme

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ABSTRACT

The group I self-splicing introns found in many organisms are competitively inhibited by L-arginine. We have found that L-arginine acts stereoselectively on the Pc1.LSU nuclear group I intron of *Pneumocystis carinii*, competitively inhibiting the first (cleavage) step of the splicing reaction and stimulating the second (ligation) step. Stimulation of the second step is most clearly demonstrated in reactions whose first step is blocked after 15 min by addition of pentamidine. The guanidine moiety of arginine is required for both effects. L-Canavanine is a more potent inhibitor than L-arginine yet it fails to stimulate. L-Arginine derivatized on its carboxyl group as an amide, ester or peptide is more potent than L-arginine as a stimulator and inhibitor, with di-arginine amide and tri-arginine being the most potent effectors tested. The most potent peptides tested are 10 000 times as effective as L-arginine in inhibiting ribozyme activity, and nearly 400 times as effective as stimulators. Arginine and some of its derivatives apparently bind to site(s) on the ribozyme to alter its conformation to one more active in the second step of splicing while competing with guanosine substrate in the first step. This phenomenon indicates that ribozymes, like protein enzymes, can be inhibited or stimulated by non-substrate low molecular weight compounds, which suggests that such compounds may be developed as pharmacological agents acting on RNA targets.

INTRODUCTION

Pneumocystis carinii is an opportunistic fungal pathogen which causes serious infections in patients with AIDS and other immune defects (1). All isolates of *P.carinii* characterized contain a group I self-splicing intron in their 26S rRNA genes (2,3); some isolates also contain such an intron in the genes encoding 16S rRNA (4–9). Group I self-splicing introns are ribozymes which utilize a guanosine nucleotide substrate to catalyze their own excision from RNA transcripts (for review, 10), whose reactions are diagrammatically represented in Figure 1. A variety of algae, fungi and protozoa harbor group I introns in their nuclear and mitochondrial genomes, while such introns have not been

described in mammalian cells (10,11). Therefore, agents inhibiting splicing by group I introns may have potential as antimicrobial agents (3,7,12,13).

Both competitive and non-competitive inhibitors of splicing *in vitro* by various group I introns have been identified. Non-competitive inhibitors include some aminoglycosides (12,14), tetracycline, ethidium bromide and the anti-pneumocystis drug pentamidine and a series of pentamidine analogues (3,13). Streptomycin (15) and L-arginine (16–23) have been shown to be competitive inhibitors of splicing by various group I introns, including the introns from *P.carinii* (7,13), competing with the guanosine nucleotide substrate. Although L-arginine and its amino acid analogues have been most studied as inhibitors of group I intron activity, arginine residues in proteins are also believed to function in binding to other RNA molecules (24). For example, an arginine residue in the region of the HIV-1 Tat transactivator protein appears to play a critical role in binding to the TAR RNA sequence of nascent viral transcripts (25–29). Therefore, arginine in peptide linkage is capable of binding tightly to some binding sites on RNA molecules, although this has not previously been demonstrated for group I intron RNA.

In initial studies done to confirm the sensitivity of splicing by intron Pc1.LSU to competitive inhibition by L-arginine (13), we noticed that inhibitory levels of L-arginine resulted in a greater decrease in the rate of production of splicing intermediates than in the rate of production of final splicing products. Although L-arginine has been reported to inhibit the first but not the second step of the splicing reaction (16), an inhibitor of the first step should secondarily result in inhibition of production of final products, as was found for pentamidine and its analogues (3,13). In this study we report that L-arginine actually stimulates the second step of the splicing reaction. A series of arginine analogues and oligopeptides, including some much more potent than L-arginine, were characterized with regard to their ability to alter the catalytic properties of the Pc1.LSU ribozyme (3). These results indicate that ribozymes, like protein enzymes, can be altered in their catalytic properties by interaction with small molecules.

MATERIALS AND METHODS

Ribozyme assays

A radioactive 660 nt E1-I-E2 precursor RNA (P) and the 356 nt guanylylated linear intron Pc1.LSU were produced by SP6 RNA

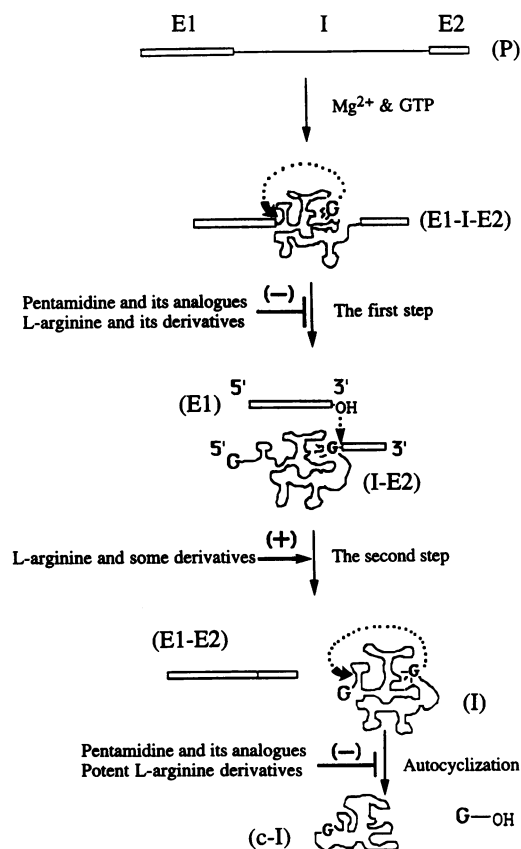*To whom correspondence should be addressed

