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6 **Interaction of the Tetracyclines with Double-Stranded RNAs of Random**
7 **Base Sequence: New Perspectives on the Target and Mechanism of**
8 **Action**

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14 **ABSTRACT**

15 The 16S rRNA binding mechanism proposed for the antibacterial action of the tetracyclines does not
16 explain their mechanism of action against non-bacterial pathogens. Also, several contradictory base
17 pairs have been proposed as their binding sites on the 16S rRNA. This study investigated the binding
18 of minocycline and doxycycline to short dsRNAs of random base sequences. These tetracyclines
19 caused a dose-dependent decrease in the fluorescence intensities of FAM-labelled dsRNA and EtBr
20 stained dsRNA, indicating that both drugs bind to dsRNA of random base sequence in a manner that
21 is competitive with the binding of ethidium bromide and other nucleic acid ligands often used as
22 stains. This effect was observable in the presence of Mg²⁺. The binding of the tetracyclines to dsRNA
23 changed features of the fluorescence emission spectra of the drugs and the circular dichroism spectra
24 of the RNA, and inhibited RNase III cleavage of the dsRNA. These results indicate that the double-
25 stranded structures of RNAs may play a more important role in their interaction with the tetracyclines
26 than the specific base pairs which had hitherto been the subject of much investigation. Given the
27 diverse functions of cellular RNAs, the binding of the tetracyclines to their double-stranded helixes
28 may alter the normal processing and functioning of the various biological processes they regulate.

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29 This could help to explain the wide range of action of the tetracyclines against various pathogens and
30 disease conditions.

31 **Keywords:** Double-stranded RNA, minocycline, doxycycline, mechanism of action, drug binding,
32 molecular interactions.

33 1. INTRODUCTION

34 The tetracyclines are a group of broad-spectrum antibiotics that are known to act by inhibiting the
35 binding of aminoacyl-tRNA to the mRNA-ribosome complex, thereby inhibiting protein synthesis ^{1,2}.
36 They are generally believed to bind to the 16S ribosomal RNA, which is composed of a 1540
37 nucleotide RNA. In addition to their use as antibacterial agents, the tetracyclines are also known to be
38 effective in the treatment of non-bacterial infections e.g. in the treatment of protozoan diseases such
39 as giardiasis ³, and viral diseases such as West Nile fever ⁴. They also possess anti-inflammatory ^{5,6},
40 anti-apoptotic ⁷ and neuroprotective properties ⁸. There is little indication of the mechanism(s) of
41 action that underlie many of the reported activities ^{5,9,10}. Because of the similarities between the
42 bacterial ribosome and mitochondrial ribosome, it was believed that the anti-protozoal activities of the
43 tetracyclines were mediated via a similar interaction with the mitochondrial ribosome of these
44 parasites ¹¹. However, the susceptibility of other protozoan species which lack mitochondria (e.g.
45 *Trichomonas vaginalis*, *Giardia lamblia*, *Entamoeba histolytica*) ^{1,3}, as well as viruses and helminths
46 raises further questions about the exact target site(s) and molecular mechanism(s) of action of the
47 tetracyclines.

48 Several studies have explored the binding of the tetracyclines to the small ribosomal subunit of
49 different bacterial species with a view to identifying the exact target site ¹². A number of binding sites
50 have been identified on the 16S rRNA through photoaffinity labelling and chemical footprinting,
51 indicating certain bases as contributing to the binding pocket ¹³⁻¹⁵. However, there have been varied
52 and sometimes conflicting reports with regards to which bases within the 16S rRNA form the core
53 target site(s) ^{14,16}. It has been reported that tetracyclines induce stabilization of various cellular
54 mRNAs in bacteria, indicating a more generalised interaction/effect on RNA ¹⁷. Interestingly, the

55 viruses against which the tetracyclines have shown some efficacy are RNA viruses (e.g. West Nile
56 fever virus ⁷, Japanese encephalitis virus ¹⁸, human immunodeficiency virus ¹⁹⁻²¹). Bearing in mind
57 that the 16S ribosomal RNAs (like most cellular RNAs) fold to form double-stranded secondary
58 structures which are potential sites for interaction with small molecules ^{22,23}; and that the flat
59 polycyclic structure and size of the tetracyclines confers potential for intercalation between base pairs
60 of nucleic acids, the binding of the tetracyclines to dsRNA is worth considering as a possible **target**
61 **site on the 16S rRNA (other than the proposed conflicting base pairs) that could allow the drugs bind**
62 **to and act against microbes that lack the 16S ribosomal RNA (such as viruses and some protozoa). If**
63 **such binding exists irrespective of the RNA base pairs (which is highly conserved in bacterial rRNA),**
64 **it could account** for the wide range of action of the tetracyclines. In this study, the interactions of
65 minocycline (C23H27N3O7) and doxycycline (C22H24N2O8) with short double stranded RNAs of
66 random base sequences were investigated, with a view to identifying the nature and essential elements
67 of their interactions.

