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Interaction of the antibiotics clindamycin and lincomycin with *Escherichia coli* 23S ribosomal RNA

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ABSTRACT

Interaction of the antibiotics clindamycin and lincomycin with *Escherichia coli* ribosomes has been compared by chemical footprinting. The protection afforded by both drugs is limited to the peptidyl transferase loop of 23S rRNA. Under conditions of stoichiometric binding at 1 mM drug concentration *in vitro*, both drugs strongly protect 23S rRNA bases A2058 and A2451 from dimethyl sulphate and G2505 from kethoxal modification; G2061 is also weakly protected from kethoxal. The modification patterns differ in that A2059 is additionally protected by clindamycin but not by lincomycin. The affinity of the two drugs for the ribosome, estimated by footprinting, is approximately the same, giving K_{diss} values of 5 μM for lincomycin and 8 μM for clindamycin. The results show that *in vitro* the drugs are equally potent in blocking their ribosomal target site. Their inhibitory effects on peptide bond formation could, however, be subtly different.

INTRODUCTION

The majority of antibiotics inhibit cell growth by binding directly to functionally important sites on ribosomes (1, 2). These drugs interact with essential rRNA structures. The inhibitory characteristics of an antibiotic are determined by its ability to gain access to its target site on the ribosome, its binding affinity at that site, and the manner in which it perturbs the rRNA structure. The major antibiotic target within the 50S ribosomal subunit is the site of peptide bond formation, termed the peptidyl transferase loop, in domain V of 23S rRNA.

Lincosamide antibiotics, such as lincomycin and its modified derivative clindamycin (7-chloro-7-deoxylincomycin), block peptide bond formation (1, 2). Of the two antibiotics, clinda mycin is the more potent inhibitor of Gram negative bacteria. This is at least in part due to the higher lipid solubility of clindamycin that enables it to permeate the outer membrane of these bacteria. The two antibiotics could possibly bind at the same ribosomal target site in a similar or identical manner if access were unimpeded. Here, an *in vitro* chemical footprinting approach was employed to determine how these two drugs interact with 23S rRNA in *E.coli* ribosomes. Both drugs gave clear footprinting patterns within a limited region of 23S rRNA under the physiological conditions employed here. Dissociation constants for both drug-ribosome interactions were calculated from the nucleotide protection data measured over a range of drug concentrations.

MATERIALS AND METHODS

Growth of cells in antibiotics

The minimal inhibitory concentrations of clindamycin (Upjohn) and lincomycin (Sigma) were determined for *Escherichia coli* strain DH1 (3), that has an intact, wild-type outer membrane, and for *E.coli* strain AS19, that has a more permeable outer membrane (4). The strains were grown at 37°C in liquid LB medium (3) containing clindamycin at 0 to 200 mg/l, or lincomycin at 0 to 5 g/l.

Isolation of ribosomes

DH1 cells were grown at 37°C in LB medium in the absence of antibiotics, and harvested in early log phase. Cell walls were broken by sonication, and tight-couple ribosomes were isolated by centrifugation (5).

Binding of antibiotics and rRNA footprinting

The antibiotics clindamycin or lincomycin were incubated at concentrations of 0.1 to 1000 μM with 6 pmol ribosomes in 100μl of 80 mM-potassium cacodylate (pH 7.2), 20 mM-$MgCl_2$, 100 mM-NH_4Cl, 1 mM-dithiothreitol, 0.5 mM-EDTA for 30 min at 37°C. Ribosomes were probed with dimethyl sulphate (DMS) (2μl of a 1:6 dilution in 96% (v/v) ethanol) for 5 to 15 min at 37°C, or with 5 μl kethoxal (37 mg/ml 20% (v/v) ethanol) for 10 min at 37°C. The reactions were stopped and RNA was extracted with phenol/chloro form, precipitated and resuspended as described by Moazed and Noller (6).

Primer extension with reverse transcriptase

Eleven deoxynucleotide primers complementary to the 23S rRNA sequences 417–433, 617–635, 888–906, 1169–1187, 1347–1363, 1687–1703, 1906–1922, 2141–2157, 2234–2251, 2607–2624, and 2888–2904 (7) were used to screen the entire 23S rRNA molecule. Primer extension of modified rRNA with reverse transcriptase (AMV, Life Sciences) was carried out as described in Stern *et al* (8), using 5′-[^{32}P]-labelled primers. Detection of guanine N-7 methylations required aniline scission of the RNA chain prior to primer extension (9). Extension products were run on 6% poly

acrylamide/7M urea gels alongside sequencing reactions performed on an unmodified rRNA template. Gel autoradiograms were scanned with an LKB Ultrascan XL enhanced laser densitometer.