Figure 1. Diagram showing the two sequential transesterification steps of group I intron splicing and the autocyclization of the excised linear intron. P indicates RNA precursor E1-I-E2; E1 and I-E2 are intermediates resulting from the first (cleavage) step; E1-E2 and I represent the final products of splicing from the second (ligation) step. Circular intron (c-I) is produced by the autocyclization reaction. The targets of action by small molecule effectors are also indicated, with inhibition represented by (–) and stimulation by (+).

polymerase (Promega) transcription of polymerase chain reaction products of a cloned genomic fragment, and were gel purified as previously described (3,13). Self-splicing and autocyclization reactions were performed at 37°C in the presence of 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$ and 0.4 mM spermidine; for splicing reactions GTP (5 μM or as indicated) was included. Reactions were stopped by addition of 4 M urea and 5 mM EDTA and were analyzed by polyacrylamide gel electrophoresis (5% acrylamide, 0.1% bis-acrylamide) run in the presence of 8 M urea. Gels were dried and bands were visualized by autoradiography and radioactivity in each band was measured with an AMBIS Gel Scanner.

Kinetic analysis

Hanes–Woolf plots (17,30) derived from data for splicing assays were used to calculate K_i values for inhibitors, where K_i was calculated (at $[GTP] \ll K_m$) by the equation:

$$\frac{v_o}{v_i} = 1 + \frac{[I]}{K_i} \qquad (1)$$

In this equation v_o is the reaction rate without inhibitors, and v_i is the rate in the presence of inhibitor I at the indicated concentration. At different concentrations of inhibitors, competitive inhibitors result in lines parallel to that for reaction run in the absence of inhibitor, and non-competitive inhibitors result in lines converging on the x-axis.

Chemicals

Pentamidine, BBE, benzamidine and p-aminobenzamidine were provided by R. R. Tidwell (University of North Carolina). All dipeptides, dipeptide amides and tripeptides were from Bachem Bioscience and arginine and its monomer derivatives and guanidine–HCl were from Sigma Chemical Company.

RESULTS

Bidirectional effects of arginine

Comparison of splicing reactions run in the presence and absence of L-arginine (5 mM) demonstrated that arginine causes greater reduction in accumulation of splicing intermediates (E1 + I-E2) than of splicing products (E1-E2 + I), as seen in Figure 2A. Quantitation of similar data (Fig. 2B) confirms the visual impression that L-arginine (20 mM) reduces the rate of the first step of splicing, yet results in an increased yield of splicing products. This result is consistent with L-arginine acting as a stimulator of the second step of the splicing reaction, although analysis is difficult because both reactions are occurring simultaneously.

In order to study the second step of the splicing reaction, we developed a 'two-step splicing assay', based on the ability of high concentrations of pentamidine or BBE [trans-1,2-bis(5-amidino-2-benzimidazolyl)ethene] to totally block the first step of the splicing reaction (3,13). In this assay (Fig. 3), a splicing reaction is run for 15 min and then pentamidine (300 μM) or BBE (5 μM) is added at a concentration sufficient to totally block the first step of splicing, followed by continued incubation for 15 min. Figure 3A (lane 7) demonstrates that without added arginine, pentamidine addition allows the intermediates (E1 + I-E2) to convert to a limited extent to products (E1-E2 + I); quantitation of bands proves that no additional precursor (P) is processed after pentamidine addition. Addition of L-arginine just after pentamidine (lanes 8–10) increases the production of products from intermediates (Fig. 3B), while quantitation demonstrates that the block by pentamidine of the first step of the splicing reaction is not reversed by arginine. In all two-step splicing reactions, quantitation of precursor RNA confirmed that inhibition of the first step remained complete during the assay.

Structural requirements for inhibition and stimulation of splicing

As indicated in Table 1, the structural requirements for amino acid inhibition of splicing are similar to those for other group I introns. The other 19 naturally occurring L-amino acids at 3 mM failed to inhibit the splicing reaction, as is the case for other group I introns (16,17). Similarly, L-citrulline failed to show detectable inhibition at 20 mM, indicating the requirement for the guanidino group for inhibitory activity. However, the guanidino group alone was not sufficient for activity, since guanidine–HCl was inactive as an inhibitor at 40 mM. N^G-Methyl-L-arginine showed a modest increase in inhibitory activity relative to L-arginine, suggesting
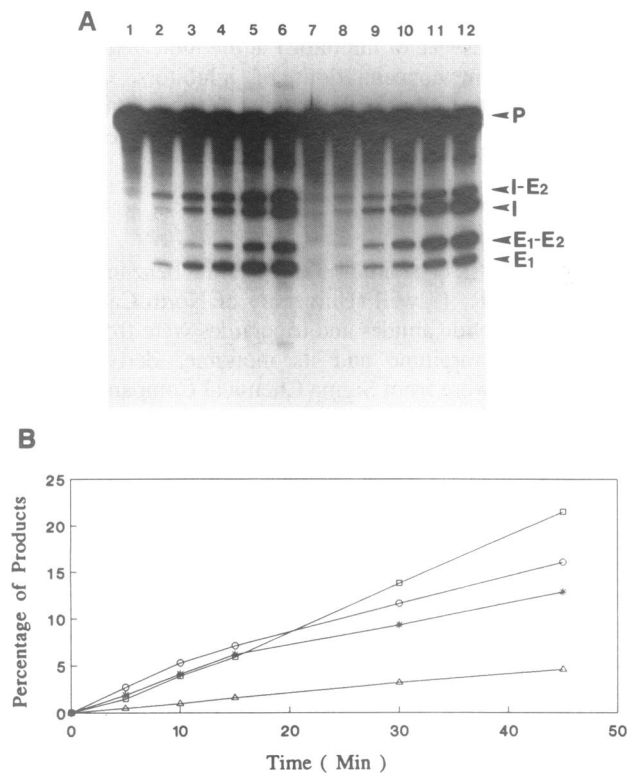
Figure 2. Time course of splicing showing the bidirectional effects of L-arginine. (**A**) Denaturing 5% polyacrylamide gel electrophoresis run in the presence of 8 M urea. The splicing was performed under the conditions as described in Materials and Methods in the absence (lanes 1–6) or presence (lanes 7–12) of 5 mM L-arginine for 0 (lanes 1 and 7), 2 (lanes 2 and 8), 5 (lanes 3 and 9), 10 (lanes 4 and 10), 20 (lanes 5 and 11) and 45 min (lanes 6 and 12). (**B**) Graphical comparison of the accumulation of splicing intermediates (I-E2 + E1) between reactions without (○) and with (△) 20 mM L-arginine and the yield of products (I + E1-E2) without (*) and with (□) 20 mM L-arginine, expressed as the percentage of the total radioactivity (P + I-E2 + I + E1-E2 + E1) in each reaction. At all times the total reaction products (intermediates plus products) was greater in the control reaction, with the level in the presence of 20 mM L-arginine gradually increasing from 43.5 (5 min) to 90.0% (45 min) of the control value.