68

69 2. MATERIALS AND METHODS

70 Nucleic acids used in this study include a small interfering RNA (siRNA) labelled with 6-
71 carboxyfluorescein (FAM-labelled dsRNA), 27 base pair RNA/DNA 1 and 2 formed from randomly
72 selected complementary base sequences (5'-cauucgcaugaugccagugguacuaac-3'), and poly I:C. FAM-
73 labelled dsRNA (siGLO® transfection indicator siRNA) was purchased from Thermo Fisher
74 Scientific (Dharmacon #D-001630-01-05). The rest were synthesized by Sigma® Aldrich. 27bp
75 RNA/DNA 1 and 2 were reconstituted to 100µM stock solution in 1x PBS, and aliquots stored at -
76 20°C. To obtain the unlabelled 27bp dsRNA and dsDNA, equal volumes of the complementary single
77 strand stock solutions were mixed, then heated to 65°C for 5mins to denature any secondary structures
78 formed during storage, and allowed to cool slowly to enhance efficient annealing. Aliquots of the
79 27bp dsRNA and dsDNA were then stored at -20°C. Radio-labelled 27bp dsRNA was obtained by
80 labelling one strand of the 27bp RNA with ³²P (to a final concentration of 100nM) using Adenosine

81 5'-triphosphate [γ -³²P] (PerkinElmer) and T4 Polynucleotide Kinase (Fermentas) according to
82 manufacturer's instructions. The radio-active strand was then washed with QIAquick nucleotide
83 removal kit and kept at 4°C. It was subsequently annealed with the complementary strand just before
84 use. Minocycline, doxycycline and magnesium sulphate were also purchased from Sigma® Aldrich.
85 Adenosine 5'-triphosphate [γ -³²P] for radioactive labelling was obtained and T4 Polynucleotide
86 Kinase. Nucleotide removal was done with QIAquick nucleotide removal kit (QIAGEN #28304). All
87 buffers and reagent solutions for experiments involving RNA were reconstituted in DEPC-treated
88 water to inactivate RNases.

89 **2.1. Gel electrophoresis binding studies**

90 The interaction of minocycline and doxycycline with dsRNA was studied by gel electrophoresis using
91 either 1% agarose gels in 1X TBE or 10% native polyacrylamide gel^{24,25}. The effect of this interaction
92 on dsRNA band intensity was initially investigated with 200nM FAM-labelled dsRNA in 1X
93 NEBuffer 2 (New England Biolabs), which contains 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂,
94 1 mM Dithiothreitol, pH 7.9 at 25°C. After Mg²⁺ was identified as the critical component of the buffer
95 for an observable effect on band intensity, subsequent experiments were performed with unlabelled
96 random 27bp dsRNA (500nM) in 0.5x PBS + 5mM MgSO₄. For these experiments, a range of
97 concentrations of minocycline or doxycycline (0-1000μM) were added to 1.5 ml microcentrifuge
98 tubes containing the dsRNA in buffer. The mixtures were incubated for 15-30 min at room
99 temperature before loading in the gel. Binding competition assays were done by adding 1X EtBr
100 (0.5μg/ml ethidium bromide, ≈1.27μM) into the sample either before or after adding
101 minocycline/doxycycline. Electrophoresis was performed at 5v/cm in 0.5X TBE (30V for 90mins
102 using Bio-Rad PowerPac and electrophoresis apparatus). For gel mobility shift assays²⁶,
103 electrophoresis was done using fine resolution gel (10% native polyacrylamide gels) that would detect
104 small differences in migration undetectable with agarose gels, and running the gel for longer (3-4hrs
105 at 150V) to ensure adequate separation of the bands. When unlabelled dsRNA were used in the
106 samples, gels were stained with EtBr (unless otherwise stated) by soaking the gels in 1x EtBr solution
107 (0.5μg/ml) for 15-20mins after electrophoresis and destained in distilled water for 10mins. Images

