Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 2549–2556

BIOORGANIC & MEDICINAL CHEMISTRY

A Tetracycline-binding RNA Aptamer

Christian Berens[†,‡], Alison Thain[†,§] and Renée Schroeder*

Institute of Microbiology and Genetics, Vienna Biocenter Dr. Bohrgasse 9/4, A-1030 Vienna, Austria

Received 23 February 2001; revised 9 March 2001; accepted 9 March 2001

Abstract—Aptamers are perfect tools to study the interaction of small ligands with RNA. To study the mode of interaction of tetracycline with RNA, we isolated aptamers with high affinity to this antibiotic via in vitro selection. One of the selected aptamers, cb28, which has a comparable affinity to tetracycline as the small ribosomal subunit, was characterised in more detail. Cb28 binds only to typical tetracyclines, while atypical tetracyclines are not recognised. The hydroxyl group at position 6 is an essential determinant for recognition, while modifications at positions 4, 5 and 7 do not interfere with RNA binding. Binding of tetracycline to cb28 is magnesium dependent. The secondary structure of cb28 was determined by lead cleavage and DMS modification. Upon tetracycline binding, nucleotides in J2/3 and the P5 stem-loop are protected from cleavage by lead, indicating a conformational change in the RNA. This conformational change was confirmed by tetracycline dependent changes in the DMS modification pattern. Photo-induced affinity incorporation of tetracycline into cb28 resulted in a crosslink to position G76, a residue in L5. The mode of binding of tetracycline to the cb28 aptamer resembles its interaction with the primary binding site on the small ribosomal subunit.

Introduction

Tetracycline is an antibiotic which inhibits prokaryotic translation by interfering with binding of the aminoacyl-tRNA to the ribosomal A-site.[1,2] It is a widely used therapeutic agent of low toxicity, active against most common pathogens, which has also been used at subtherapeutic levels in animal feed to stimulate weight gain, as well as for prophylactic disease control.[3]

In spite of its prevalent usage, the mode of action of tetracycline remains poorly understood.[4] Recently, the atomic structure of the 30S ribosomal subunit, the target site of tetracycline, complexed with the antibiotic was described.[5] Two molecules of tetracycline were detected in the crystal, one site is located near the acceptor site for the aminoacylated tRNA between the head and the body of the small subunit and the second tetracycline molecule was found between three RNA domains, helices H11, H44 and H27, a region involved in the regulation of translation accuracy.[6] Besides, tetracycline is known to have several non-specific binding sites on both ribosomal subunits.

Tetracycline was also reported to inhibit the activity of ribozymes in vitro. Splicing of the *Pneumocystis carinii* group I intron is inhibited with a K_i of 27 μM.[7] For the group II αI5γ intron of the *cox*I gene from yeast mitochondria 50 μM tetracycline resulted in complete inhibition of splicing.[7] In contrast, the self-cleaving activity of the hammerhead ribozyme is only inhibited by 50% at 420 μM tetracycline.[8] A similar concentration of tetracycline is needed to interfere with the activity of the ribozyme derived from the human Hepatitis delta virus.[9] This ribozyme is however inhibited by the atypical tetracycline derivatives 4-epi-anhydrochlor-tetracycline (20 μM) and chelocardin (10 μM).[9] Recently, chelocardin was also found to inhibit the hammerhead ribozyme in a fluorescence-based high throughput screen.[10] Similarly, two types of tetracycline binding sites have been determined for the ribosome: a high affinity binding site with a K_d between 1 and 20 μM and several low affinity sites with K_ds in the high micromolar range.[11,12]

It is very well established how tetracycline interacts with the Tet-repressor protein, but how this antibiotic interacts with RNA is less well understood.[13] To address this question, we selected small tetracycline-binding RNA aptamers aiming for a low micromolar K_d similar to the affinity to the small ribosomal subunit. Several other

*Corresponding author. Tel.: +43-1-4277-54611; fax: +43-1-4277-9546; e-mail: renee@gem.univie.ac.at
†The two authors contributed equally to this work.
‡Present address: Institut für Mikrobiologie, Biochemie und Genetik der Friedrich-Alexander Universität Erlangen-Nürnberg, Germany.
§Present address: Division of Gene Expression and Regulation Wellcome Trust Biocentre, Dow Street, University of Dundee, Dundee DD1 5EH, UK.

0968-0896/01/$ - see front matter
PII: S0968-0896(01)00063-3

antibiotic-binding RNA aptamers have already been selected, like for tobramycin,[14] neomycin B,[15] lividomycin and apramycin,[16] chloramphenicol[17] and viomycin.[18] Recently, hammerhead ribozymes which are allosterically responsive to doxycycline have been selected.[19] Antibiotic binding aptamers are not only amenable to structural determination by NMR and to elucidate their mode of interaction,[20,21] but they have also been used as tools to control gene expression or to purify RNA-binding proteins.[22,23]

Results and Discussion

In vitro selection of RNAs with high affinity to tetracycline

Starting from an RNA pool with 74 nucleotides of random sequence tetracycline-binding RNAs were selected as previously described with several modifications as outlined in the methods section.[15,24] After 15 rounds of selection and amplification, the enriched pool bound with high affinity to the tetracycline affinity column and could be eluted specifically with tetracycline. Aptamers from cycles 13 and 14 were cloned and 44 individual binders were sequenced. Figure 1 shows the sequences of the selected molecules. Eight clones were unique, two clones were isolated twice, one sequence three times, one four times, two five times, one six times and one clone nine times. All clones, which were isolated more than once contained several variations, which probably occurred during the amplification steps.