Figure 3. 'Two-step splicing assay' to measure the stimulatory activity of L-arginine. (**A**) Lane 1 is a control reaction without GTP. Lane 2 shows a splicing reaction run with 5 μM GTP for 15 min, which was incubated for another 15 min in the absence (lanes 3–6) or presence (lanes 7–10) of 300 μM pentamidine with 0 (lanes 3 and 7), 2 (lanes 4 and 8), 5 (lanes 5 and 9) and 10 mM L-arginine (lanes 6 and 10). (**B**) Graphical presentation of (A) showing the stimulatory effects of L-arginine, either in the presence (△, intermediates E1 + I-E2; ○, products E1-E2 + I) or absence (*, intermediates; □, products) of 300 μM pentamidine, on the second step of splicing. Total yield of products was lower in the presence of pentamidine due to its complete inhibition of further splicing from precursor.

that N-substituents on the guanidino moiety of L-arginine may have increased its ability to interact with the ribozyme. The inhibitory effect of arginine is stereospecific, with the K_i for D-arginine being 2.7 times that of L-arginine. The α-amino group appears to be replaceable by a hydroxyl moiety, since L-argininic acid is approximately as inhibitory as is L-arginine. On the other hand, addition of an acetyl moiety to the α-amino group in N-α-acetyl-L-arginine results in loss of inhibitory activity. Substitution of O for the last CH_2 in the arginine side chain in L-canavanine increases the inhibitory activity.

Some derivatives of arginine are more potent inhibitors of the splicing reaction. The methyl and ethyl esters and amide of L-arginine have K_i values at least 450-fold below that of L-arginine. The increased inhibitory potency of the esters and amide relative to the parent compound for the reaction catalyzed by Pc1.LSU was greater than reported for the tetrahymena group I intron reaction (16). N^G-Nitro-L-arginine methyl ester was more potent an inhibitor than L-arginine but the nitrate moiety on the guanidino group clearly raised the K_i relative to that of L-arginine methyl ester.
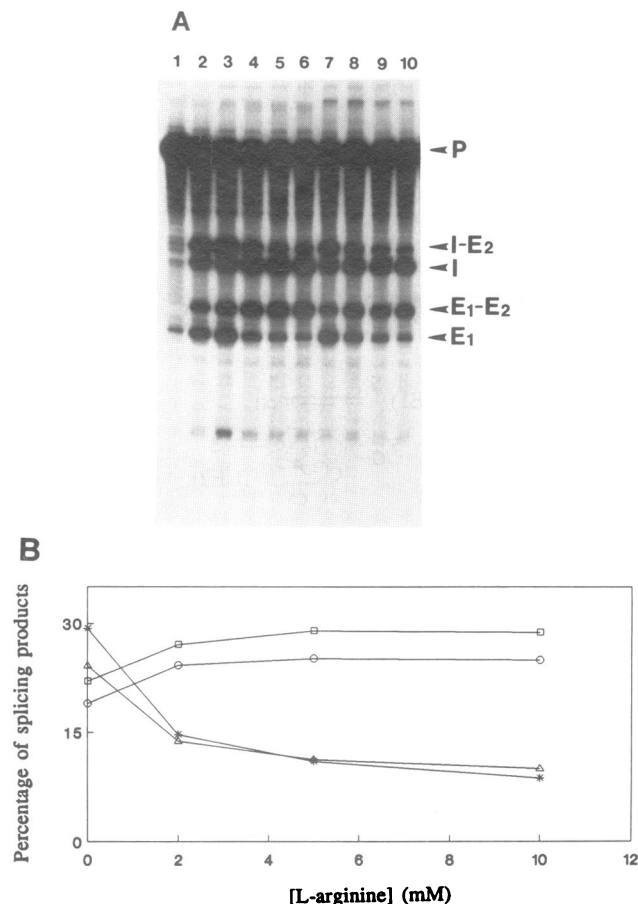
Hanes–Woolf plots for all of the active inhibitors discussed above demonstrated competitive inhibition, as previously reported for L-arginine (13). When the two-step splicing assay was performed to measure stimulation of the second step of the splicing reaction using these compounds (as shown for L-arginine in Fig. 3), the non-inhibitory compounds L-citrulline and N-α-acetyl-L-arginine also failed to show stimulatory activity. To quantitate stimulatory potency in the two-step splicing assay, we measured the effect of each stimulator by the stimulation index, defined as the increase due to the stimulator in the production of splicing products relative to intermediates plus products at a given concentration of stimulator:

$$\text{Stimulation index} = \frac{(I + E1\text{-}E2)_s - (I + E1\text{-}E2)_o}{E1 + I\text{-}E2 + I + E1\text{-}E2} \times 100$$

