Antibiotic interactions with the hammerhead ribozyme: tetracyclines as a new class of hammerhead inhibitor

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A screening of a range of common laboratory antibiotics for inhibition of the hammerhead ribozyme has shown that in addition to certain aminoglycosides (most notably neomycin B) the tetracyclines are also effective inhibitors, with chlorotetracycline being more effective than tetracycline. Inhibition by chlorotetracycline is not as strong as that by neomycin B but is more complicated, with at least two binding sites apparent. As with hammerhead inhibition by neomycin B, chlorotetracycline inhibition can be overcome by raising the concentration of the Mg^{2+} ion cofactor. We find that around six Mg^{2+} ions will displace neomycin B, compared with twelve for chloro-

tetracycline. Inhibition observed in the presence of mixtures of neomycin B and chlorotetracycline is consistent with separate binding sites on the hammerhead for these two classes of antibiotic. Under certain conditions of the mixing order and low concentration of chlorotetracycline, enhancement of single-turnover hammerhead cleavage by up to 20% is observed, with higher concentrations of antibiotic being inhibitory. We have also found that the presence of 2.5% (v/v) DMSO causes a 30% enhancement of the single-turnover cleavage. These results thus extend the range of known inhibitors of hammerhead cleavage, and also demonstrate how the cleavage can be accelerated.

INTRODUCTION

Satellite RNA species of certain plant viruses and a number of viroids contain a self-cleaving domain of around 40 bases known as the 'hammerhead' [1], which has also been found in the RNA transcript of newt satellite II DNA [2]. Viroids are circular, single-stranded RNA species and are the smallest infectious agents so far characterized, and are the causative agents of several plant diseases [3]. The hammerhead is one of several distinct classes of ribozyme that have so far been discovered. It consists of three helices meeting in a central core region, where most of the 13 conserved nucleotides reside (Figure 1). The isolated hammerhead cleaves *in vitro* in the presence of certain divalent metal ions; the ribose phosphate backbone is broken to yield a 2',3'-cyclic phosphate and 5'-hydroxyl group [4].

Experimental evidence strongly suggests that circular, plant-pathogenic RNA species (viroids and virusoids) replicate by rolling circle mechanisms *in vivo* [5]. Such mechanisms require site-specific RNA cleavage, which can be achieved in the ham-

merhead domain. It has been demonstrated that hammerhead self-cleavage occurs *in vivo* during the replication of the minus strand of the virusoid of lucerne transient streak virus [6]; this was the first direct evidence that the hammerhead cleavage observed *in vitro* also operates *in vivo*. Hammerhead-containing RNA plant pathogens, such as the avocado sunblotch viroid [7], the peach latent mosaic viroid [8], and the agent that probably causes carnation stunting [9], cause diseases.

The hammerhead can be assembled from one [10], two [4] or three [11] separate RNA strands. A number of studies have been reported that used chemical modifications to probe the roles of various functional groups within the hammerhead domain (reviewed in [12,13]). Recently two X-ray crystal structures of non-cleavable hammerhead systems have been reported, one with a DNA substrate analogue strand [14], and another with an all-RNA substrate strand with a 2'-O-methyl group at the cleavage site [15]. The two structures are very similar, based on a wishbone shape with a number of non-Watson-Crick and non-wobble base-pair interactions in the conserved core.

There have so far been few reports of antibiotic interactions with ribozymes. Certain aminoglycoside antibiotics, especially neomycin B, have been observed to inhibit the activity of the group I self-cleaving intron [16,17]. Neomycin B has also recently been found to inhibit hammerhead cleavage [18]. The pH and Mg^{2+} dependence of this inhibition have also been investigated [19]. Hammerhead inhibition provides a quantitative assay for assessing the potential RNA-binding properties of ligands, which is a potentially valuable tool for the rational design of RNA-binding drugs. Here we investigate some additional antibiotics for hammerhead inhibition, and also conditions that cause activation of hammerhead cleavage.

Figure 1 Secondary structure of the hammerhead ribozyme

The structure shown here is composed of a 19-mer ribozyme strand and 24-mer substrate strand. Conserved nucleotides are boxed.