108 were captured using SynGene G:Box camera and GeneSnap image software. For experiments
109 involving radio-labelled dsRNA, the concentration of minocycline or doxycycline and dsRNA used
110 were proportionately reduced from the concentrations used for the FAM-labelled and EtBr-stained
111 experiments to acceptable levels for working safely with radioactivity (5nM dsRNA and 0-10 μ M
112 minocycline/doxycycline). Samples were incubated at 37°C for 30mins before loading in wells of
113 10% native acrylamide gel. Gels were dried in a gel dryer after electrophoresis and exposed in a film
114 cassette overnight. Images were taken with a Fujifilm image reader (FLA-3000 series v1.8) and
115 analysed using Multiguage v3.0 image software.

116 **2.2. Fluorescence spectroscopy**

117 The changes in the absorbance and fluorescence emission spectra of minocycline/doxycycline upon
118 binding to RNA were studied by fluorescence spectroscopy²⁷⁻²⁹. A range of concentrations of both
119 dsRNA (0-20 μ M) and minocycline/doxycycline (0-500 μ M) in Tris buffer (pH 8) + 5mM MgSO₄
120 were used in a 96 well plate. The absorbance and fluorescence emission spectra of the samples were
121 monitored using a SpectraMax M2 microplate reader operated with SOFTmax PRO data collection
122 software. The optimal excitation wavelength of minocycline/doxycycline was first determined by
123 measuring the absorbance and excitation spectra. The fluorescence emission spectra were
124 subsequently monitored with the excitation wavelength fixed at the pre-determined maximum
125 absorption wavelength.

126 **2.3. Circular dichroism**

127 The binding of the tetracyclines to dsRNA was also studied by circular dichroism as described by
128 Khan and Musarrat 2003^{28,30} using poly I:C titrated against a range of concentrations of
129 minocycline/doxycycline (0-50 μ M). The CD spectra of a fixed concentration of poly I:C (50 μ M) was
130 first obtained, as well as that of the highest concentrations of minocycline and doxycycline used. The
131 fixed concentration of poly I:C was then titrated against increasing concentrations of the drugs and the
132 CD spectra of the dsRNA upon interaction with the given concentrations of minocycline or
133 doxycycline was recorded.

134 **2.4. RNase protection assay**

135 The effect of doxycycline on RNase III cleavage and consequent degradation of dsRNA was studied
136 by using ShortCut® RNase III to digest 27bp dsRNA in the absence and presence of 1mM
137 doxycycline following the manufacturer's protocol. Samples were collected from the reaction mixture
138 at given time points. Upon sample collection, the reaction was stopped by transferring collected
139 samples to tubes containing 5X EDTA on ice before loading in 1% agarose gels. Electrophoresis was
140 carried out at 30V for 120mins, and the gels were stained with ethidium bromide (EtBr). Images were
141 taken with SynGene G:Box camera using GeneSnap image software.

142 **2.5. Data Analysis**

143 RNA bands were quantified using the image processing software, GeneTools from SynGene.
144 Statistical analysis (multiple regression) was done using IBM SPSS Statistics version 19.

145

146 *3. RESULTS*

147 **3.1. Effects of the tetracyclines on fluorescence intensity of dsRNA bands**

148 Agarose gel electrophoresis of FAM-labelled dsRNA to which a range of concentrations of
149 minocycline or doxycycline had been added showed a decrease in the fluorescence intensity of the
150 dsRNA bands with increasing amounts of minocycline or doxycycline (Figure 1A). This indicates
151 some kind of interaction between the tetracyclines and dsRNA. To investigate the nature of these
152 interactions, the experiments were repeated using the non-labelled 27bp dsRNA of random sequence,
153 and the gel was stained with EtBr after electrophoresis. Again, there was decreasing band intensity
154 with increasing concentration of tetracycline (Figure 1B). The observed decrease in band intensity of
155 dsRNA with increasing concentrations of tetracycline appears similar to the band intensity of lower
156 amounts of dsRNA in the absence of the tetracyclines (Figure 1C). This suggests that the interaction
157 of the tetracyclines with dsRNA effectively reduces the amounts of dsRNA (binding sites) available

158 for EtBr binding in the samples during staining, even though the same amount of dsRNA was used in
159 these samples.

