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

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ABSTRACT

The 16S rRNA binding mechanism proposed for the antibacterial action of the tetracyclines does not explain their mechanism of action against non-bacterial pathogens. Also, several contradictory base pairs have been proposed as their binding sites on the 16S rRNA. This study investigated the binding of minocycline and doxycycline to short dsRNAs of random base sequences. These tetracyclines caused a dose-dependent decrease in the fluorescence intensities of FAM-labelled dsRNA and EtBr stained dsRNA, indicating that both drugs bind to dsRNA of random base sequence in a manner that is competitive with the binding of ethidium bromide and other nucleic acid ligands often used as stains. This effect was observable in the presence of Mg^{2+}. The binding of the tetracyclines to dsRNA changed features of the fluorescence emission spectra of the drugs and the circular dichroism spectra of the RNA, and inhibited RNase III cleavage of the dsRNA. These results indicate that the double-stranded structures of RNAs may play a more important role in their interaction with the tetracyclines than the specific base pairs which had hitherto been the subject of much investigation. Given the diverse functions of cellular RNAs, the binding of the tetracyclines to their double-stranded helixes may alter the normal processing and functioning of the various biological processes they regulate.

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

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

1. INTRODUCTION

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

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

viruses against which the tetracyclines have shown some efficacy are RNA viruses (e.g. West Nile fever virus [7], Japanese encephalitis virus [18],human immunodeficiency virus [19-21]). Bearing in mind that the 16S ribosomal RNAs (like most cellular RNAs) fold to form double-stranded secondary structures which are potential sites for interaction with small molecules [22,23]; and that the flat polycyclic structure and size of the tetracyclines confers potential for intercalation between base pairs of nucleic acids, the binding of the tetracyclines to dsRNA is worth considering as a possible target site on the 16S rRNA (other than the proposed conflicting base pairs) that could allow the drugs bind to and act against microbes that lack the 16S ribosomal RNA (such as viruses and some protozoa). If such binding exists irrespective of the RNA base pairs (which is highly conserved in bacterial rRNA), it could account for the wide range of action of the tetracyclines. In this study, the interactions of minocycline ($C_{23}H_{27}N_3O_7$) and doxycycline ($C_{22}H_{24}N_2O_8$) with short double stranded RNAs of random base sequences were investigated, with a view to identifying the nature and essential elements of their interactions.

2. *MATERIALS AND METHODS*

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

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

2.1. Gel electrophoresis binding studies

The interaction of minocycline and doxycycline with dsRNA was studied by gel electrophoresis using either 1% agarose gels in 1X TBE or 10% native polyacrylamide gel [24,25].The effect of this interaction on dsRNA band intensity was initially investigated with 200nM FAM-labelled dsRNA in 1X NEBuffer 2 (New England Biolabs), which contains 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.9 at 25°C. After Mg^{2+} was identified as the critical component of the buffer for an observable effect on band intensity, subsequent experiments were performed with unlabelled random 27bp dsRNA (500nM) in 0.5x PBS + 5mM $MgSO_4$. For these experiments, a range of concentrations of minocycline or doxycycline (0-1000µM) were added to 1.5 ml microcentrifuge tubes containing the dsRNA in buffer. The mixtures were incubated for 15-30 min at room temperature before loading in the gel. Binding competition assays were done by adding 1X EtBr (0.5µg/ml ethidium bromide, ≈1.27µM) into the sample either before or after adding minocycline/doxycycline. Electrophoresis was performed at 5v/cm in 0.5X TBE (30V for 90mins using Bio-Rad PowerPac and electrophoresis apparatus). For gel mobility shift assays [26], electrophoresis was done using fine resolution gel (10% native polyacrylamide gels) that would detect small differences in migration undetectable with agarose gels, and running the gel for longer (3-4hrs at 150V) to ensure adequate separation of the bands. When unlabelled dsRNA were used in the samples, gels were stained with EtBr (unless otherwise stated) by soaking the gels in 1x EtBr solution (0.5µg/ml) for 15-20mins after electrophoresis and destained in distilled water for 10mins. Images

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

2.2. Fluorescence spectroscopy

The changes in the absorbance and fluorescence emission spectra of minocycline/doxycycline upon binding to RNA were studied by fluorescence spectroscopy [27-29]. A range of concentrations of both dsRNA (0-20μM) and minocycline/doxycycline (0-500μM) in Tris buffer (pH 8) + 5mM $MgSO_4$ were used in a 96 well plate. The absorbance and fluorescence emission spectra of the samples were monitored using a SpectraMax M2 microplate reader operated with SOFTmax PRO data collection software. The optimal excitation wavelength of minocycline/doxycycline was first determined by measuring the absorbance and excitation spectra. The fluorescence emission spectra were subsequently monitored with the excitation wavelength fixed at the pre-determined maximum absorption wavelength.