One to two members from each group of clones isolated at least twice (Fig. 1) were in vitro transcribed and tested on the affinity column for binding. Of these, cb04, cb05, cb12, cb25, cb26, cb28 and ev05 were binders with an affinity in the low micromolar range.

The cb28 aptamer binds tetracycline with a K_d of approximately 1 μM (see below), which is also the affinity range in which tetracycline binds to the *E. coli* ribosome. Furthermore, the lead cleavage pattern of this RNA changed significantly upon addition of tetracycline, suggesting that cb28 changes conformation upon tetracycline binding. Therefore, we decided to characterise this aptamer in more detail.

Lead-cleavage of cb28 with increasing amounts of tetracycline

Pb^{2+} induced backbone cleavage is an excellent method for probing RNA secondary structure. In general, base

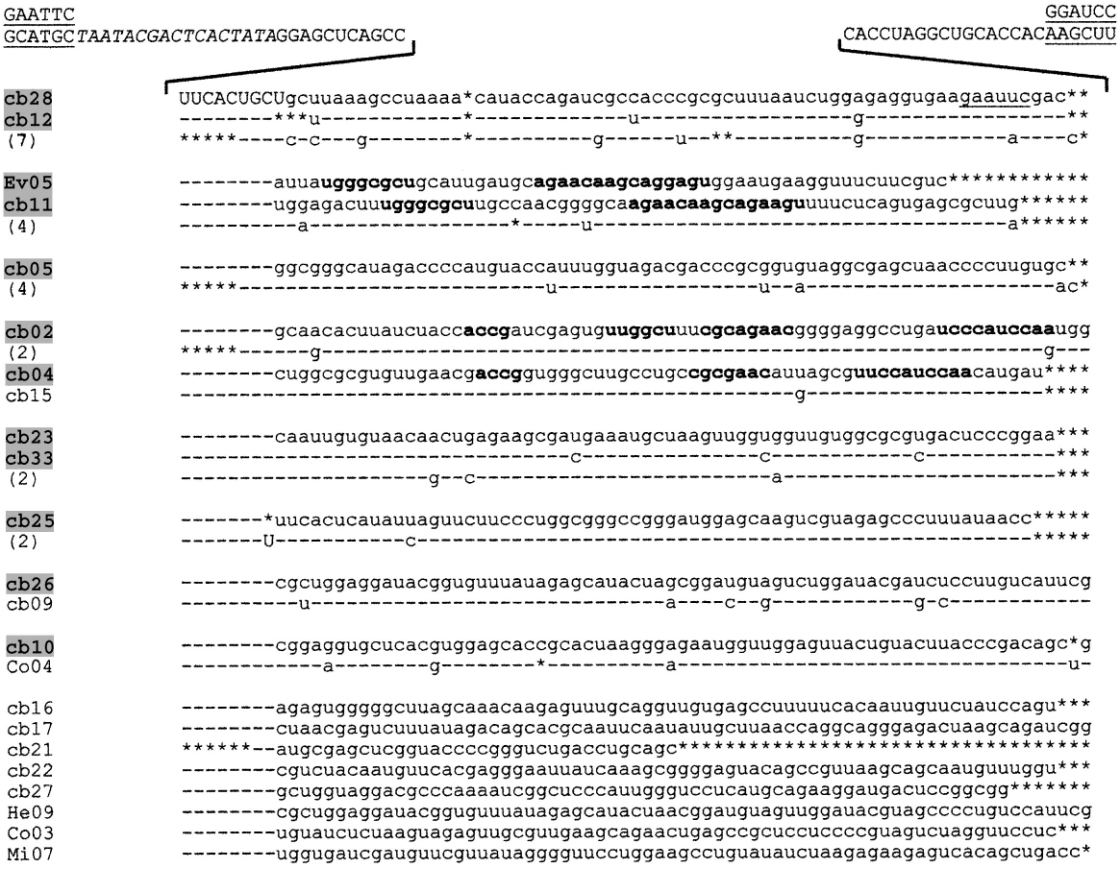

Figure 1. Nucleotide sequences of tetracycline-agarose selected clones. Fourty four individual clones from the pool after 13 and 14 rounds of selection are shown with their designations at the left-hand side. Nucleotides that are invariant in all sequenced clones are shown above the individual sequences. Lower case letters indicate the 74 nucleotides that where random at the beginning of the selection, upper case letters represent the conserved flanking nucleotides. Restriction sites are underlined, the T7 promoter is in italics. In the individual sequences, a dash represents an identical nucleotide, an asterisk a deletion. Sequence patterns in several clones are highlighted in bold face. Numbers in parentheses show how often a clone was isolated. Shaded numbers indicate that the respective clone was tested on the affinity column for tetracycline binding.

paired nucleotides are not cleaved, whereas unstructured flexible regions are cleaved.[25] Additionally, structural changes upon addition of ligands can be easily detected with this method.[26] First, the cb28 RNA was folded with the program RNA Structure 3.5 and the secondary structure was compared to the Pb^{2+} cleavage pattern.[27] Five paired regions, termed P1 to P5, three loops, L2, L4 and L5 and joining regions J1/2, J2/3, J4/3 and J3/5 are proposed. Figure 2A lanes 4 and 6 show the lead cleavage sites in the absence of tetracycline, in the absence (lane 4) and presence of 5 mM $MgCl_2$ (lane 6). The cleavage pattern coincides with the secondary structure proposed by the RNA Structure 3.5 program (Fig. 3A).