MATERIALS AND METHODS

Synthesis and purification of oligoribonucleotides

Solid-phase synthesis and purification of the substrate (24-mer) and ribozyme (19-mer) oligoribonucleotides were performed as

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previously described [20]. The substrate RNA strand was radiolabelled at the 5' terminus in a 20 μ l volume containing 50 mM Tris/HCl, pH 8, 10 mM MgCl₂, 5 mM DDT, 1 μ M ATP, 0.1 μ M RNA, 20–50 μ Ci γ -[³²P]ATP and 15 units of polynucleotide kinase. Reactions were incubated for 30 min at 37 °C. The reactions were quenched with an equal volume of RNA loading buffer (25 mM EDTA, 8 M urea). The radiolabelled RNA was then purified by denaturing PAGE. All antibiotics were purchased from Sigma with the exception of the tetracyclines, which were purchased from Janssen Chimica. Antibiotic stock solutions were always freshly prepared.

Hammerhead single-turnover kinetics

All experiments were performed at 37 °C. Two procedures were used, differing in the way in which the RNA strands were mixed with MgCl₂ and the antibiotics.

Method 1

A mixture of ribozyme and radiolabelled (100 000 c.p.m. per 15 μ l) substrate in 50 mM Tris/HCl, pH 7.5, was heated at 90 °C for 1 min, allowed to cool to 37 °C. The appropriate quantity of antibiotic was then added, the solution was centrifuged at 13 600 rev./min for 5 min at 25 °C, and then it was incubated at 37 °C for 10 min. The cleavage reaction was initiated by the addition of 6 μ l of MgCl₂ solution and incubated at 37 °C. The reaction mixture then contained ribozyme (1 μ M), substrate (0.2 μ M) and (unless stated otherwise) 10 mM MgCl₂ in a volume of 30 μ l. At intervals, aliquots of 2 μ l were withdrawn and quenched with 5 μ l of 50 mM EDTA in 8 M urea and then subjected to denaturing PAGE. The radiolabelled bands were revealed and quantified with a FUJIX BAS1000 Phosphorimager. Experiments were performed in at least duplicate. Semi-logarithmic plots of the proportion of substrate cleaved against time were used to determine the half-lives of the hammerhead. The linear correlation coefficients were in the range $r = 0.99$ – 0.97 .

Method 2

Details are as for method 1, except that the ribozyme and substrate strands were annealed separately, both in the presence of MgCl₂; after cooling to 37 °C an appropriate amount of antibiotic solution was added to each solution. After incubation at 37 °C for 10 min, the cleavage reaction was initiated by mixing the two solutions.

When DMSO was included in the reaction mixture it was added in place of antibiotics with the use of method 1 mixing procedure.

Quoted error margins for dissociation constants are standard deviations; data points are averages of two experiments.

RESULTS AND DISCUSSION

The version of the hammerhead used in this study is that originally reported by Uhlenbeck [4], consisting of a 19-mer ribozyme strand and 24-mer substrate strand (Figure 1). This combination has been kinetically well characterized and used in a number of studies [4,21–23]. We have previously shown it to obey Michaelis–Menten kinetics under the conditions of pH and temperature used here [23].

A screening of a range of antibiotics for hammerhead cleavage inhibition has recently been reported [18] but with a different ribozyme (38-mer) and substrate (17-mer) strand combination from the one used here. For our screening we have used a number of antibiotics that have not previously been reported to

Table 1 Inhibition of hammerhead ribozyme cleavage by a range of common antibiotics

Inhibition is expressed as the percentage reduction in the observed rate constant for single-turnover hammerhead cleavage with respect to that observed in the absence of antibiotic, with the use of method 1 (see the Materials and Methods section) for mixing.

Antibiotic	Inhibition with antibiotic concentration of 500 μ M (%)
Tetracycline	56
Chlorotetracycline	100
Gentamicin*	86
Kanamycin A	32
Paromomycin	76
Neomycin B	100
Lincomycin	10
Chloramphenicol	0
Spectinomycin	0
Streptomycin	0
Erythromycin	0
Ampicillin	0

* Mixture of gentamicins C₁ and C₂.