RESULTS

Cell growth in antibiotics

It was first determined how effectively the antibiotics arrested cell growth. The minimal concentrations of clindamycin and lincomycin that inhibited growth of *E.coli* DH1 overnight cultures in liquid medium were 100 mg/l and 2 g/l, respectively. Growth of the more permeable strain AS19 was inhibited by 50 mg/l clindamycin and 500 mg/l lincomycin in liquid medium.

Footprinting of clindamycin and lincomycin on 23S rRNA

The accessibility of unpaired adenines, cytosines and guanines were charted for 23S rRNA in 70S ribosomes using DMS and kethoxal. These have previously been shown to be the most versatile reagents for detecting rRNA-drug interactions (10–12). DMS methylates N-1, N-3 and N-7 positions of accessible adenines, cytosines and guanines, respectively. Kethoxal modifies accessible, unpaired guanines at N-1 and N-2. The entire 23S rRNA was screened with the eleven primers selected here and the base modification pattern observed is in good agreement with previously published data (13). Changes in this pattern resulting from binding of clindamycin and lincomycin were limited to the peptidyl transferase loop in domain V.

The highly accessible base, adenine 2058, was shielded from DMS modification by both clindamycin and lincomycin. This effect was first discernible at a 1 μM concentration of either drug, the protection became more distinct with increasing drug concentra tions and was almost complete at 1 mM (Figures 1a and 2). The drug concentrations required for 50% binding (Figure 2), and thus the apparent K_{diss}, are approximately 5 and 8 μM for lincomycin and clindamycin, respectively.

Clindamycin also reduced the accessibility of adenine 2059, but not as markedly as at A2058. Lincomycin afforded no protection at A2059. At 1 mM, both drugs completely protected A2451 (not shown) and G2505 (Figure 1b) from weak modification with DMS and kethoxal, respectively. The drugs also partially shielded G2061 (Figure 1b) from kethoxal modification.

Screening the rest of the 23S rRNA structure showed that there were no additional effects at other accessible adenine and guanine N-1 positions. The accessibility of cytosine N-3 and guanine N-7 positions throughout the 23S rRNA were unaffected by drug binding.

DISCUSSION

Drug interaction with 23S rRNA

Clindamycin and lincomycin bind to the 50S ribosomal subunit and reduce the accessibility of phylogenetically conserved bases in the peptidyl transferase loop of 23S rRNA (Figure 3). The peptidyl tranferase loop undoubtedly forms a complex tertiary structure, probably involving the adjacent hairpin loop containing position 2032 (14, 15). A transition mutation at position 2032 confers lincomycin resistance in tobacco chloro plast (16) and clindamycin resistance in *E.coli* (14). This hairpin loop seems to be tightly structured as it is inaccessible to the chemical probes,