Joining region 3/5 is protected upon the addition of magnesium. Titration of tetracycline in the presence of lead results in protection of nucleotides C35 and U37 in J2/3, nucleotides A67-A69 in J3/5, and loop L5 (Figure 2A, lanes 7–18). Protection becomes clearly visible at a concentration of 1 μM tetracycline. These results suggest that stem-loop P5 with joining regions J2/3 and J3/5 change conformation upon binding of tetracycline.

Tetracycline coordinates a magnesium ion through a ketoenolate group at positions 11 and 12 (Fig. 4). This Mg^{2+} ion is involved in binding of tetracycline to its primary binding site on the 30S subunit, whereas no metal ion was found at the secondary binding site.[5] This metal ion is also essential for mediating tetracycline binding to the Tet-repressor protein.[13] In order to test whether magnesium is also essential for tetracycline binding to the cb28 aptamer, binding and cleavage experiments were done in the absence of magnesium. No binding and no changes in the lead cleavage pattern could be observed in the absence of magnesium (data not shown).

Tetracycline binding results in protections and enhancements in the binding domain during DMS modification

Dimethylsulfate modifies unpaired cytosines and adenines and is thus an excellent chemical to probe RNA secondary structure. We determined the accessibility of As and Cs to DMS in the absence and presence of increasing amounts of tetracycline. Figures 2B and 3 show the results of the chemical probing experiments.

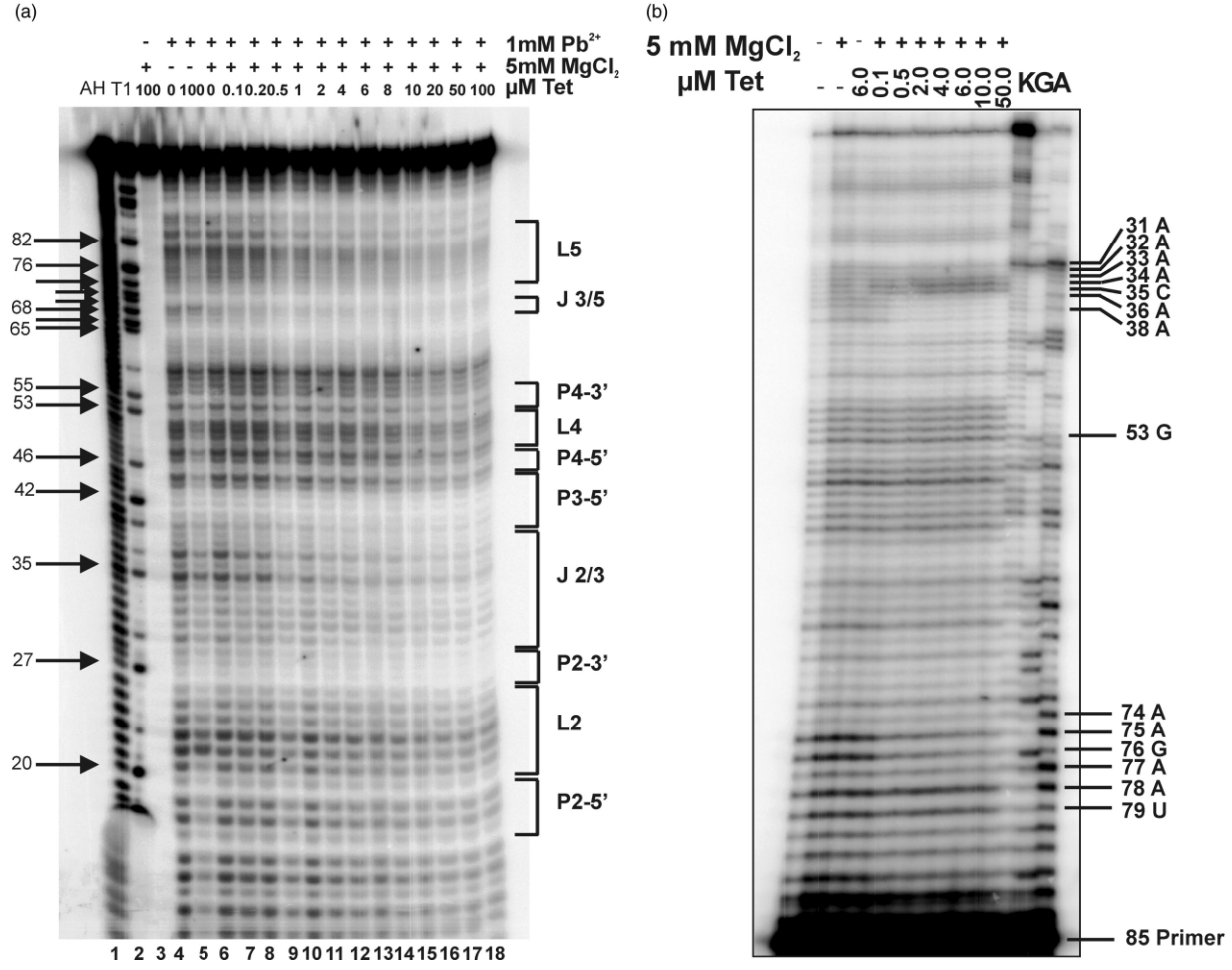

Figure 2. Structural probing of the cb28 aptamer by **A:** lead induced backbone cleavage. 5′ end labeled cb28 RNA was incubated with lead in the presence of increasing amounts of tetracycline as indicated. AH is the alkaline hydrolysis and T1 the RNase T1 digestion lane. P indicates double stranded stems, L loops and J junction regions as depicted in Fig. 3A. Numbers on the left indicated positions in the sequence of cb28. **B:** DMS modification. Reverse transcription analysis of DMS modified cb28 RNA in the presence of increasing amounts of tetracycline. K is the control without DMS treatment, G and A represent sequencing lanes. Numbers on the right indicate positions in the sequence of cb28.