2.3. Circular dichroism

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

2.4. RNase protection assay

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

2.5. Data Analysis

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

3. RESULTS

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

Agarose gel electrophoresis of FAM-labelled dsRNA to which a range of concentrations of minocycline or doxycycline had been added showed a decrease in the fluorescence intensity of the dsRNA bands with increasing amounts of minocycline or doxycycline (Figure 1A). This indicates some kind of interaction between the tetracyclines and dsRNA. To investigate the nature of these interactions, the experiments were repeated using the non-labelled 27bp dsRNA of random sequence, and the gel was stained with EtBr after electrophoresis. Again, there was decreasing band intensity with increasing concentration of tetracycline (Figure 1B). The observed decrease in band intensity of dsRNA with increasing concentrations of tetracycline appears similar to the band intensity of lower amounts of dsRNA in the absence of the tetracyclines (Figure 1C). This suggests that the interaction 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 Mg^{2+} 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 Mg^{2+}, the gel showed decreasing dsRNA band intensity with increasing
166 concentration of minocycline or doxycycline in the samples that contain Mg^{2+}. This decrease in
167 dsRNA band intensity was obscure in the absence of Mg^{2+}, and was enhanced with increasing
168 concentrations of Mg^{2+} (Figure 2). Similar results were also obtained for minocycline. These results
169 indicate that Mg^{2+} 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

3.4. Tetracycline inhibits binding of nucleic acid ligands to dsRNA

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

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

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

The absorbance and emission spectra of the tetracyclines were monitored with increasing concentrations of dsRNA. Again, the presence of Mg^{2+} was found to be essential to generate

measurable changes in the fluorescence spectra of the drugs, even though it caused a massive quenching of the fluorescence of both doxycycline and minocycline (>200 folds) and an increase in the maximum absorption and emission wavelengths (not shown). Therefore, these experiments were done in Tris buffer (pH 8), with 5mM $MgSO_4$. In these experimental conditions, the maximum absorption wavelength of doxycycline was found to be 370-375nm, while that of minocycline was 380-385nm (350nm for both without Mg^{2+} in the buffer). The absorbance peak of both drugs was found to increase slightly (about 8% for doxycycline and 3% for minocycline) upon the addition of RNA, although a clear dose-response effect was not observed.

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

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

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

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

3.7. Tetracycline inhibits RNase III activity *in vitro*

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

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

4. DISCUSSION

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

Mg^{2+} was suspected to be the critical component of the reaction buffer for an observable effect because it has been shown to enhance the cross-linking of bases in 30S ribosomal subunit [32]. The role of divalent metal ions in the binding of tetracyclines to DNA have been explored by Kohn [33], who noted that little or no tetracycline became bound to DNA in the absence of divalent metal ions, and magnesium was most effective in enhancing the binding of tetracycline to the nucleic acid. There has also been speculation that the active drug species that binds to the ribosome is a magnesium-tetracycline complex [1,34]. Therefore these results concur that divalent cations like Mg^{2+} is an essential factor in the interaction of the tetracyclines with dsRNA, as the observed effects were enhanced in the presence of increasing concentrations of Mg^{2+}.