160 **3.2. The effects of Mg²⁺ on the binding of the tetracyclines to dsRNA**

161 When the previous experiments involving FAM-labelled dsRNA were conducted with water or PBS
162 in place of NEBuffer 2, there was no observable effect of tetracycline on the band intensity. Hence,
163 the component of the buffer essential for the observed effect on band intensity was investigated.
164 When the experiments were conducted in PBS buffer in the absence or presence of increasing
165 concentrations of Mg²⁺, the gel showed decreasing dsRNA band intensity with increasing
166 concentration of minocycline or doxycycline in the samples that contain Mg²⁺. This decrease in
167 dsRNA band intensity was obscure in the absence of Mg²⁺, and was enhanced with increasing
168 concentrations of Mg²⁺ (Figure 2). Similar results were also obtained for minocycline. These results
169 indicate that Mg²⁺ enhances the interaction of the tetracyclines with dsRNA. In the samples containing
170 doxycycline, a diffuse fluorescence increasing in a dose-dependent manner was observed above the
171 dsRNA bands at higher drug concentration. This was not observed in the samples containing
172 minocycline. This diffuse fluorescence was subsequently found to be coming from free doxycycline,
173 as it was not observed when the samples were run without the antibiotics, and became more diffuse
174 and widespread in antibiotic samples run without dsRNA. This therefore suggests that the
175 tetracyclines may interact with dsRNA by binding, leaving the unbound drug molecules or molecules
176 that dissociate from dsRNA to fluoresce above the RNA bands.

177

178 **3.3. Effects of tetracycline on dsRNA migration (electrophoretic mobility shift assay)**

179 The observed decrease in dsRNA band intensity in the presence of increasing amounts of the
180 tetracyclines could result from precipitation of the RNA or differential migration of bound and
181 unbound dsRNA. This would give rise to smearing of the RNA bands instead of a single/sharp band
182 that would ordinarily be seen. To eliminate this possibility, gel mobility shift assay was done. The

183 results showed no difference in the migration level of the bands from the top of the wells, and no
184 accumulation of precipitates on the top of the wells (Figure 3A) in the samples containing both
185 minocycline and doxycycline. These results suggest that the decrease in band intensity of dsRNA
186 observed with the tetracyclines is not due to changes in the mobility of the complex formed by the
187 interaction through the gel during electrophoresis. On the other hand, doxycycline was found to
188 accumulate on the top of the wells at high drug concentrations. Again, this accumulation of drug
189 molecules on the top of the wells was not observed for minocycline. This further indicates that
190 doxycycline binds to dsRNA and migrates alongside dsRNA through the gel during electrophoresis,
191 leaving only the unbound drug molecules at higher concentrations to accumulate in the wells.

192 To further test the possibility of the tetracyclines binding to dsRNA and keep track of the RNA
193 molecules while avoiding the complications of interference from drug fluorescence, radioactively-
194 labelled RNA was used. The result showed that the primary dsRNA band intensity remained fairly
195 consistent (increased slightly with increasing drug concentrations), indicating that the dsRNA is not
196 precipitated out of the sample during electrophoresis (Figure 3B). This suggests that the earlier
197 observed decrease in band intensity of FAM-labelled and EtBr-stained dsRNA in the presence of
198 increasing concentrations of the tetracyclines is not due to loss of RNA during electrophoresis.

199 However, there was a marked decrease in the intensity of the smaller secondary and tertiary bands
200 (which are degradation fragments of the radioactively-labelled 27bp dsRNA). This suggests that the
201 binding of the tetracyclines to dsRNA may protect dsRNA from radioactivity-induced degradation.

202 The slight increase in band intensity with increasing drug concentration appears to suggest a slightly
203 higher amount of dsRNA. However, given that the same amount of dsRNA was used in all the
204 samples, the secondary and tertiary bands with higher intensity at lower drug concentrations may
205 account for this slight difference.

206

207 **3.4. Tetracycline inhibits binding of nucleic acid ligands to dsRNA**

208 If tetracycline binds to dsRNA, it would be expected to compete with the binding of other molecules
209 that are known to bind dsRNA. The effect of the interaction of the tetracyclines with dsRNA on the
210 binding of a known nucleic acid ligand (EtBr) and the relative strength/affinity of the binding of
211 tetracycline to dsRNA was investigated by adding 1X EtBr (0.5µg/ml) to the sample mixture before
212 or after the addition of tetracycline. The result showed that the presence of the tetracyclines led to a
213 dose-dependent decrease in dsRNA band intensity, irrespective of which of the two compounds was
214 first added to the sample (Figure 4A, B). These results indicate that the tetracyclines reduce the
215 binding of EtBr to dsRNA, and may be able to displace EtBr bound to dsRNA. Although these
216 experiments were done with high tetracycline-EtBr molar ratios in the samples (for safety concerns),
217 the gels were further soaked in EtBr solution to ensure maximum detection of dsRNA bands. After
218 staining with EtBr, the gels still showed a dose-dependent decrease in band intensity with tetracycline,
219 suggesting that EtBr is not able to completely displace bound tetracycline from dsRNA.