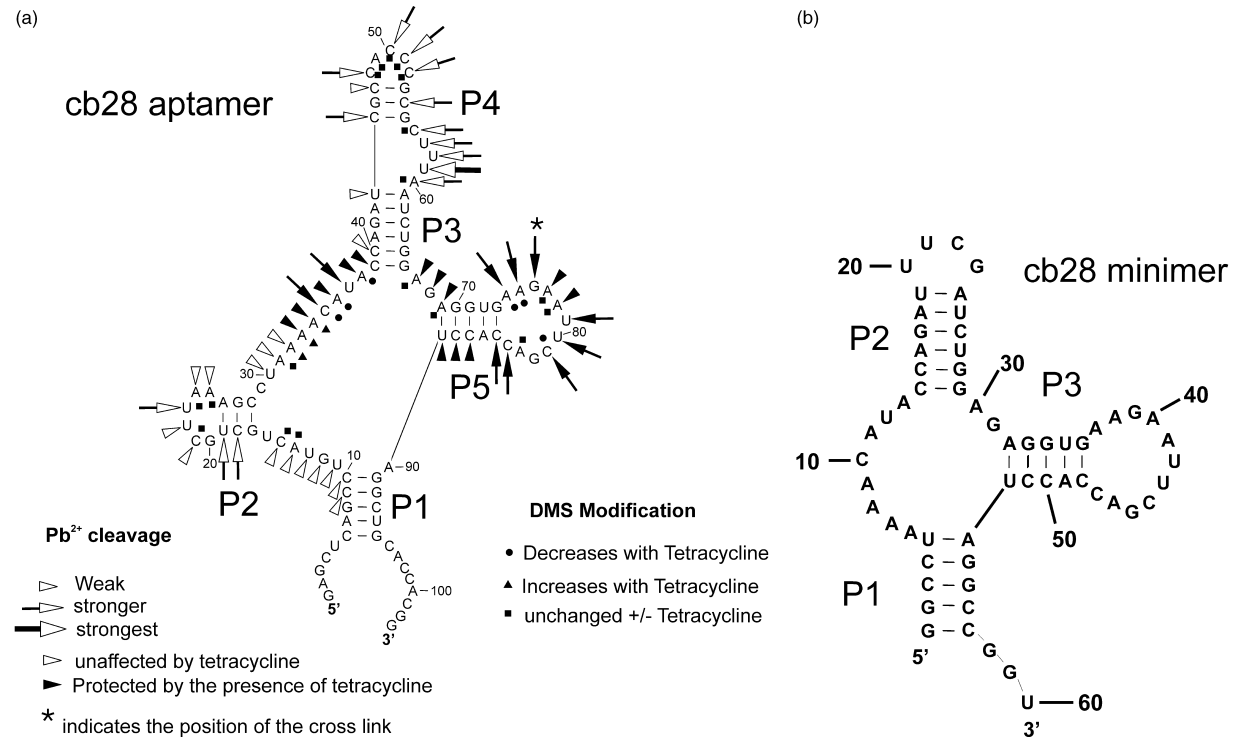

Figure 3. Secondary structural model of **A:** cb 28 aptamer and **B:** cb28 minimer. Structure probing results are indicated by arrow heads for lead cleavage, by squares, triangles and dots for DMS modification. The asterisk indicates the position of the tetracycline crosslink.

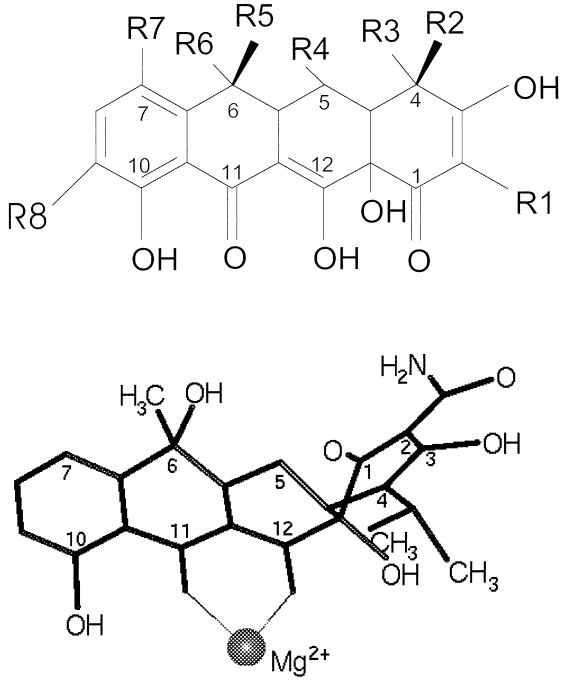

Figure 4. Structure of tetracycline. Upper drawing shows the variable groups of the tetracycline derivatives tested in this study. Lower drawing indicates the position of Mg^{2+} ion binding to the ketoenolate group 011/012 (indicated by thin lines). The coordinates were taken from the crystal structure of the TetR(D)-tetracycline complex 2TRT.[31]

Again, the DMS modification pattern in the absence of tetracycline confirmed the secondary structure proposed by the RNA structure program and the lead cleavage experiments. Upon addition of tetracycline protection of positions C35, A36 and A38 in junction 2/3 as well as of positions A74, A75 and C81 in loop 5 were detected at a concentration of 0.5 μM tetracycline. Modification at positions A33 and A34 becomes strongly enhanced and slightly enhanced at position A32 suggesting that, after tetracycline binding, these positions are more accessible to DMS modification.