EtBr is used as a stain for nucleic acids because its fluorescence increases several-fold upon binding (by intercalation) to nucleic acids, allowing detection of the nucleic acids by UV illumination. It then follows that when more EtBr is bound to a nucleic acid molecule, the band intensity will increase (up to a saturation point), and vice versa. Therefore, the simplest explanation for the tetracycline effect on dsRNA band intensity is that the number of dsRNA binding sites available for EtBr binding (used for staining) was reduced by the presence of tetracycline. In other words, the tetracyclines occupy the binding sites of EtBr in dsRNA, leaving fewer binding sites available for EtBr during staining. Hence less EtBr is bound to the dsRNA in the presence of the tetracyclines, which is seen as lower band intensity for the same concentration of dsRNA. As EtBr is a well characterised nucleic acid ligand, these observations therefore suggest that the tetracyclines also bind to dsRNA. Again, similar effects were observed with SYBR Green, SYBR Gold and SYTOX Green. A decrease in fluorescence intensity has been reported for the binding of SYBR green to dsDNA and total RNA at higher dye/base pair ratios (dbpr) above the optimal, and this effect was found to be significantly enhanced by divalent cations [31]. Since both the nucleic acid stains (dye) and dsRNA were used at the same concentrations in all the samples in this experiment, the decrease in fluorescence intensity observed with increasing tetracycline could therefore imply that the presence of tetracycline reduces the number of base pairs available for the stains to bind, thereby effectively increasing the dye/base pair ratio. The results of the binding competition assay between doxycycline/minocycline and EtBr indicate that the tetracyclines not only compete with EtBr for their binding sites on dsRNA, but are able to displace EtBr from its binding sites in dsRNA. These results therefore suggest that the binding of the tetracyclines to dsRNA is relatively strong compared to that of EtBr, hence their ability to displace EtBr from the binding sites on dsRNA and the inability of EtBr to displace the tetracyclines from dsRNA. They also indicate that the tetracyclines bind to dsRNA in a way that may be similar to the binding of EtBr, SYBR Green, SYBR Gold and SYTOX Green to DNA; probably by intercalation and/or electrostatic surface binding, hence their ability to compete with these ligands for binding sites on the nucleic acids.

Tetracyclines are naturally fluorescent, and binding to larger molecules such as RNA alter their fluorescence properties [35]. The fluorescence intensities of some fluorescent molecules are known to either increase or decrease upon binding to nucleic acids, depending on factors such as dye/base pair ratios and the presence of cations [31,36,37], as was also observed in this study**.** It has been reported that the fluorescence intensity of tetracycline and doxycycline decreases upon binding to DNA, with doxycycline fluorescence being less quenched than that tetracycline [28]. In this study however, the fluorescence intensity of minocycline was found to increase upon interaction with dsRNA while that of doxycycline decreased. This could be due to differences in the reactive functional groups of the drugs, which would invariably affect their binding characteristics. Although the fluorescence intensity of minocycline has been reported to be lower than that of doxycycline [35], the huge gap in the maximum emission wavelength of doxycycline and minocycline (515 and 415 nm respectively) observed in this study may account for the nearly non-existent fluorescence of minocycline recorded by Glette et al [35] since they only measured emission at 520 nm.

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

RNase III generally degrades double stranded RNAs both *in vitro* and *in vivo* [39]. Inhibition of this process could have wide-ranging implications in living cells. In bacteria, for example, RNase III is essential for the initial processing of precursor rRNA transcripts for further maturation into the functional ribosomal RNA necessary for protein synthesis [40]. In addition to the fact that the viruses against which the tetracyclines are effective are RNA viruses (West Nile fever virus [7], Japanese encephalitis virus [18], human immunodeficiency virus [19-21]), most viruses produce dsRNA structures

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

5. CONCLUSION

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

6. ACKNOWLEDGEMENTS

This work was funded by the Commonwealth Scholarship Commission in the UK.