Chlorotetracycline

Neomycin-B

Figure 2 Chemical structures of neomycin B and chlorotetracycline

The chlorine at position 7 in chlorotetracycline is replaced by hydrogen in tetracycline.

have been tested for hammerhead inhibition, namely lincomycin, spectinomycin, erythromycin, tetracycline and chlorotetracycline. The effects on single turnover of the hammerhead of antibiotic concentrations of 500 μ M are summarized in Table 1. Under these conditions complete inhibition (i.e. less than 1% cleavage observed after 30 min of incubation) was observed for neomycin B and chlorotetracycline, members of two distinctly different classes of antibiotic (Figure 2). Less strong, but still notable, inhibition was apparent for paromomycin, tetracycline and gentamicin. Moderate inhibition was observed with

Figure 3 Variation of inhibition of single-turnover hammerhead activity in the presence of various concentrations of neomycin B

Protocols used for mixing the RNA strands, $MgCl_2$ and antibiotic of the reaction mixture: Δ , method 1; $\circ$, method 2.

kanamycin A and lincomycin. The levels of inhibition that we found for neomycin B, paramomycin, gentamicin and kanamycin A are broadly consistent with the findings of Stage et al. [18], as are the lack of inhibition by chloramphenicol, ampicillin and spectinomycin. We found streptomycin to be inactive, although it has been reported [18] to be a modest inhibitor; this difference could be due to the alternative hammerhead constructs but most probably arises from the mixing procedures used, as discussed below. Neomycin B and paromomycin are closely related, and within a different group of aminoglycosides kanamycin A and gentamicin are also related. Lincomycin is a member of another distinct group of aminoglycosides. The tetracyclines have not, to our knowledge, previously been noted as ribozyme inhibitors.

Further investigations of the dependence of the observed first-order rate constant for hammerhead cleavage (single turnover) on the concentration of antibiotic were performed for the most potent aminoglycosides tested, neomycin B and gentamicin. The resulting curve for neomycin B (Figure 3) is a single hyperbola, consistent with a single binding site (or a number of identical sites) on the hammerhead, with a K_d of $31.4 \pm 3 \mu M$. Similar results were obtained for gentamicin with a K_d of $304 \pm 64 \mu M$ (results not shown). These values were obtained with a protocol (method 1; see the Materials and methods section) in which antibiotics were added to a pre-annealed ribozyme/substrate mixture (which thus contained intact hammerhead complex) before initiation of the reaction by the addition of Mg^{2+} . With an alternative order of mixing (method 2; see the Materials and methods section) in which antibiotic was added to both the ribozyme and substrate strands, each in the presence of Mg^{2+} , before they were mixed, a different curve is obtained (Figure 3) from which a K_d for neomycin B of $12.6 \pm 1.2 \mu M$ was obtained. This is in close agreement with the $13.5 \mu M$ obtained for neomycin B inhibition of single-turnover hammerhead cleavage by Stage et al. [18] under the same conditions of pH and Mg^{2+} concentration but with a different hammerhead construct. Those authors also noted that the extent of hammerhead inhibition was independent of the order of mixing of the various components present in the assay. Evidently, for the particular hammerhead used here, the apparent K_d for neomycin B inhibition is somewhat dependent on the order of mixing of the components. The simplest explanation is that where antibiotic is added to both ribozyme and substrate strands before their mixing, some or all

Figure 4 Variation of rate constant for single-turnover hammerhead activity in the presence of various concentrations of chlorotetracycline ($\circ$) and tetracycline (Δ) with method 1, and chlorotetracycline ($\square$) with method 2

of any initial antibiotic–RNA interactions must presumably be overcome before the hammerhead complex can form after mixing. The antibiotic thus causes apparently increased inhibition compared with the case when the antibiotic is mixed with the preformed hammerhead complex. (It should be noted that in experiments devoid of antibiotics, within experimental error, we obtained the same cleavage rates when using method 1 and method 2, as is evident in Figure 4.)