Design of a cb28 minimer

Since no changes in the lead cleavage pattern were observed in regions J1/2, P2, P3, P4 and J4/3 (Fig. 2A), we assumed that these parts of the RNA were not involved in the formation of the tetracycline-binding pocket. We therefore deleted P2 and replaced P4 and J4/3 by a UUCG tetraloop to construct a small RNA of 60 nucleotides as shown in Figure 3B. The elution profiles of both minimer and full size cb28 from a tetracycline column were identical (Fig. 6A and B). Lead cleavage of the cb28 minimer confirmed the secondary structure proposed in Fig. 3B (data not shown).

Tetracycline crosslinks to G76 in loop L5

Tetracycline can be activated via a short exposure to UV light with a wavelength of 366 nm and becomes covalently attached to ribosomal proteins and rRNA or to the tetracycline repressor protein TetR(D).[28–30] In TetR(D), tetracycline is crosslinked to two peptides containing residues which are in direct contact to tetracycline as determined by X-ray structural analysis.[31] Irradiating tetracycline in the presence of the ribosome resulted in crosslinks with both the ribosomal protein S7,[28] and the 16S rRNA indicating that tetracycline

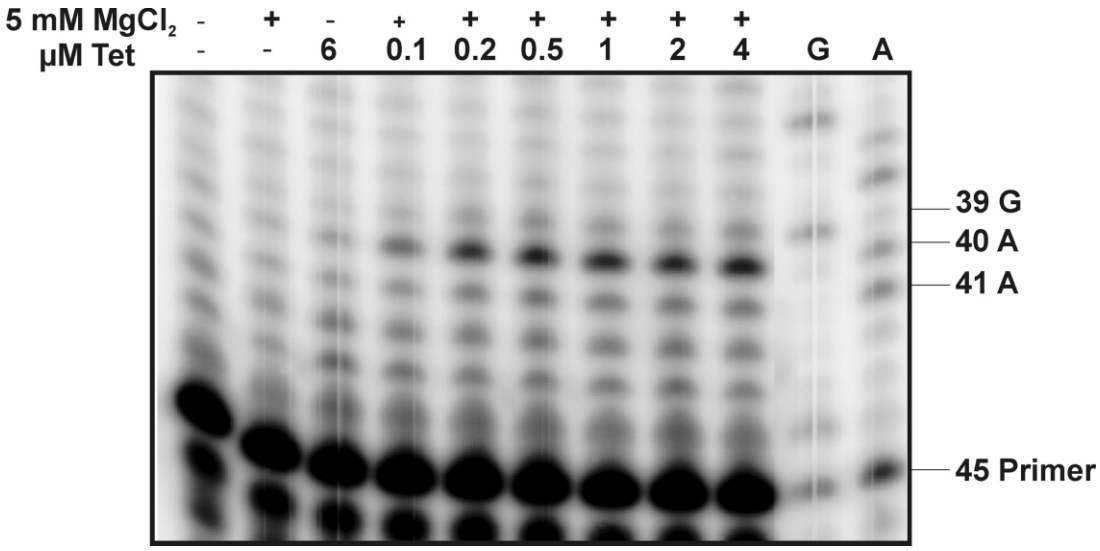

Figure 5. Photoinduced tetracycline crosslinking to the cb28 minimer. Reverse transcription of cb28 RNA, which was UV irradiated in the presence of increasing amounts of tetracycline. G and A indicate sequencing lanes.

might bind to several non-specific sites on the ribosome.[29] We used photoactivation to determine the binding site of tetracycline to the cb28 aptamer.

Cb28 aptamer and cb28 minimer RNAs were irradiated in the presence and absence of tetracycline for 15 seconds as described in the methods section. Covalent attachment of tetracycline into the RNA was monitored by primer extension, where a successful incorporation leads to a stop of the reverse transcription reaction. As shown in Figure 5, a strong tetracycline dependent crosslink appears at position G76 (corresponding to position G39 in the minimer numbering) in loop 5 already in the presence of 0.1 µM tetracycline. This crosslink is not observed under conditions where tetracycline binding does not occur (e.g., in the absence of magnesium, Fig. 5, lane 3). We therefore assume that loop 5 is part of the tetracycline binding site. Interestingly, all positions in the ribosomal RNA to which tetracycline crosslinked are guanosines.[29]

Binding of tetracycline derivatives

Based on their mode of action, tetracyclines have been divided in two different groups.[32] Class I, the typical tetracyclines, with compounds like tetracycline, 7-chlortetracycline, doxycycline and minocycline inhibit prokaryotic protein synthesis in cell-free extracts and alter the reactivity of bases in the 16S rRNA.[33] In contrast, class II tetracyclines, with compounds like anhydrotetracycline, 4-epi-anhydrochlortetracycline and chelocardin are less efficient as translational inhibitors, but promote cell lysis.[34] Structurally, tetracycline is a flat molecule with four fused rings carrying hydrophilic functional groups mainly on one side (Fig. 4).