7. REFERENCES

1. I. Chopra, M. Roberts, Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews* **65**, 232-260 (2001).
2. D. Schnappinger, W. Hillen, Tetracyclines: Antibiotic action, uptake, and resistance mechanisms. *Archives of Microbiology* **165**, 359-369 (1996).
3. S. K. Katiyar, T. D. Edlind, Enhanced Antiparasitic Activity of Lipophilic Tetracyclines - Role of Uptake. *Antimicrobial Agents and Chemotherapy* **35**, 2198-2202 (1991).
4. K. Dutta, A. Basu, Use of minocycline in viral infections. *Indian J Med Res* **133**, 467-470 (2011).
5. A. Y. Debrah *et al.*, Doxycycline Reduces Plasma VEGF-C/sVEGFR-3 and Improves Pathology in Lymphatic Filariasis. *PLoS Pathog* **2**, e92 (2006).
6. N. Scheinfeld, T. Berk, A review of the diagnosis and treatment of rosacea. *Postgrad Med* **122**, 139-143 (2010).
7. M. Michaelis, M. C. Kleinschmidt, H. W. Doerr, J. Cinatl, Minocycline inhibits West Nile virus replication and apoptosis in human neuronal cells. *Journal of Antimicrobial Chemotherapy* **60**, 981-986 (2007).
8. H. F. Elewa, H. Hilali, D. C. Hess, L. S. Machado, S. C. Fagan, Minocycline for short-term neuroprotection. *Pharmacotherapy* **26**, 515-521 (2006).
9. S. A. Ralph, M. C. D'Ombrain, G. I. McFadden, The apicoplast as an antimalarial drug target. *Drug Resistance Updates* **4**, 145-151 (2001).
10. E. L. Dahl *et al.*, Tetracyclines specifically target the apicoplast of the malaria parasite Plasmodium falciparum. *Antimicrobial Agents and Chemotherapy* **50**, 3124-3131 (2006).
11. T. D. Edlind. in *Biochemical protozoology* (eds Coombs, G. H.;North, M. J.) 569-586 (Taylor & Francis Ltd, London, 1991).

12. L. Nonaka, S. R. Connell, D. E. Taylor, 16S rRNA mutations that confer tetracycline resistance in Helicobacter pylori decrease drug binding in Escherichia coli ribosomes. *J Bacteriol* **187**, 3708-3712 (2005).
13. R. Oehler, N. Polacek, G. Steiner, A. Barta, Interaction of tetracycline with RNA: photoincorporation into ribosomal RNA of Escherichia coli. *Nucleic Acids Res* **25**, 1219-1224 (1997).
14. M. Pioletti *et al.*, Crystal structures of complexes of the small ribosomal subunit with tetracycline, edeine and IF3. *EMBO J* **20**, 1829-1839 (2001).
15. D. E. Brodersen *et al.*, The Structural Basis for the Action of the Antibiotics Tetracycline, Pactamycin, and Hygromycin B on the 30S Ribosomal Subunit. *Cell* **103**, 1143-1154 (2000).
16. M. M. Anokhina, A. Barta, K. H. Nierhaus, V. A. Spiridonova, A. M. Kopylov, Mapping of the second tetracycline binding site on the ribosomal small subunit of E.coli. *Nucleic Acids Research* **32**, 2594-2597 (2004).
17. Y. Wei, D. H. Bechhofer, Tetracycline induces stabilization of mRNA in Bacillus subtilis. *Journal of Bacteriology* **184**, 889-894 (2002).
18. M. K. Mishra, A. Basu, Minocycline neuroprotects, reduces microglial activation, inhibits caspase 3 induction, and viral replication following Japanese encephalitis. *J Neurochem* **105**, 1582-1595 (2008).
19. G. L. Szeto *et al.*, Minocycline Attenuates HIV Infection and Reactivation by Suppressing Cellular Activation in Human CD4+ T Cells. *Journal of Infectious Diseases* **201**, 1132-1140 (2010).
20. M. C. Zink *et al.*, Neuroprotective and anti-human immunodeficiency virus activity of minocycline. *JAMA* **293**, 2003-2011 (2005).
21. K. F. T. Copeland, J. I. Brooks, A Novel Use for an Old Drug: The Potential for Minocycline as Anti-HIV Adjuvant Therapy. *Journal of Infectious Diseases* **201**, 1115-1117 (2010).
22. T. Hermann, Y. Tor, RNA as a target for small-molecule therapeutics. *Expert Opinion on Therapeutic Patents* **15**, 49-62 (2005).
23. S. J. Sucheck, C.-H. Wong, RNA as a target for small molecules. *Current Opinion in Chemical Biology* **4**, 678-686 (2000).
24. K. A. Xavier, P. S. Eder, T. Giordano, RNA as a drug target: methods for biophysical characterization and screening. *Trends in Biotechnology* **18**, 349-356 (2000).
25. D. M. J. Lilley, in *Methods in Enzymology*. (Academic Press, 2000), vol. Volume 317, pp. 368-393.
26. R. L. A. Furlan *et al.*, A rapid and sensitive method for the screening of DNA intercalating antibiotics. *Biotechnology Letters* **24**, 1807-1813 (2002).
27. N. G. Walter, J. M. Burke, in *Methods in Enzymology*. (Academic Press, 2000), vol. Volume 317, pp. 409-440.
28. M. A. Khan, J. Musarrat, Interactions of tetracycline and its derivatives with DNA in vitro in presence of metal ions. *International Journal of Biological Macromolecules* **33**, 49-56 (2003).
29. J. C. Canzoneri, A. K. Oyelere, Interaction of anthracyclines with iron responsive element mRNAs. *Nucleic Acids Research* **36**, 6825-6834 (2008).
30. T. R. Sosnick, X. Fang, V. M. Shelton, in *Methods in Enzymology*. (Academic Press, 2000), vol. Volume 317, pp. 393-409.
31. H. Zipper, H. Brunner, J. Bernhagen, F. Vitzthum, Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nucleic Acids Res* **32**, e103 (2004).
32. J. W. Noah, M. A. Dolan, P. Babin, P. Wollenzien, Effects of Tetracycline and Spectinomycin on the Tertiary Structure of Ribosomal RNA in the Escherichia coli 30 S Ribosomal Subunit. *J. Biol. Chem.* **274**, 16576-16581 (1999).
33. K. W. Kohn, Mediation of divalent metal ions in the binding of tetracycline to macromolecules. *Nature* **191**, 1156-1158 (1961).