220
221 To further investigate the nature/mechanism of the binding of tetracycline to dsRNA, a 27bp dsDNA
222 with a similar base sequence to the 27bp dsRNA was used. As with the dsRNA, a reduction in band
223 intensity with increasing tetracycline concentrations was also observed. Also, other nucleic acid
224 ligands were used to stain the gel after electrophoresis in place of EtBr. Similar results to that of EtBr
225 were obtained with SYBR Green, SYBR Gold and SYTOX Green staining (Figure 4 C-F). These
226 results indicate that the tetracyclines bind to dsRNA in a way that is competitive with the binding of
227 EtBr, SYBR Green, SYBR Gold and SYTOX Green to dsRNA, and similar to the binding of these
228 ligands to DNA.

229 **3.5. Effects of the interaction with dsRNA on the fluorescence spectra of the** 230 **tetracyclines**

231 The absorbance and emission spectra of the tetracyclines were monitored with increasing
232 concentrations of dsRNA. Again, the presence of Mg²⁺ was found to be essential to generate

233 measurable changes in the fluorescence spectra of the drugs, even though it caused a massive
234 quenching of the fluorescence of both doxycycline and minocycline (>200 folds) and an increase in
235 the maximum absorption and emission wavelengths (not shown). Therefore, these experiments were
236 done in Tris buffer (pH 8), with 5mM MgSO₄. In these experimental conditions, the maximum
237 absorption wavelength of doxycycline was found to be 370-375nm, while that of minocycline was
238 380-385nm (350nm for both without Mg²⁺ in the buffer). The absorbance peak of both drugs was
239 found to increase slightly (about 8% for doxycycline and 3% for minocycline) upon the addition of
240 RNA, although a clear dose-response effect was not observed.

241 The wavelength at which the maximum fluorescence emission was observed for doxycycline and
242 minocycline was 515nm 410-415nm respectively. The emission peak of doxycycline was found to
243 decrease with increasing concentrations of dsRNA. On the other hand, the emission peak of
244 minocycline was found to increase with increasing concentrations of dsRNA (Figure 5A). These
245 changes are indicative of a binding interaction, and the extent of increase or decrease may be
246 suggestive of the binding modes (depending on the dye/base pair ratio). These results therefore
247 suggest that both minocycline and doxycycline bind to dsRNA, but may do so via slightly different
248 modes such as different degrees of intercalation and surface /groove binding.

249 **3.6. Effects of the interaction with tetracyclines on the circular dichroism (CD)** 250 **spectra of dsRNA**

251 The effect of the interaction of the tetracyclines with dsRNA on the dsRNA structure and
252 conformation was assessed by monitoring the CD spectra of a given concentration of dsRNA (poly
253 I:C) in the absence and presence of increasing concentrations of minocycline and doxycycline.
254 Double-stranded poly I:C (50μM) showed a characteristic CD spectrum with two positive bands at
255 240 and 278 nm respectively, and no negative band. The interaction of both drugs with dsRNA led to
256 an increase in the positive band wavelength (red shift). With increasing amounts of minocycline (0-
257 50μM), there was an increase in the ellipticity of both positive bands until saturation at 45-50μM
258 minocycline, with a shift in the wavelength of the bands to 245 and 295 nm respectively (Figure 5B).

259 Concomitant with these changes in the intrinsic CD of the dsRNA, two negative bands which also
260 increased in ellipticity with increasing amounts of minocycline were also observed at 265 and 340 nm
261 respectively. When titrated with doxycycline, the positive band at 240 nm was no longer observed and
262 there was a shift in the second positive band from 278 to 290 nm (Figure 5B). The ellipticity of this
263 positive band also increased with increasing amounts of doxycycline (up to a maximum at 40 μ M, then
264 decreased). However, the maximum band intensity was still lower than the intrinsic dsRNA band. In
265 addition, a negative band with similar characteristic was also observed at 265 nm. These changes in
266 the CD spectra of the dsRNA molecules upon interaction with minocycline or doxycycline are
267 suggestive of structural alterations of the dsRNA in complex with the drugs. These results therefore
268 indicate that the tetracyclines bind to dsRNA to induce structural alterations. They also further
269 indicate that there may be differences in the specific binding modes of minocycline and doxycycline
270 respectively to dsRNA.