We tested a set of compounds from both classes for binding to the cb28 aptamer. The results are shown in Figure 6 and Table 1. Class II tetracyclines bind only poorly to the cb28 aptamer and could not elute the RNA from the affinity column. Chelocardin is shown as an example in Figure 6C. From the class I tetracyclines, 4-epi-tetracycline, oxytetracycline and 7-chlortetracycline (Fig. 6D) bind very well to the cb28 aptamer, whereas the derivatives doxycycline (Fig. 6E) and minocycline (Fig. 6F) were able to elute the aptamer only poorly at concentrations of 10 to 100 µM. Doxycycline contains a hydroxyl group at position 5 and lacks the hydroxyl group at position 6 as the sole differences to tetracycline, suggesting that the hydroxyl at position 6 is an important component for binding, since the efficient elution observed with oxytetracycline demonstrates that the hydroxyl group at position 5 is not important for binding. This is in good agreement with the results of the class II tetracyclines, which all lack this hydroxyl group. The other poor-binding typical tetracycline, minocycline, lacks both hydroxyl and methyl groups at position 6, and carries an additional $-N(CH_3)_2$ group at position 7. This analysis suggests that binding of tetracyclines to cb28 is both different from its binding to the ribosome or its inhibition of ribozyme activity. Doxycycline and minocycline are efficient inhibitors of protein biosynthesis,[33] but do not bind cb28, while 4-epi-tetracycline efficiently binds the aptamer, but fails to inhibit translation. On the other hand, the more lipophilic minocycline and the atypical tetracyclines which do not bind to cb28, inhibit the HDV ribozyme strongly.[9] Clearly, further experiments are needed to elucidate the similarities as well as the differences in tetracycline interactions with the ribosome, and the ribozymes.

Tetracycline binding modes to RNA

Tetracycline is a flat poly-cyclic molecule with one aromatic ring. On one face the molecule is able to make ionic interactions while the other face allows either hydrophobic or stacking interactions (Fig. 5). Several aptamers directed against aromatic compounds have been structurally characterised. Intercalation, a relatively unspecific process, has not been observed. The association of aromatic ligands to their respective apta-