Here the subscript 's' refers to products produced with the addition of stimulator, and 'o' to products produced without stimulator. Although the stimulation index is not a standard

Table 1. Summary of the inhibitory and stimulatory activity of the arginine-related compounds

Compound	Structure		Ki^a(mM)	Cs^b(mM)	Ki/Cs
	NH$_2$-CH-CH$_2$CH$_2$CH$_2$-NH-C-NH$_2$ R1 R2				
L-arginine	R1=COOH	R2=NH	9.0	1.7	5.3
D-arginine	COOH	NH	24	30	0.80
L-citrulline	COOH	O	(-) 20	(-) 20	-
agmatine	H	NH	1.5	1.1	1.4
L-arginine methyl ester	COOCH$_3$	NH	0.016	0.035	0.46
L-arginine ethyl ester	COOCH$_2$CH$_3$	NH	0.017	0.043	0.40
L-arginine amide	CONH$_2$	NH	0.020	0.042	0.48
N-α-acetyl L-arginine	CH$_3$C-NH-CH-CH$_2$CH$_2$CH$_2$-NH-C-NH$_2$ O COOH NH		(-) 20	(-) 20	-
L-argininic acid	HO-CH-CH$_2$CH$_2$CH$_2$-NH-C-NH$_2$ COOH NH		7.5	8.4	0.89
L-canavanine	NH$_2$-CH-CH$_2$CH$_2$- O -NH-C-NH$_2$ COOH NH		1.1	(-) 10	-
N^G-methyl L-arginine	NH$_2$-CH-CH$_2$CH$_2$CH$_2$-NH-C-NH-CH$_3$ COOH NH		2.3	0.94	2.4
N^G-nitro-L-arginine methyl ester	NH$_2$-CH-CH$_2$CH$_2$CH$_2$-NH-C-NH-NO$_2$ COOCH$_3$ NH		1.5	1.9	0.79
L-Ala-L-Arg			(-) 10	(-) 10	-
L-Arg-L-Ala			0.24	0.45	0.53
L-Arg-L-Ala amide			0.027	0.12	0.23
L-Arg-L-Ser			0.22	0.90	0.24
L-Arg-L-Ser amide			0.016	0.059	0.27
L-Arg-L-Ser-L-Arg			0.0083	0.15	0.055
L-Arg-L-Arg			0.011	0.069	0.16
L-Arg-L-Arg amide			0.0008	0.020	0.040
L-Arg-L-Arg-L-Arg			0.0015	0.0043	0.35
guanidine-HCl			(-) 40	(-) 40	-
benzamidine			(-) 4	(-) 2	-
p-aminobenzamidine	NH$_2$-C$_6$H$_5$-C-NH$_2$ NH		1.3	(-) 2	-

Modifications on some of the monomeric compounds are indicated in bold.
$^a K_i$ values were calculated based on the data derived from Hanes–Woolf plots as described in the text; (–) indicates no inhibition was detected at concentration indicated.
$^b C_s$, termed the stimulation constant, is the concentration of the compound which results in a stimulation index of 10 in the 'two–step splicing assay'; (–) indicates no stimulation was detected at concentration indicated.

Figure 4. Comparison of stimulatory effect of arginine derivatives. Stimulatory index values are compared for D-arginine (✗), L-arginine (○), L-Arg-L-Ala (◆), L-Arg-L-Ser-L-Arg (+), L-Arg-L-Arg (*), L-arginine methyl ester (star), L-Arg-L-Arg amide (△), and L-Arg-L-Arg-L-Arg (□).

kinetic parameter, it represents a quantitative measurement of stimulation of conversion of reaction intermediates to products in the two-step splicing assay. To define a parameter for stimulatory potency of specific compounds, we used the stimulation constant, C_s, which is defined here as the concentration of the compound which results in a stimulation index of 10. The stimulation index measurements used to calculate C_s values are shown in Figure 4. In Table 1, the C_s values of the compounds tested are listed, along with the ratio K_i/C_s. A high ratio indicates a compound which is relatively more active as a stimulator than as an inhibitor, and a low ratio indicates the opposite.