In addition to certain aminoglycosides, chlorotetracycline and tetracycline were also shown by our screening to be effective inhibitors of hammerhead cleavage. The dependence of this inhibition on the concentrations of tetracycline antibiotics was investigated in a manner similar to that used for neomycin B. Under single-turnover conditions, and using method 1 of mixing the components, plots as shown in Figure 4 were obtained. The curves are not simple hyperbolae and are thus quite different from the neomycins. At low concentrations of chlorotetracycline (below about $250 \mu M$) the curve is a rough fit to a simple binding curve with a K_d of approx. $70 \mu M$, but this covers only a few data points. With increasing chlorotetracycline concentration, the curve actually rises slightly before declining in a nearly linear fashion. This plot is difficult to analyse but it does suggest that more than one binding site is involved.

For chlorotetracycline, 50% inhibition occurred at approx. $300 \mu M$ and complete (100%) inhibition was observed at approx. $500 \mu M$. Tetracycline had similar effects, with 50% inhibition at approx. $420 \mu M$ and complete inhibition at approx. $1250 \mu M$. ‘Complete’ inhibition means that for both tetracycline and chlorotetracycline there was no trace of product even after prolonged incubation and PAGE analysis with a sensitive phosphorimager. In contrast, at high concentrations of neomycin B, a trace of product was always detectable, although at a very low (and difficult to quantify) level. Having noted that the order of mixing the components influenced the K_d obtained for neomycin B, we repeated the experiments for chlorotetracycline but using method 2 for the mixing. The resulting data are also shown in Figure 4, the relevant plot demonstrating that at low concentrations of chlorotetracycline (below approx. $75 \mu M$) the cleavage reaction is actually accelerated by as much as 20%; this effect is reproducible and well in excess of experimental error. Increasing chlorotetracycline concentration then neutralizes this effect, followed by increasing inhibition, with 50% inhibition at approx. $125 \mu M$ and complete (100%) inhibition at approx.

450 μM , i.e. somewhat lower concentrations than with method 1. Thus altering the order of mixing, as with neomycin B, does affect the extent of inhibition but chlorotetracycline interactions are obviously more complicated than those of neomycin B. If the 'effective' chlorotetracycline concentration is taken as the actual value minus 75 μM to remove the activating amount of antibiotic and leave the inhibitory contribution, the relevant curve of Figure 4 (i.e. using method 2) is a satisfactory fit to a simple hyperbola with a K_d of $63 \pm 5 \mu\text{M}$. The simplest interpretation of this is that there is one relatively strong binding site for chlorotetracycline, occupation of which enhances hammerhead cleavage, and a second 'inhibitory' site of weaker affinity.

Enhancement of hammerhead cleavage by methods other than chemical modification is not without precedent. Significant (10–20-fold) acceleration of hammerhead cleavage for both single and multiple turnover has been previously noted by the addition of a protein derived from the p7 nucleocapsid protein of HIV-1 [24]. We have also found that it is possible to accelerate hammerhead cleavage by the addition of an organic solvent, DMSO (using method 1, but with DMSO added in place of antibiotic), with a final proportion of DMSO of 2.5% (v/v), so that an enhancement of the rate of single turnover of hammerhead cleavage of 30% was observed. To our knowledge this effect has not previously been reported. Because we are using single-turnover activity and the hammerhead complex is preformed, only the cleavage event itself is rate-limiting; rates of substrate binding and product release are not manifested. The enhanced cleavage rate is likely to be due to the induction of a small amount of denaturation of the hammerhead complex, as this is the effect one would normally expect of the presence of an organic solvent on a nucleic acid structure. The promotion of cleavage by the introduction of some degree of flexibility is consistent with an optimum temperature of approx. 50 $^{\circ}\text{C}$ [4] for single-turnover cleavage of the hammerhead configuration used here; this is similar to the thermal melting temperature observed in the absence of Mg^{2+} but presence of 1 M NaCl [25].