271 **3.7. Tetracycline inhibits RNase III activity *in vitro***

272 In view of the results from the radioactively-labelled RNA (section 3.3), the possibility that the
273 binding of the tetracyclines to dsRNA could offer some form of protection against degradation and/or
274 cleavage was assessed by measuring the rate of RNase III degradation of dsRNA in the absence and
275 presence of increasing amounts of doxycycline. Degradation of the 27bp dsRNA was observed as a
276 decrease in fluorescence intensity of the band in gel electrophoresis, since it is too short to form
277 distinct bands of RNase degradation products. Whereas RNA degradation began almost immediately
278 in the sample without the drug and proceeded quite rapidly, onset of degradation was delayed in the
279 sample containing doxycycline, and eventually occurred very slowly. Within the time studied in these
280 experiments (1hr), RNase III degraded only about half of the dsRNA in the sample containing
281 doxycycline, in contrast to a near complete degradation in the sample without the drug (Figure 6).
282 Statistical analysis shows that doxycycline significantly reduced the rate of degradation of dsRNA
283 (multiple regression analysis resulted in p value of 0.001 at 0.05 significance level, $R^2= 0.640$). These
284 results indicate that doxycycline inhibits dsRNA cleavage and degradation by RNase III, suggesting
285 that doxycycline may interfere with RNase III processing/degradation of dsRNA. However, the

286 eventual and slow degradation of dsRNA in the presence of doxycycline suggests that the binding of
287 the drug to dsRNA may be reversible, and that the enzyme may eventually evade the protective effect
288 of the drug.

289 4. DISCUSSION

290 Different base pairs have been indicated by several studies as the binding site of the tetracyclines to
291 the 16S ribosomal RNA [14-18]. In view of the variations in these reports as to the exact target site(s),
292 it is possible that the specific base pairs may not necessarily be the essential targets for the binding of
293 the tetracyclines to rRNA. This study therefore investigated the binding of doxycycline and
294 minocycline to short double-stranded RNAs (seen in most cellular RNAs). The observed decrease in
295 band intensity of the FAM-labelled dsRNA following electrophoresis in the presence of increasing
296 amounts of the tetracyclines suggests an interaction with dsRNA. It is possible that the tetracyclines
297 could quench FAM fluorescence when they interact with the FAM-labelled dsRNA. However, the
298 observation of similar effect with unlabeled dsRNA stained with EtBr rules out this possibility. It is
299 also possible that the binding of the tetracyclines to dsRNA could result in precipitation of the RNA,
300 or a variable degree of mobility shift between bound and unbound dsRNA, as has been reported for
301 some dsDNA ligands^{26,31}. This would give rise to smearing of the RNA bands instead of a
302 single/sharp band that would ordinarily be seen. However, neither precipitation nor smearing of RNA
303 bands was observed in this study.

304 Mg^{2+} was suspected to be the critical component of the reaction buffer for an observable effect
305 because it has been shown to enhance the cross-linking of bases in 30S ribosomal subunit³². The role
306 of divalent metal ions in the binding of tetracyclines to DNA have been explored by Kohn³³, who
307 noted that little or no tetracycline became bound to DNA in the absence of divalent metal ions, and
308 magnesium was most effective in enhancing the binding of tetracycline to the nucleic acid. There has
309 also been speculation that the active drug species that binds to the ribosome is a magnesium-
310 tetracycline complex^{1,34}. Therefore these results concur that divalent cations like Mg^{2+} is an essential
311 factor in the interaction of the tetracyclines with dsRNA, as the observed effects were enhanced in the
312 presence of increasing concentrations of Mg^{2+} .