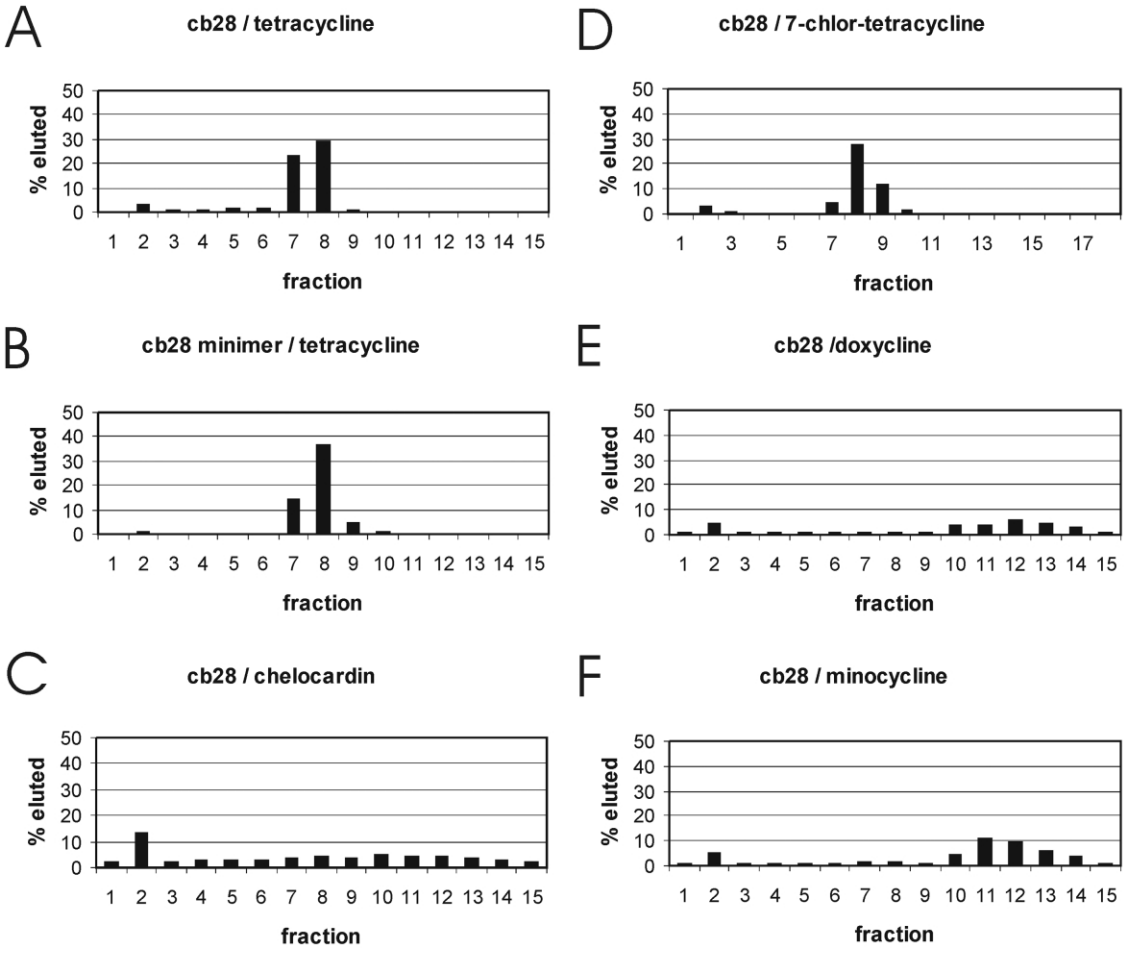

Figure 6. Tetracycline derivatives. Cb28 aptamer and minimer were tested for binding to tetracycline and its derivatives by affinity chromatography using tetracycline coupled agarose and tetracycline or derivatives for affinity elution. In fractions 7–9 elution was induced by addition of 1 μM tetracycline (derivative), in fractions 10–12 the concentration of the eluant was 10 μM and in fractions 13–15 100 μM. The RNA species and the tetracycline derivative used are indicated in the headings.

Table 1.

Derivative	Type	R1	R2	R3	R4	R5	R6	R7	R8	cb28 elution
Tetracycline (tc)	c	$CONH_2$	H	$N(CH_3)_2$	H	OH	CH_3	H	H	+ +
4-epi-tc	c		$N(CH_3)_2$	H						+ +
5-hydroxy-tc (oxy-tc)	c				OH					+ +
7-chlor-tc	c							Cl		+ +
Doxycycline	c				OH	H				+/−
Minocycline	c					H	H	$N(CH_3)_2$		+/−
Anhydrochlor-tc	a					CH_3	—	Cl		+/−
4-epi-anhydrochlor-tc	a		$N(CH_3)_2$	H		CH_3	—	Cl		+/−
β-chelocardin	a	$COCH_3$	NH_2	H		—			CH_3	+/−

Tetracycline derivatives, c: typical, a: atypical derivative

mers results in highly specific contacts mediated by a combination of stacking interactions and hydrogen-bonding.[21] Tetracycline-binding pockets composed of RNA are not simple stem-loops with widened grooves as observed for the aminoglycoside antibiotics; from the two binding sites found on the small ribosomal subunit and from the results obtained with cb28, it is evident that tetracycline binds to interhelical regions, probably via ionic interactions with the charged RNA backbone. Cb28 accommodates tetracycline in a three-way junction reminiscent of the hammerhead ribozyme structure, which is also inhibited by this antibiotic.[8] The same is true for the in vitro selected allosteric hammerhead ribozyme, where the binding domain of doxycycline consists of two stem loops, which most probably fold into an interhelical structure.[19] There are no conserved sequence homologies between the doxycycline binding motif and cb28, but the structural complexity of the motives is common to both.

From biochemical data, it was proposed that tetracycline inhibits binding of the aminoacyl-tRNA to the

A-site, probably not by competition through direct interaction with the decoding site, but more probably by allosterical weakening of the ribosomal A-site. Tetracycline also alters the UV crosslinking pattern at several positions leading to inhibitions and enhancements, suggesting that this antibiotic promotes a conformational change in the ribosomal RNA.[35] However, no significant structural changes were observed between the crystal structures of the empty ribosome and the 30S subunit complexed with tetracycline.[5] The change in the lead cleavage pattern of cb28 RNA, which is predominantly protection from cleavage after binding of tetracycline, could be interpreted as an increase in rigidity. Lead preferentially cleaves at flexible regions of the RNA and a loss in flexibility results in protection from lead cleavage. The enhancements in the DMS modification pattern, however, cannot be explained by a loss of flexibility, but only by an increased accessibility or an altered local p*K*a of the functional groups of the modified base. The effect of tetracycline binding to RNA can now be addressed by time resolved monitoring of RNA folding after tetracycline binding with spectroscopic methods.

Material and Methods

Tetracycline-sepharose coupling and affinity chromatography

Five grams of epoxy-activated sepharose 6B (Amersham Pharmacia Biotech AB) was hydrated according to the manufacturers instructions. The sepharose 6B was incubated with 2 mM tetracycline in 10 mM NaOH (pH 11.5–12), in a total volume of 50 mL at 28 °C overnight. The sepharose was then washed with alternate 60 mL volumes of 0.1 M NaOAc pH 4.0 and 0.1 M Tris–HCl pH 8.0 three times each and finally resuspended in 50 mL of 10 mM Tris–HCl pH 8.0. This can be stored at 4 °C for up to 3 weeks. 220,000 cpm of 5′ end labelled RNA and 5 μg unlabelled RNA in a total volume of 500 μl H_2O were denatured at 90 °C for 3 min and renatured at RT for 5 min. The volume was increased to 1 mL with equilibration buffer (final conditions of 10 mM Tris–HCl pH 7.6, 5 mM $MgCl_2$, 250 mM NaCl). After washing a 1 mL bed volume of Tetracycline-sepharose with 10 volumes of 1× equilibration buffer, the sample was applied and washed with 5 column volumes. The specifically bound RNA was eluted step wise with 3 column volumes each of 1, 10 and 100 μM tetracycline (or derivatives) successively. 1 mL elution volumes were collected and quantified in a scintillation counter.