The data in Figure 4 and Table 1 indicate that the stereospecificity of stimulation is even greater than that of inhibition, with C_s of D-arginine being over 17 times that of L-arginine. The monomeric arginine derivatives having both inhibitory and stimulatory activities tend to show a correlation between these activities; the most potent inhibitors also tend to be the most potent stimulators. Although some variation is seen, the other monomeric compounds all seem to have a higher ratio of inhibitory to stimulatory activity than does L-arginine (K_i/C_s = 5.3), with K_i/C_s ratios varying from 0.40 to 2.4. One exception to the correlation between inhibitory and stimulatory potency is
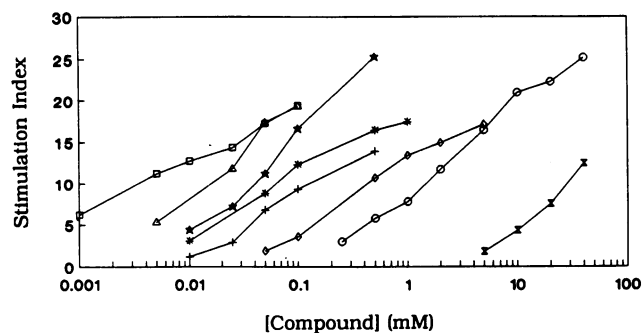
L-canavanine, which is a more potent inhibitor than L-arginine (K_i = 1.1 mM), but for which stimulatory activity could not be detected at 10 mM.

Peptides interacting with the group I intron

For intron Pc1.LSU, some arginine-containing peptides were far more potent inhibitors than L-arginine (Table 1). The inhibitory binding site on the ribozyme appears to accommodate bulky substituents on the carboxyl group but not on the α-amino group, since 10 mM L-alanyl-L-arginine failed to inhibit splicing yet L-arginyl-L-alanine had a K_i over 37-fold below that of L-arginine. L-Arginyl-L-serine was about as inhibitory as L-arginyl-L-alanine, while the amides of both dipeptides had much lower K_i values, indicating that carboxyl-amidation of these dipeptides increases their inhibitory activity. L-Arginyl-L-arginine was even more potent, and L-arginyl-L-arginine amide and L-arginyl-L-arginyl-L-arginine were the most potent inhibitors tested, with K_i values ~1/10 000 that of L-arginine. L-Arginyl-L-seryl-L-arginine had a K_i over 26-fold below that of L-arginyl-L-serine and 5.5 times that of L-arginyl-L-arginyl-L-arginine. Therefore, the inhibitory potency of peptides of up to three amino acids in length can be increased by the presence of arginine in each position, with amino-terminal arginine apparently optimal for inhibitory activity. Since the twenty naturally occurring amino acids can be combined to produce 400 dipeptides and 8000 tripeptides, complete analysis of their effect on the ribozyme is beyond the scope of this work.

The di-and tri-peptides which had lower K_i values than L-arginine for the splicing reaction also had lower C_s values based on the two-step splicing assay (Fig. 4 and Table 1). For the peptides tested, the K_i/C_s ratio varied from 0.53 to 0.040. The peptide L-arginyl-L-arginine amide displayed the lowest K_i/C_s ratio (0.040), indicating that this most inhibitory (lowest K_i) peptide displayed the highest ratio of inhibitory to stimulatory activity. The inhibitory and stimulatory effects of L-arginine and L-arginyl-L-arginyl-L-arginine on introns Pc2.LSU and Pc3.LSU resemble those described for Pc1.LSU in this report (data not shown).

In a standard splicing assay, L-alanyl-L-arginine (10 mM) was the only compound tested showing modest stimulation of production of both the intermediates and products (Fig. 5). In contrast, L-alanyl-L-arginine (10 mM) showed no inhibitory activity in the standard splicing assay and no stimulatory activity
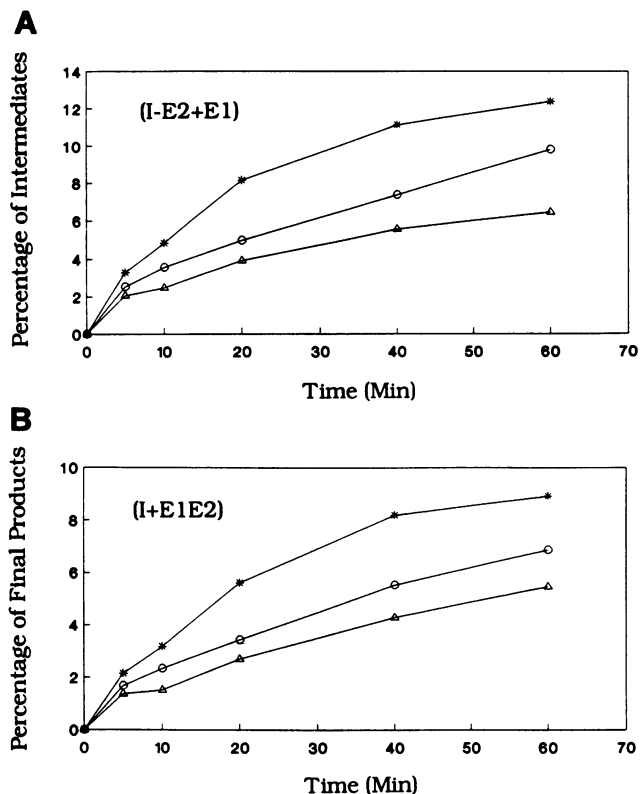
A

B

Figure 5. Time course showing the effects of L-canavanine and L-Ala-L-Arg on both steps of splicing. (**A**) Accumulation of intermediates (I-E2 + E1) and (**B**) yield of products (I + E1-E2) in reactions without (○) or with 1 mM L-canavanine (△) or 10 mM L-Ala-L-Arg (*), expressed as in Figure 2B.

in the two-step splicing assay (Table 1). L-Canavanine (1 mM) inhibited production of both intermediates and products (Fig. 5), as would be expected from its lack of stimulatory activity in the two-step assay (compare Fig. 2B).