313 EtBr is used as a stain for nucleic acids because its fluorescence increases several-fold upon binding
314 (by intercalation) to nucleic acids, allowing detection of the nucleic acids by UV illumination. It then
315 follows that when more EtBr is bound to a nucleic acid molecule, the band intensity will increase (up
316 to a saturation point), and vice versa. Therefore, the simplest explanation for the tetracycline effect on
317 dsRNA band intensity is that the number of dsRNA binding sites available for EtBr binding (used for
318 staining) was reduced by the presence of tetracycline. In other words, the tetracyclines occupy the
319 binding sites of EtBr in dsRNA, leaving fewer binding sites available for EtBr during staining. Hence
320 less EtBr is bound to the dsRNA in the presence of the tetracyclines, which is seen as lower band
321 intensity for the same concentration of dsRNA. As EtBr is a well characterised nucleic acid ligand,
322 these observations therefore suggest that the tetracyclines also bind to dsRNA. Again, similar effects
323 were observed with SYBR Green, SYBR Gold and SYTOX Green. A decrease in fluorescence
324 intensity has been reported for the binding of SYBR green to dsDNA and total RNA at higher
325 dye/base pair ratios (dbpr) above the optimal, and this effect was found to be significantly enhanced
326 by divalent cations³¹. Since both the nucleic acid stains (dye) and dsRNA were used at the same
327 concentrations in all the samples in this experiment, the decrease in fluorescence intensity observed
328 with increasing tetracycline could therefore imply that the presence of tetracycline reduces the number
329 of base pairs available for the stains to bind, thereby effectively increasing the dye/base pair ratio. The
330 results of the binding competition assay between doxycycline/minocycline and EtBr indicate that the
331 tetracyclines not only compete with EtBr for their binding sites on dsRNA, but are able to displace
332 EtBr from its binding sites in dsRNA. These results therefore suggest that the binding of the
333 tetracyclines to dsRNA is relatively strong compared to that of EtBr, hence their ability to displace
334 EtBr from the binding sites on dsRNA and the inability of EtBr to displace the tetracyclines from
335 dsRNA. They also indicate that the tetracyclines bind to dsRNA in a way that may be similar to the
336 binding of EtBr, SYBR Green, SYBR Gold and SYTOX Green to DNA; probably by intercalation
337 and/or electrostatic surface binding, hence their ability to compete with these ligands for binding sites
338 on the nucleic acids.

339 Tetracyclines are naturally fluorescent, and binding to larger molecules such as RNA alter their
340 fluorescence properties³⁵. The fluorescence intensities of some fluorescent molecules are known to
341 either increase or decrease upon binding to nucleic acids, depending on factors such as dye/base pair
342 ratios and the presence of cations^{31,36,37}, as was also observed in this study. It has been reported that
343 the fluorescence intensity of tetracycline and doxycycline decreases upon binding to DNA, with
344 doxycycline fluorescence being less quenched than that tetracycline²⁸. In this study however, the
345 fluorescence intensity of minocycline was found to increase upon interaction with dsRNA while that
346 of doxycycline decreased. This could be due to differences in the reactive functional groups of the
347 drugs, which would invariably affect their binding characteristics. Although the fluorescence intensity
348 of minocycline has been reported to be lower than that of doxycycline³⁵, the huge gap in the
349 maximum emission wavelength of doxycycline and minocycline (515 and 415 nm respectively)
350 observed in this study may account for the nearly non-existent fluorescence of minocycline recorded
351 by Glette et al³⁵ since they only measured emission at 520 nm.

352 The observed changes in CD spectra of dsRNA upon interaction with the tetracyclines indicate
353 structural alterations due to binding of the drugs. Increase in ellipticity is a characteristic feature of the
354 elongation of the duplex as a result of intercalation of the planar molecules between the stacked base
355 pairs of nucleic acids and subsequent helix opening^{28,38}. Again, the differences in the nature and
356 magnitude of the CD spectral changes on interaction with minocycline and doxycycline respectively
357 indicate differences in their binding characteristics, probably due to differences in their reactive
358 functional groups.

359 RNase III generally degrades double stranded RNAs both *in vitro* and *in vivo*³⁹. Inhibition of this
360 process could have wide-ranging implications in living cells. In bacteria, for example, RNase III is
361 essential for the initial processing of precursor rRNA transcripts for further maturation into the
362 functional ribosomal RNA necessary for protein synthesis⁴⁰. In addition to the fact that the viruses
363 against which the tetracyclines are effective are RNA viruses (West Nile fever virus⁷, Japanese
364 encephalitis virus¹⁸, human immunodeficiency virus¹⁹⁻²¹), most viruses produce dsRNA structures

365 during replication. This could explicate the mechanism of their wide range of actions against various
366 microbes.