In vitro selection procedure

The random sequence pool consists of a 74-nt long randomized region flanked by constant regions (total length 113 nucleotides) to allow transcription, reverse transcription, and amplification by polymerase chain reaction.[24] The selection procedure was as described previously with the following modifications.[15] The number of column washes prior to elution was increased from five column volumes (rounds 1–4), to eight (rounds 5, 6), twelve (rounds 7–11) and twenty column volumes (rounds 12–15). Selection pressure was increased from round nine by decreasing the tetracycline concentration to 10 μM in the affinity buffer. After fifteen rounds of enrichment, PCR fragments from rounds thirteen and fourteen were either cloned into pGem3Z using *Eco*RI and *Bam*HI (non cbxx-clones in Fig. 1) or into pQE30Δ*EcoBam* (constructed by Dr. Régis Stentz) using *Sph*I and *Hin*dIII as restriction enzymes (cbxx-clones in Fig. 1). Sequencing reactions were performed with the T7 sequencing kit (Pharmacia) or according to the protocol provided by Perkin–Elmer for cycle sequencing and analyzed with an ABI PRISM 310 genetic analyzer (PE Applied Biosystems).

Preparation of aptamer RNA

200 ng of template DNA was amplified in a total volume of 100 μl with 100 pmol each of forward and reverse primers (M38.27 and H20.106, respectively) with 5 U Promega *Taq* DNA polymerase. Final conditions of 0.2 mM dNTPs, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, at 95 °C 1 min, 50 °C 1 min, 72 °C 1 min, for 30 cycles with a final 72 °C incubation of 5 min. RNA transcription was carried out using one tenth of the product from a PCR reaction in a total volume of 100 μl in 5 mM NTPs, 10 mM DTT, 40 mM Tris–HCl, pH 7.5, 26 mM $MgCl_2$, 3 mM spermidine, with 200 U T7 RNA polymerase at 37 °C o/n. The RNA was purified on an 8% denaturing polyacrylamide gel (20:1) and eluted from the gel slices by shaking for 6 h at rt in 10 mM Tris–HCl pH 7.5, 0.1% SDS, 2 mM EDTA, 250 mM NaOAc. After precipitation, the RNA was resuspended in chromatography grade H_2O (Merck).

RNA dephosphorylation and 5′ end labelling

Dephosphorylation of 100 pmol of cb28 was carried out in 0.1 M Hepes–KOH pH6.7, with 10 U Calf Intestinal Phosphatase (NEB) in a total volume of 20 μl at 37 °C for 1 h. After phenol chloroform isoamylalcohol extraction and precipitation, 10 pmol of RNA were 5′ end labelled with 30 μCi (= 10 pmol) [γ-^{32}P]-ATP and T4 polynucleotide kinase (NEB, 10 U) in a total volume of 10 μl at 37 °C for 30 min in T4 polynucleotide kinase buffer (NEB), then precipitated and gel purified.

Pb^{2+} RNA cleavage

25,000 cpm 5′ end labelled RNA in the presence of 200 ng (6 pmol cb28, 10 pmol cb28-min) unlabelled RNA in a total volume of 50 μl, was incubated at rt for 15 mins in final conditions of 50 mM Tris–HCl, pH 7.5, 250 mM NaCl, 5 mM $MgCl_2$, 0.1–100 μM tetracycline, 1 mM lead(II)-acetate. After precipitation, the RNA fragments were resolved on 8% denaturing polyacrylamide gel (20:1), run at 40 W. RNA samples treated by alkaline hydrolysis and RNase T1 were run as sequence ladders.

UV crosslinking

200 ng (6 pmol cb28, 10 pmol cb28-min) of RNA per sample was prepared as for the Pb^{2+} cleavage experiments, and then irradiated for 15 s, 5 Amp (+/-75 Volts, +/- 500 Watts), on ice, using a short arc mercury lamp HBO 500 W/2 from OSRAM. Samples were irradiated in vertical tubes at a distance of ~200 mm from the lamp in the outer focal point (average luminance of 300 cd/cm^2). After precipitation, 0.3 pmol of ^{32}P 5′ end labelled primer was hybridised to 0.6 pmol of irradiated RNA in 25 mM Tris–HCl pH 8.4, 30 mM KCl in a total volume of 10 µl at 42 °C for 30 min, after an initial 2 min at 96 °C. Extension reactions were carried out in a total volume of 20 µl, in 100 mM Tris–HCl pH 8.4, 10 mM $MgCl_2$, 10 mM DTT, 250 µM dNTPs, and 0.4 U AMV reverse transcriptase (Life Sciences) at 42 °C for 45 min. Sequencing reactions contained 7 µM ddNTP additionally. Reactions were stopped by the addition of 2 volumes of stop solution (4 M NH_4OAc/20 mM EDTA). After precipitation, the RNA fragments were resolved on 8% denaturing polyacrylamide gel (20:1) run at 40 W.

DMS modification

200 ng (6 pmol cb28, 10 pmol cb28-min) of RNA per sample was prepared as for the Pb^{2+} cleavage experiments, and then incubated with 1 µl of a 1:8 fold dilution of DMS in EtOH at 37 °C for 10 min. Reactions were stopped by the addition of 1 µl of a 1:5 dilution of β-mercaptoethanol. After precipitation, 5′ end labelled primer was annealed and primer extension performed as described above.

Acknowledgements

We thank Régis Stentz for pQE30Δ*EcoBam*, Beatrix Tiebel for help with sequencing and Norbert Polacek for help with the crosslinking experiments. We thank Oliver Mayer, Christina Waldsich and Herbert Wank for comments on the manuscript. This work was funded by the European community grant FMRX-CT97-0154 to RS.

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