The two X-ray crystallographic structures of hammerhead systems [14,15] both contain a conformation in the vicinity of the cleavage site that is inconsistent with the requisite 2'-hydroxyl in-line nucleophilic attack on the adjacent phosphodiester [14,15]. Although this might be due to crystal packing forces favouring such a non-cleaving conformation, it is possible that this conformation is in fact a significantly populated solution state. Photocross-linking experiments have suggested that the hammerhead exists in several conformations in solution under cleavage conditions [26], and it seems unlikely that more than one of these can be catalytically active. Some degree of extra flexibility, be it thermally or solvent-induced, then increases the accessibility of the active conformation and thus promotes cleavage. Alternatively a specific structural perturbation caused by the binding of a ligand may cause the cleavable conformation to become favoured. This line of reasoning suggests that a molecule of chlorotetracycline intrudes into the hammerhead, inducing a small amount of structural perturbation and thus promoting cleavage. Subsequently a second chlorotetracycline binds but is inhibitory. The initial binding of the activating ligand in the appropriate manner would then be possible only as the hammerhead assembles from the component strands; the ligand is unable to bind in the same way if the hammerhead complex has been preformed. This explains why activation is observed only with method 2 and not method 1. This is a hypothesis based on the results presented here; further work is in progress to test it. The stronger inhibition observed at higher chlorotetracycline concentrations with method 2 than with method 1 matches the general pattern observed for neomycin B.

Figure 5 Variation of observed rate constant for single turnover of hammerhead cleavage with increasing Mg^{2+} concentration in the absence of antibiotics (□), in the presence of 400 μM neomycin B (Δ) and in the presence of 400 μM chlorotetracycline (○)

All experiments used method 2 for mixing.

An alternative explanation for the enhancement of the hammerhead reaction is that, instead of affecting the ground state (i.e. increasing the population of the particular state compatible with cleavage), binding of chlorotetracycline (or the presence of organic solvent) might induce increased stabilization of the transition state of the cleavage. This possibility cannot be excluded at this stage. It should also be noted that in our experiments with DMSO the presence of organic solvent could alter the chemical activity, rather than the concentration, of Mg^{2+} , as well as possibly changing the effective pH, complicating the comparison with the reaction rate in the absence of the solvent.

We have noted above that complete inhibition of hammerhead cleavage is achieved (with method 1) by 500 μM chlorotetracycline and 1250 μM tetracycline. Interestingly, the ratio of these values is similar to that generally observed in the relative bactericidal activities of chlorotetracycline and tetracycline [27]. These two compounds differ only by the single atomic substitution of hydrogen for chlorine at position 7 (Figure 2), to which interaction with the hammerhead is evidently sensitive. The current consensus view of the interaction of tetracyclines with bacterial ribosomal RNA is that there is one strong binding site on the 70 S ribosome (with a K_d of between 1 and 30 μM), with many more weaker binding sites (K_d approx. 60 mM) [28]. This pattern has some obvious similarities with chlorotetracycline interactions with the hammerhead.

Previous reports have suggested that hammerhead inhibition by neomycin B is due to competition with Mg^{2+} ions, such that about five such ions displace the antibiotic [19]. Furthermore the inhibition increases as the pH is lowered, which is consistent with successive protonation of the various amino groups of neomycin B and consequent ionic interactions with the hammerhead. With tetracyclines, also found here to be effective inhibitors, it is of obvious interest to compare their mode of inhibition with that of neomycin B. Thus the effects of the presence of neomycin B and chlorotetracycline on the Mg^{2+} dependence of hammerhead cleavage were investigated. By using strongly inhibitory (at 10 mM Mg^{2+}) antibiotic concentrations of 400 μM , and method 2 for mixing (because this gives rise to slightly stronger inhibition than method 1), the curves in Figure 5 were obtained. Increasing the Mg^{2+} concentration overcomes inhibition by both neomycin

Figure 6 Hill plots derived from the data in Figure 5

Symbols: □, in the absence of antibiotics; △, in the presence of 400 μM neomycin B; ○, in the presence of 400 μM chlorotetracycline.