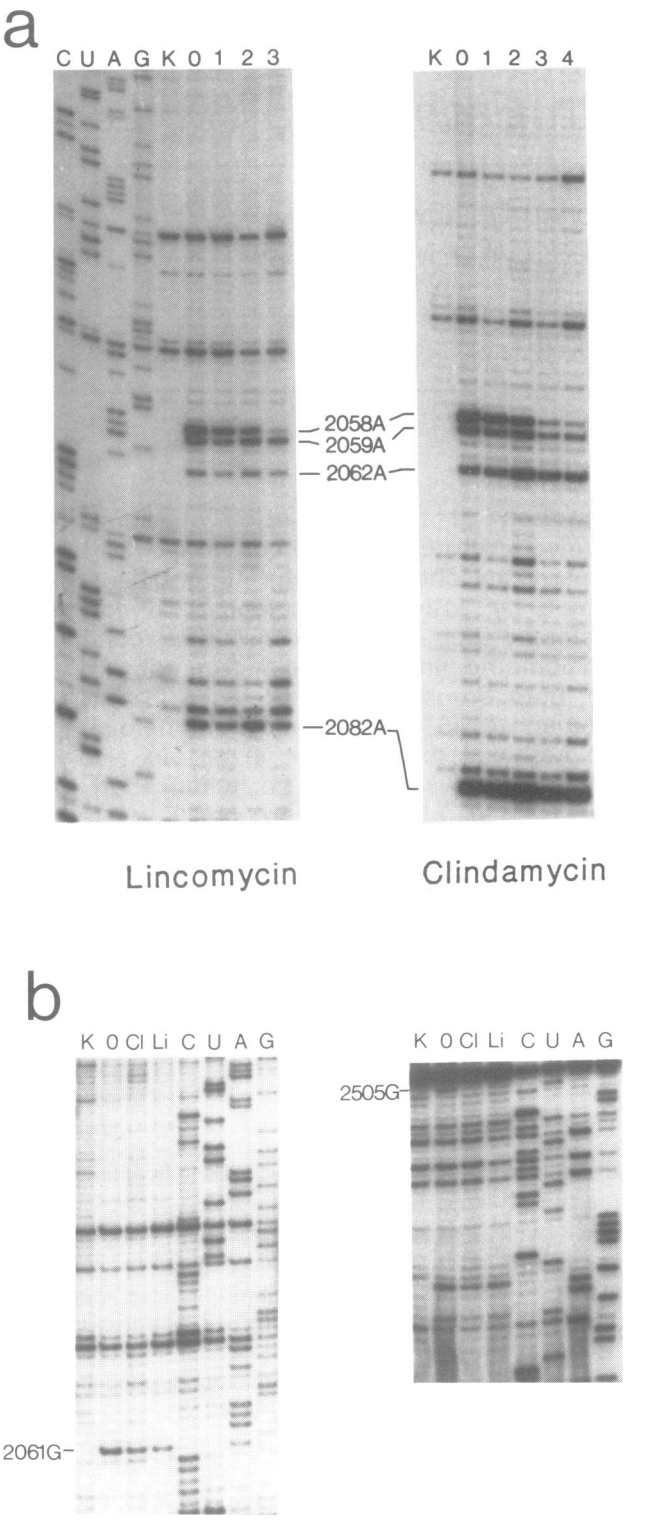

Figure 1. Autoradiograms of gel separation of primer extension reactions on 23S rRNA after drug binding to 70S ribosomes followed by chemical modification. (a) Extension from the 2141–2157 primer on unmodified (lane K) and DMS modified RNA templates (lanes 0 to 4), after binding of 0, 1, 10, 100, or 1000 μM antibiotic (lanes 0 to 4, respectively). (b) Extension from the 2141–2157 (left) and 2607–2624 primers on unmodified (lane K) and kethoxal modified templates (lanes 0, Cl and Li); modification was in the absence of drugs (lane 0), or after binding of 1 mM clindamycin (Cl) or 1 mM lincomycin (Li). RNA sequencing lanes are shown.

and consequently it could not be screened for drug interactions. The most straightforward interpretation of the data presented here is that clindamycin and lincomycin interact directly with bases in the peptidyl transferase loop.

Bases A2451 and G2505 appear to be particularly important in the peptidyl transferase reaction. The aminoacyl moiety of charged tRNA protects A2451 in the A-site, and A2451 plus G2505 in the P-site (17), and both these positions are completely protected by clindamycin, lincomycin (Figure 3), celesticetin (18), carbomycin and chloramphenicol (10). All these drugs inhibit the peptidyl transferase reaction (1, 2). The drugs could act by perturbing an essential RNA tertiary structure involving A2451 and G2505, or by hampering correct alignment of the peptidyl and aminoacyl substrates at the peptidyl transferase catalytic site.

Drug binding affinities estimated by footprinting

Adenosine 2058 is a highly accessible base that becomes almost totally protected (Figure 1a) and this position was therefore used to quantitatively compare binding of the drugs. The degree of protection at A2058 is interpreted here as correlating directly with the proportion of ribosomes binding the drug. The data in Figure 2 reproducibly showed similar binding affinities for clindamycin and lincomycin.

The K_{diss} value of 5 μM found here for lincomycin agrees well with previous estimates of 3.9 μM and 6.2 μM determined by ethanol sedimentation of *E.coli* run-off ribosomes and polysomes free of nascent peptides, respectively (19). However, a higher value of 34 μM (20) was obtained by equilibrium dialysis under physiological conditions similar to those used in this study. Similar footprinting experiments on erythromycin bound to ribosomes gave a value for K_{diss} of 10^{-8} M (5, and unpublished data), in good agreement with values of 10^{-8} and 10^{-7} M obtained by other methods (reviewed in ref. 1). The data in these cases show that footprinting is a reliable means of estimating the affinity of drug-ribosome interactions.