Figure 6 demonstrates the time course of a standard splicing assay performed in the presence of 5 μM L-arginyl-L-arginyl-L-arginine, where it can be seen that inhibition of the first step of splicing initially slows production of both intermediates and products, but stimulation of the second step allows products to accumulate by 60 min with a higher yield than seen in the control reaction.

Although all of the arginine-related compounds inhibited splicing by acting competitively with GTP, GTP at concentrations as high as 1 mM failed to block the stimulatory effects of L-arginine (2 mM) or L-arginyl-L-arginyl-L-arginine (50 μM) observed in the two-step splicing reaction (data not shown).

Distinction between arginine and pentamidine effects

Pentamidine contains two amidino groups and all of the active pentamidine analogues previously tested for effects on intron splicing have paired positively charged amidino or imidazolyl moieties (13). Therefore, the kinetic differences between the non-competitive inhibition by pentamidine and competitive inhibition by L-arginine might be attributed to the bifunctional nature of pentamidine and its analogues, as opposed to the single functional guanidino group on L-arginine and its monomeric analogues.
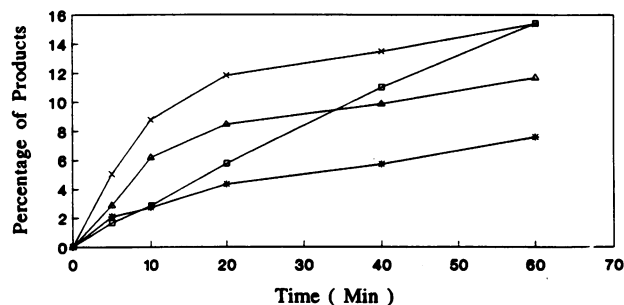

Figure 6. Time course showing the bidirectional effects of L-Arg-L-Arg-L-Arg on splicing. The accumulation of intermediates (I-E2 + E1) in reactions run without (×) or with (*) 5 μM L-Arg-L-Arg-L-Arg and the yield of products (I + E1-E2) in reactions without (△) or with (□) 5 μM L-Arg-L-Arg-L-Arg, expressed as in Figure 2B.

Although some arginine-containing peptides with multiple guanidino groups are more potent inhibitors and stimulators of the splicing reaction than L-arginine (Table 1), all are still competitive inhibitors, as shown in Figure 7A for L-arginine-L-arginyl-L-arginine. This contrasts with the non-competitive inhibition shown by pentamidine and its analogues (13). The compound *p*-aminobenzamidine resembles a mono-functional pentamidine derivative, having a single aromatic ring bearing amino and amidino groups. Like pentamidine, *p*-aminobenzamidine inhibits splicing *in vitro*, but has a K_i of 1.3 mM, as opposed to 0.075 mM for pentamidine (13). Kinetic analysis demonstrates that *p*-aminobenzamidine acts as a non-competitive inhibitor (Fig. 7B). This compound demonstrates no stimulation in the two-step splicing assay. Thus it appears that the arginine and pentamidine-related classes of compounds act on group I intron ribozymes by distinct mechanisms, whose distinction is not based upon the number of positively charged moieties on each molecule.

Inhibition of autocyclization

The lack of inhibition by L-arginine of the second step of the splicing reaction or autocyclization of the tetrahymena group I intron has been cited as evidence that L-arginine inhibition is limited to the initial reaction of free guanosine nucleotide with the ribozyme (16). Although the second step of splicing does not involve participation of free guanosine, the autocyclization reaction is believed to proceed by nucleophilic attack on a 5′-proximal site by the 3′-OH of the 3′-terminal G of the excised linear 5′-guanylylated intron (31–33). We were unable to demonstrate inhibition of autocyclization of intron Pc1.LSU by L-arginine at concentrations as high as 20 mM. However, Figure 8 demonstrates that the more potent splicing inhibitors, such as pentamidine and L-arginyl-L-arginyl-L-arginine, did inhibit this reaction. Inhibition has also been demonstrated by L-canavanine, L-arginine methyl ester, L-arginyl-L-arginine and L-arginyl-L-arginine amide (data not shown). This inhibition indicates that arginine-related inhibitors do act on autocyclization, although they may be less able to compete with the 3′-terminal G residue of the excised intron than with free guanosine substrate, both of which probably interact with the same binding site on the ribozyme. Pentamidine and BBE, which act on the first step of splicing as non-competitive inhibitors (13), inhibit autocycliz-
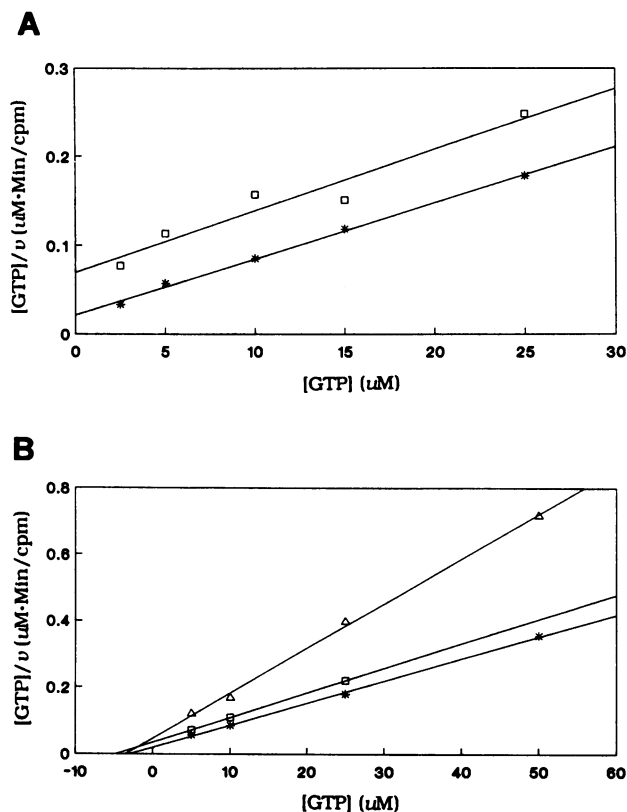
A