B and chlorotetracycline. This can be expressed by the derived values of K_{Mg} (Mg^{2+} concentrations giving half the maximum rate), which are 9.4 ± 0.5 mM in the absence of antibiotics, 23.7 ± 0.5 mM in the presence of 400 μM neomycin B, and 44.9 ± 0.2 mM in the presence of 400 μM chlorotetracycline. The value of K_{Mg} in the absence of antibiotics is similar to values ranging from 5.3 to 13 mM reported previously under the same or similar experimental conditions [23,29–32]. The plots in Figure 5 have very good fits to sigmoidal curves, so these data can be analysed further to show the change in the co-operativity of Mg^{2+} binding caused by the antibiotics. A Hill plot of these data (Figure 6) yields a Hill coefficient of 2.1 ± 0.2 in the absence of the antibiotics. Values of 2–3 have previously been reported under the same experimental conditions [29]; non-co-operativity has also been observed [19] but it has been noted that the curve-fitting can be ambiguous [32]. The Hill coefficients here are markedly increased by the presence of both neomycin B and chlorotetracycline to 6.0 ± 0.1 and 12.0 ± 0.8 respectively.

For the Hill coefficient to equal the number of sites there should be no stable intermediate states. Thus the hammerhead should bind either no Mg^{2+} ions or the full complement n . However, if $n-1$, $n-2$, etc., states are also populated, the Hill coefficient will be reduced. This is one of the main reasons why the Hill coefficient generally indicates a lower limit for the number of binding sites. Thus at least two Mg^{2+} ions are required for cleavage in the absence of antibiotics. About six Mg^{2+} ions can alleviate the inhibitory effect of 400 μM neomycin B, whereas about twice this number, twelve, will do so for 400 μM chlorotetracycline. This does not necessarily mean that these numbers are relevant to catalysis. In the simplest model, they are the numbers of Mg^{2+} ions that will displace the antibiotics from the hammerhead.

Our observation of the Hill coefficient in the presence of neomycin B is in reasonable agreement with the five Mg^{2+} ions previously reported to displace neomycin B with a different hammerhead construct, slightly lower pH (7.0) and a different analytical method [19]. More Mg^{2+} ions are apparently required to displace chlorotetracycline, but it may be that twelve metal ions displace two chlorotetracyclines from the hammerhead. The tetracyclines are noted as being strong chelators of metal cations [27,28] although the physiological role of this feature is not clear. (The large excess of Mg^{2+} over the antibiotic in our experiments means that such complexation is insufficient to make the ions

Table 2 Inhibition of hammerhead ribozyme in the presence of neomycin B and chlorotetracycline, both individually and as mixtures

All numbers are percentages of inhibition derived from the first-order rate constant for single-turnover hammerhead cleavage in the presence of one or both of the antibiotics chlorotetracycline and neomycin B, compared with cleavage rate in the absence of antibiotics. Method 2 was used for mixing throughout.

Inhibition by chlorotetracycline alone	Inhibition by neomycin B alone	Inhibition by chlorotetracycline–neomycin B mixture	Expected inhibition by mixture based on	
			Single-site model	Independent sites
12 ± 2	22 ± 2	34 ± 2	29	31
35 ± 2	70 ± 2	84 ± 1	74	81
49 ± 4	82.7 ± 2.6	92 ± 1	85	91

unavailable to the hammerhead.) Chlorotetracycline may be binding to the hammerhead as a metal ion chelate or the uncomplexed form, or both. Alternatively, the displacement of chlorotetracycline by Mg^{2+} ions could be due to formation of chelates that have reduced affinity for the hammerhead, so the ‘simplest model’ mentioned above would not apply. Because of these various possibilities, no conclusion about ionic interactions between the hammerhead and chlorotetracycline can be made at this stage. However, in that chlorotetracycline inhibition can be suppressed by increasing the concentration of Mg^{2+} ions, there is a clear resemblance to inhibition by neomycin B.