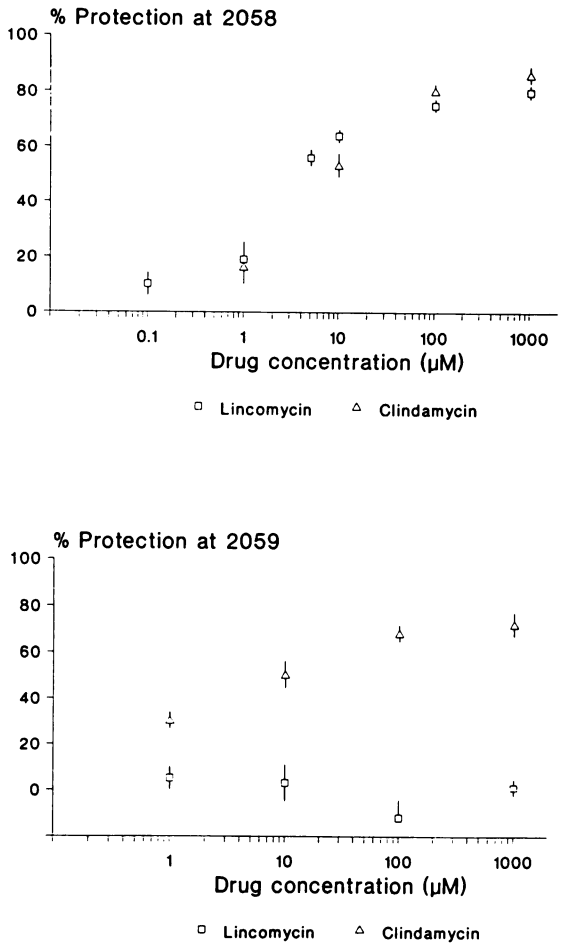

Figure 2. Graphs showing how the protection by clindamycin and lincomycin against DMS modification at A2058 and A2059 varies with drug concentration. Band intensities in each lane were normalised relative to A2082, and the percentage protection was calculated relative to control lanes modified in the absence of antibiotic. Each point on the curve is an average of at least three experiments. Standard errors of the mean are shown as vertical bars.

A difference in the interaction of clindamycin and lincomycin with ribosomes

The two drugs used in this study differ chemically in that the configuration of the lincomycin side chain has been altered in clindamycin by introduction of a chlorine atom on the 7-carbon (21). As a consequence of this, clindamycin is approximately 20 times more effective than lincomycin in inhibiting the growth of *E.coli*. The increased susceptibility to lincomycin of a more permeable *E.coli* strain suggests that the toxicological difference is (at least in part) the result of less efficient assimilation of lincomycin.

This study was designed to determine whether there are also differences in the manner in which the two drugs interact with ribosomes. The data show that lincomycin binds at least as tightly as clindamycin to *E.coli* ribosomes, and that both drugs interact with the same specific ribosomal target site. Does this mean that both drugs when bound are equally effective at inhibiting protein synthesis? An earlier study on lincomycin suggests that it does not, as this drug did not inhibit *in vitro* protein synthesis by *E.coli* ribosomes (22). There is a difference in the manner in which the two antibiotics slot into the peptidyl transferase site—adenine 2059 is shielded from modification by clindamycin but not by lincomycin. However, lincomycin resistance is conferred by a transition mutation at A2059 in tobacco chloroplast 23S rRNA

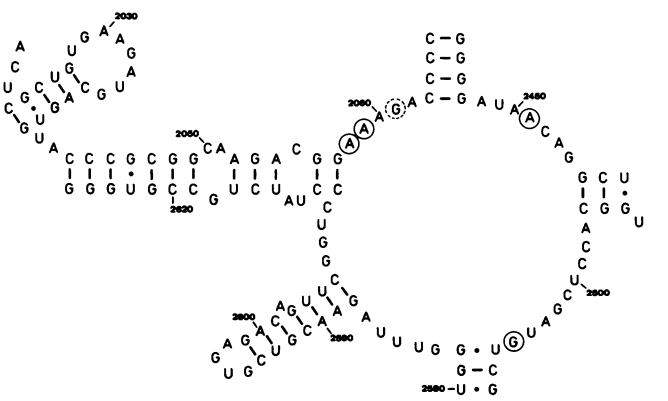

Figure 3. Secondary structure model of the peptidyl transferase loop in *E.coli* 23S rRNA (7). Accessible bases that are protected strongly (circles) or partially (broken circle) from chemical modification by the antibiotics are shown.

(16), so it remains possible that lincomycin interacts here without altering the accessibility of the N-1 position of the base. Parallel observations have been made for micrococcin and thiostrepton, two sulphur-containing peptide antibiotics that interact in an almost identical manner with the GTPase region of 23S rRNA (12). Accessibility of adenine 1067 is, however, increased by micrococcin but reduced by thiostrepton (12), and this correlates with the opposite effects the drugs have on ribosomal GTP hydrolysis (23). Mutagenesis or methylation of A1067 confers drug resistance (24).

In conclusion, the data presented here show that lincomycin binds at least as tightly as clindamycin to *E.coli* ribosomes. Both drugs interact with a discrete region of 23S rRNA within the peptidyl transferase loop, but in a slightly different manner. It is not yet clear to what extent this difference affects how the drugs inhibit protein synthesis.

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