B

Figure 7. Kinetics (Hanes–Woolf plots) of inhibition of splicing. (**A**) Competitive inhibition of splicing by L-Arg-L-Arg-L-Arg. Reactions were run without (*) or with (□) 2.0 μM L-Arg-L-Arg-L-Arg. (**B**) Non-competitive inhibition by p-aminobenzamidine. Reactions were run without (*) or with 1.25 mM (□) or 1.50 mM p-aminobenzamidine (△).
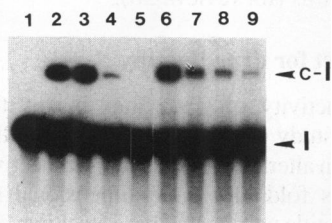

Figure 8. Inhibition of autocyclization reaction. Lane 1 shows the reaction without magnesium. The linear intron were incubated for 30 min under the conditions described in Materials and Methods in the presence of 0, 200, 300 and 500 μM pentamidine (lanes 2–5), 20 mM L-arginine (lane 6), or 25, 100, and 500 μM L-Arg-L-Arg-L-Arg (lanes 7–9).

ation at about the same concentrations that affect the splicing reaction.

DISCUSSION

Interaction of low molecular weight compounds with ribozymes

Our results suggest that low molecular weight compounds can interact with a ribozyme to alter its folding, resulting in changed catalytic properties. A variety of compounds act as competitive

or non-competitive inhibitors of splicing by group I introns *in vitro*, acting on the first step of the splicing reaction. In addition, a series of arginine derivatives and oligopeptides act on the ribozyme to increase its ability to carry out the second step of the splicing reaction. These results suggest that ribozymes, like protein enzymes, might be targets for physiological or pharmacological regulation by small molecules other than their required substrates or co-factors. Physical studies will be needed to determine if the stimulatory effect is mediated by an alteration in ribozyme conformation induced by the stimulatory small molecules.

Structural requirements for interaction of arginine and related compounds with RNA

The inhibitory arginine-binding site of the Pc1.LSU intron ribozyme appears to have properties similar to those described for other group I introns (16,17,21). The site to which arginine binds to stimulate the second step of the splicing reaction appears to share many properties with the inhibitory site, based on comparison of the spectrum of compounds interacting with both sites. Previous site-directed mutagenesis studies of the tetrahymena group I intron have confirmed that the same ribozyme nucleotides are required for inhibition by arginine and interaction with guanosine nucleotides (18,21). However, the lack of requirement for participation of guanosine in the second step of splicing and the differences seen in inhibitory and stimulatory potency of the compounds tested in Table 1 indicate that despite their similarities, distinctions exist between the inhibitory and stimulatory arginine binding sites. These differences may reflect conformational changes occurring in the same region of the ribozyme during catalysis, since the RNA structure required for guanosine nucleotide binding and attack at the 5′ end of the intron, resulting in cleavage at the 5′ exon–intron junction, may be distinct from that required for cleavage at the intron–3′-exon junction and ligation of the two exons. A conformational change bringing the 3′ terminal guanosine nucleotide of the intron close to the guanosine nucleotide binding site has been shown to occur for the second step of the splicing reaction (18,34,35). Since the same site apparently functions as the guanosine binding site and inhibitory arginine binding site during the first step of the splicing reaction, the conformational change required to bring the guanosine binding site near the 3′ terminal guanosine of the intron may also alter the binding site itself, thus affecting the spectrum of its affinity for different arginine derivatives.

The chiral preference for L-arginine by the inhibitory binding site on group I intron ribozymes resembles the chiral preference for L-amino acids in protein synthesis. Such chiral preference is not required for all RNA binding by arginine, since an RNA binding site selected from among random sequences has been shown to prefer D-arginine (36). For intron Pc1.LSU, while inhibition by arginine showed a 2.7-fold preference for the L-enantiomer, similar to that described for other group I introns (17), the C_s value for D-arginine was 17.6 times that for L-arginine, making the stimulatory binding site the most stereospecific amino acid binding site on RNA yet described.

Both the inhibitory and stimulatory effects require the guanidino group. The non-competitive inhibition and lack of stimulation displayed by p-aminobenzamidine indicates that an amidino group will not replace the guanidino moiety for either effect. The lack of response to guanidine–HCl indicates that guanidine alone is not

sufficient for either interaction. L-Canavanine is more potent than L-arginine as an inhibitor of splicing by Pc1.LSU, yet fails to show stimulatory activity. This dramatic distinction suggests that either the steric effect or altered charge distribution caused by the oxygen for carbon substitution in L-canavanine potentially eliminates its ability to interact with the stimulatory site. In contrast, methylation of one guanidino nitrogen of L-arginine appears to modestly increase its potency in both assays.

The α-amino group of L-arginine can be replaced by a hydroxyl group without loss of effect on the ribozyme, as previously reported for the tetrahymena group I intron (16). However, α-amino derivatization by acetyl or L-alanyl moieties eliminated both effects on the ribozyme, indicating that the α-amino group plays a role in these interactions and must not be more distant from the guanidino group than it is in L-arginine.