With two distinct classes of antibiotic being effective in inhibiting the hammerhead, it is of interest to determine whether their effects are additive. Thus in the presence of concentrations of neomycin B and chlorotetracycline that individually cause 50% inhibition, if the inhibitions are entirely independent (different binding sites, with occupation of either not altering the affinity of the other) the observed inhibition will be 75%. Alternatively if these two antibiotics compete for the same site the observed inhibition will be 66%. Experiments of this type were performed with mixtures of neomycin B and chlorotetracycline at three different concentration ratios. The resulting inhibition levels are shown in Table 2, together with the levels determined in the presence of each antibiotic alone and those expected from the simple one-site and independent-sites models. At lower levels of antibiotic inhibition the distinction between the two models is somewhat marginal, but at higher levels the observed values clearly support the independent-sites model. This conclusion is the simplest that fits the data; it ignores complications such as the possible effects of the presence of two chlorotetracycline binding sites and the order of mixing the components. Further work is under way to clarify the combined effects of these two inhibitors. Because the structures of neomycin B and chlorotetracycline are very different (Figure 2), one might expect them to occupy different sites; chlorotetracycline might intercalate whereas neomycin B might bind to grooves and clefts.

Tetracyclines are liable to isomerization; chlorotetracycline readily converts to isochlorotetracycline on heating at pH 7.5 [33]. To minimize this possibility we used fresh solutions and avoided excessive heating of the antibiotics before initiating the cleavage reactions.

Conclusions

Aspects of neomycin B inhibition investigated here are in general agreement with previous reports [18,19] but tetracyclines have

not to our knowledge previously been reported as ribozyme inhibitors. The interaction of chlorotetracycline with the hammerhead is complicated; there seem to be at least two different binding sites, occupation of one of which confers enhancement of ribozyme activity under certain experimental conditions. The complexity of the hammerhead-chlorotetracycline interaction is such that we are able to make only preliminary explanations and speculations based on the results presented here. A considerable number of additional experiments such as variation of pH, temperature, the identity of the divalent metal ion cofactor, the permutations of mixing of assay components, and the use of multiple-turnover experiments are all obviously appropriate for further characterization of these interactions.

We have shown two methods for enhancing the rate of hammerhead cleavage. At present the precise mechanism of the catalysed reaction (i.e. the roles of metal ions and RNA functional groups) is not known although some speculation based on crystal data has been made [15]. It is thus unclear whether chlorotetracycline-induced and DMSO-induced rate enhancements have the same underlying cause.

The availability of X-ray crystallographic structures of the hammerhead means that molecular modelling of docking of antibiotic structures to the hammerhead would be practicable. This would be complicated for aminoglycosides such as neomycin B because they have a number of internal degrees of freedom. However, the tetracyclines are far more rigid, with only a limited number of possible tautomeric forms [33]. It is possible that chemical modifications would produce a more potent tetracycline-type hammerhead inhibitor than the two members of the tetracycline class tested here.

There is some prospect that the tetracyclines will provide the basis for chemical treatments for plant diseases caused by infection by hammerhead-containing viroids. Aminoglycosides are generally toxic to plants, as exemplified by the use of resistance to neomycin B as a genetic marker [34]. Plants are more tolerant of tetracyclines; indeed there is an extensive history of the use of tetracyclines in horticulture [27,35]. For example, tetracyclines have been used against mycoplasma [36,37] and aphid [38] attack, with the antibiotics being administered by injection into the plants. Thus tetracycline inhibition of hammerhead cleavage *in vivo* could interrupt the viroid replication cycle, provided that hammerhead self-cleavage is essential and the antibiotic can infuse into the infected tissue and attain adequate local concentrations.

It is evident that under our experimental conditions neomycin B binds more tightly to the hammerhead than does chlorotetracycline. However, at antibiotic concentrations giving strong hammerhead inhibition, a higher concentration of Mg^{2+} ions is required to displace chlorotetracycline than neomycin B (the K_{Mg} values noted above differ by a factor of approx. 2). In this sense chlorotetracycline is a more potent inhibitor than neomycin B. It is interesting to note that tetracyclines are in extensive clinical

use, and in the recent past have been among the drugs administered in the greatest quantity [27].

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