367 5. CONCLUSION

368 This study showed that the tetracyclines (minocycline and doxycycline) bind to short double-stranded
369 RNAs of random base sequence and inhibit their cleavage by RNase III, indicating that the binding of
370 the tetracyclines to RNAs may inhibit their processing, and consequently, function. Since the
371 functional forms of most cellular RNAs often involve secondary and tertiary structures formed by
372 folding (leading to the formation of short double helices), it is possible that the binding of the
373 tetracyclines to these double-stranded regions of various cellular RNAs may account for the wide
374 range of therapeutic effects of the drugs.

375 6. ACKNOWLEDGEMENTS

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TITLES AND LEGENDS TO FIGURES

- 473 1. **Figure 1: Effect of the tetracyclines (minocycline and doxycycline) on dsRNA band**
474 **intensity.** Graphs show decreasing dsRNA band intensity of (A) 200nM siRNA (FAM-
475 labelled dsRNA); (B) 500nM Unlabelled 27bp dsRNA (stained with EtBr after
476 electrophoresis) with increasing concentrations of minocycline or doxycycline. (C) Band
477 intensities of various concentrations (0-1000nM) of EtBr-stained 27bp dsRNA (without
478 tetracycline) compared to 500nM dsRNA in increasing concentrations of
479 minocycline/doxycycline. (D) A sample gel image (of B). Arrow indicates dsRNA bands,
480 RFU= Relative fluorescence unit.
- 481 2. **Figure 2: Effect of Mg²⁺ on the interaction of the tetracyclines with dsRNA.**
482 Gel image and graphs show decreasing dsRNA band intensity of 200µM FAM-labelled
483 dsRNA with increasing concentrations of doxycycline, which is enhanced in the presence
484 of increasing concentrations of Mg²⁺. Note that the diffuse fluorescence increasing from
485 left to right above the dsRNA bands in the gel image is from free doxycycline.
- 486 3. **Figure 3: Effect of the tetracyclines on dsRNA migration through a high resolution gel**
487 **(10% native polyacrylamide gel).** (A) EtBr-stained gel image shows no difference in gel
488 shift/mobility of the dsRNA bands in the presence of increasing doxycycline
489 concentrations, no smearing of the bands and no accumulation of dsRNA precipitates in
490 the wells. Note the accumulation of free doxycycline may on the top of the last two
491 wells with high drug concentration. Arrow indicates lane of sample containing the
492 highest concentration of doxycycline used without dsRNA. (B) Gel and graph of relative
493 absorbance of radioactively labelled dsRNA in the presence of increasing concentrations
494 of doxycycline or minocycline. Note that the secondary and tertiary bands are
495 degradation products of the dsRNA in the primary band.
- 496 4. **Figure 4: Effect of the tetracyclines on the binding of nucleic acid ligands to dsRNA and**
497 **dsDNA:** (A,B) Competition assay graphs show decreasing band intensities of 500µM
498 27bp dsRNA with increasing minocycline/doxycycline concentration in the presence of
499 1X EtBr, irrespective of which compound was added first to the dsRNA (the tetracyclines
500 or EtBr); Decreasing band intensities of dsRNA and dsDNA with increasing
501 concentrations of doxycycline in gels stained with EtBr (C), SYBR green (D), SYBR gold (E)
502 and SYTOX green (F).

503 5. **Figure 5: Effects of the interaction of the tetracyclines with dsRNA on the biophysical**
504 **properties of the molecules.** The graphs show the fluorescence emission spectra (A) of
505 minocycline and doxycycline in the presence of increasing concentrations of 27bp dsRNA
506 (excitation wavelength was set at 375nm), and circular dichroism spectra of the dsRNA
507 (poly I:C) in the presence of increasing concentrations of the drugs (B). For minocycline:
508 25, 30, 35, 40, 45, 50 μ M (curves 1-6); for doxycycline: 10, 20, 40, 50 μ M curves 1-4). M
509 and D represent the curves for minocycline and doxycycline respectively without any
510 RNA, R represent CD spectra of 50 μ Mpoly I:C without any drug.

511 6. **Figure 6: Effect of doxycycline on RNase III degradation of dsRNA.** Gel image shows (A)
512 normal degradation of 27bp dsRNA by RNase III (in the absence of tetracycline), (B)
513 inhibition of degradation in the presence of 1mM doxycycline. Samples were taken from
514 the reaction mixture at the times (minutes) indicated above each well. The graphs show
515 the rate of RNase III degradation of dsRNA in the absence (C) and presence (D) of
516 doxycycline, with a significant difference between the two ($p=0.001$ at 0.05 significance
517 level, $R^2= 0.640$). RFU=relative fluorescence unit of dsRNA bands.

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