34. I. Chopra, P. M. Hawkey, M. Hinton, Tetracyclines, Molecular and Clinical Aspects. *Journal of Antimicrobial Chemotherapy* **29**, 245-277 (1992).
35. J. Glette, S. Sandberg, G. Hopen, C. O. Solberg, Influence of tetracyclines on human polymorphonuclear leukocyte function. *Antimicrob Agents Chemother* **25**, 354-357 (1984).
36. K. Bhadra, M. Maiti, G. S. Kumar, Interaction of isoquinoline alkaloids with polymorphic DNA structures. *Chem Biodivers* **6**, 1323-1342 (2009).
37. R. C. Yadav *et al.*, Berberine, a strong polyriboadenylic acid binding plant alkaloid: spectroscopic, viscometric, and thermodynamic study. *Bioorganic & Medicinal Chemistry* **13**, 165-174 (2005).
38. G. S. Kumar, S. Das, K. Bhadra, M. Maiti, Protonated forms of poly[d(G-C)] and poly(dG).poly(dC) and their interaction with berberine. *Bioorg Med Chem* **11**, 4861-4870 (2003).
39. A. K. Srivastava, D. Schlessinger, Mechanism and regulation of bacterial ribosomal RNA processing. *Annu Rev Microbiol* **44**, 105-129 (1990).
40. N. Nikolaev, D. Schlessinger, P. K. Wellauer, 30 S pre-ribosomal RNA of Escherichia coli and products of cleavage by ribonuclease III: length and molecular weight. *J Mol Biol* **86**, 741-747 (1974).

TITLES AND LEGENDS TO FIGURES

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

5. **Figure 5**: **Effects of the interaction of the tetracyclines with dsRNA on the biophysical properties of the molecules.** The graphs show the fluorescence emission spectra (A) of minocycline and doxycycline in the presence of increasing concentrations of 27bp dsRNA (excitation wavelength was set at 375nM), and circular dichroism spectra of the dsRNA (poly I:C) in the presence of increasing concentrations of the drugs (B). For minocycline: 25, 30, 35, 40, 45, 50µM (curves 1-6); for doxycycline: 10, 20, 40, 50µM curves 1-4). M and D represent the curves for minocycline and doxycycline respectively without any RNA, R represent CD spectra of 50µMpoly I:C without any drug.
6. **Figure 6**: **Effect of doxycycline on RNase III degradation of dsRNA.** Gel image shows (A) normal degradation of 27bp dsRNA by RNase III (in the absence of tetracycline), (B) inhibition of degradation in the presence of 1mM doxycycline. Samples were taken from the reaction mixture at the times (minutes) indicated above each well. The graphs show the rate of RNase III degradation of dsRNA in the absence (C) and presence (D) of doxycycline, with a significant difference between the two (p=0.001 at 0.05 significance level, R^2= 0.640). RFU=relative fluorescence unit of dsRNA bands.