Absence of the carboxyl group of L-arginine in agmatine or its esterification or amidation increases potency of effect on the ribozyme, with esterification or amidation increasing the inhibitory effect more than the stimulatory effect. This result suggests that the inhibitory binding site of the ribozyme is better able to accommodate a bulky carboxyl derivative than is the stimulatory site.

Some short oligopeptides are the most potent effectors of ribozyme activity yet reported. In polymers of L-arginine, both inhibition and stimulation were greatest for trimer, less for dimer and least for monomer, indicating that bulky polypeptides can be accommodated by the binding sites on the ribozyme. Arginine homopolymers are more potent inhibitors and stimulators than are heteropolymers (Table 1). These data suggest that in di- and tripeptides, L-arginine in each position contributes to ability to interact with the ribozyme. These results contrast with the weak inhibitory effect of L-arginyl-L-leucine and ineffectiveness of L-leucyl-L-arginine on the tetrahymena intron, which seems relatively unresponsive to these arginine-containing peptides (16). Recently, a series of cyclic guanidino peptides of the tuberactinomycin family have been shown to be competitive inhibitors of splicing by other group I introns with K_i values as low as 10 μM (37).

In addition to the lack of stimulatory activity by L-canavanine, the data presented indicate differences between optimal structures effective in inhibiting and stimulating the ribozyme activity. Based on the data in Table 1, L-arginine shows the highest ratio of K_i/C_s of any compound with both effects, indicating that it is relatively more potent as a stimulator than as an inhibitor. Many derivatives are more potent both as inhibitors and stimulators than is L-arginine, but the esters, amide and L-arginyl-L-alanine all have K_i/C_s ratios of 0.40–0.53, while other active L-arginine peptides have K_i/C_s ratios of 0.04–0.35. These differences in ratios indicate that those compounds with reduced K_i/C_s ratios are relatively more effective as inhibitors than as stimulators. These distinctions presumably reflect structural differences between ribozyme sites to which arginine binds to cause these two effects. Conformational differences between the ribozyme as it catalyzes the first and second steps of splicing (18,34,35) may alter the conformation of a single arginine-binding region responsible for both effects, with the 'inhibitory site' better able to accommodate bulky carboxyl-substituents on arginine than the 'stimulatory site'.

Inhibition of intron autocyclization

We have found that pentamidine and L-arginyl-L-arginyl-L-arginine, potent non-competitive and competitive inhibitors,

respectively, of the first step of the splicing reaction, also inhibit the autocyclization of the linear guanylylated excised intron Pc1.LSU. The relatively weak inhibitor L-arginine fails to inhibit autocyclization, despite the proposed mechanistic similarity of this reaction and the first step of splicing, as previously reported for the tetrahymena intron (16). Inhibition of autocyclization by the more potent inhibitors of the first step of the splicing reaction is consistent with the similarity between the attack by the 3'-OH of free guanosine nucleotide in the first step of splicing and by the 3'-OH of the 3'-terminal guanosine in autocyclization. The relative resistance of autocyclization to inhibition by the weak competitive inhibitor L-arginine may reflect the greater accessibility of 3'-terminal guanosine than free guanosine nucleotide to the active site of the ribozyme, due to proximity of the 3' terminus to the active site.

Antiribozyme compounds as antimicrobial agents

As previously proposed (3,7,12,13), compounds specifically inhibiting group I intron splicing in infectious agents might have potential as antimicrobial drugs, since humans appear to lack such introns. L-arginine is a weak competitive inhibitor of splicing by intron Pc1.LSU, and complete inhibition has not been observed at any concentration of L-arginine tested. However, this report indicates that related compounds, such as L-arginyl-L-arginine-amide and L-arginyl-L-arginyl-L-arginine are ~10 000 times more potent inhibitors than L-arginine. Although we have not tested the pharmacology or clinical efficacy of these compounds, these results indicate that systematic study of derivatives of weak ribozyme inhibitors might yield much more potent agents. These results support the possibility of using the principles of rational drug design to develop potent inhibitors of ribozymes as well as of protein enzymes. In addition to self-splicing introns, other potential targets for this approach might include other ribozymes of infectious agents, such as the self-cleaving ribozymes of hepatitis delta virus (for review, 38).

RNA as a target for drug therapy

The catalytic activity of ribozymes makes them convenient models for the study of the interaction of small molecules with RNA resulting in alteration of the biological activity of that RNA. RNA molecules fold into three-dimensional structures determined by nucleotide pairing and base stacking, as well as by their interaction with other molecular species. Any molecule binding to a specific RNA structure might alter its biological activity, whether this activity is catalytic, coding or regulatory in nature. The report that aminoglycosides, which act on ribosomes to inhibit protein synthesis as well as on group I introns, can also selectively block binding by the HIV-1 Rev regulatory protein to its RNA target indicates that this phenomenon is not limited to catalytic RNA molecules (39). The recent report of RNA molecules specifically binding to the bronchodilator theophylline further demonstrates the potential of RNA molecules to recognize specific ligands (40). The potential spectrum of RNA targets for chemotherapy is unknown, but may approach that of protein targets. The finding that regulatory RNA may act in *trans* as well as in *cis*, defining a class of RNA molecules termed riboregulators (41), may result in additional RNA targets for development of small molecule effectors. Although such effectors have been considered mainly as inhibitors of the function of their RNA targets, this report indicates that small molecules can also

stimulate RNA function. Both effects might provide a basis for development of therapeutic agents.

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