CLEROCIDIN, A TERPENOID ANTIBIOTIC, INHIBITS BACTERIAL DNA GYRASE

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DNA topoisomerases catalyze the topological interconversions of DNA molecules which are required for several essential processes in DNA metabolism including replication, recombination, transcription, and chromosome separation at mitosis[1]. They are the target for the action of potent antitumor and antibacterial agents. A bacterial type II topoisomerase, bacterial gyrase, is the target of the quinolone antibacterial agents[2,3]. The effectiveness of the quinolones indicates that bacterial DNA gyrase is a target for clinically useful antibacterial agents. This has stimulated interest in searches for non-quinolone DNA gyrase inhibitors. These searches have resulted in the discovery of 2 closely related antibacterial agents, cinodine and coumamidine[4,5]. Cinodine has been shown to be a specific inhibitor of bacterial gyrase. We have screened culture broths of bacteria, actinomycetes, and fungi to find additional chemical structures which selectively interact with bacterial gyrase. A known antibiotic, clerocidin[6,7], was identified in this screen. KAWADA *et al.*[8] have recently shown that clerocidin induces eucaryotic topoisomerase II mediated double strand breaks. In this note we show that clerocidin inhibits DNA supercoiling by bacterial gyrase and causes bacterial gyrase mediated DNA cleavage. That this interaction is the primary mode of action of clerocidin is indicated by the selection of a mutation in *Escherichia coli* causing resistance to clerocidin which is in the *gyrA* gene.

A component in a fermentation broth of *Fusidium viride* was identified in a screen for agents which interact with *E. coli* gyrase by the same mechanism as nalidixic acid. This component was isolated and shown to be identical to clerocidin (Fig. 1). Clerocidin had previously been identified as a cytotoxic agent[5,6], and has recently been shown to stabilize the eucaryotic topoisomerase II cleavable complex[8].

The effect of clerocidin on supercoiling by bacterial gyrase was tested. Fig. 2 shows a series of supercoiling assays using relaxed pBR322 as a substrate for *E. coli* gyrase. These data clearly show inhibition of the gyrase mediated supercoiling at the higher concentrations of clerocidin (lanes 8, 9, 10 and 11) and by control drugs (nalidixic acid, lane 4 and ciprofloxacin, lane 3). In these experiments supercoiling was inhibited 50% by approximately 10 μM clerocidin.

Nalidixic acid and oxolinic acid are potent inhibitors of DNA gyrase and appear to stabilize the DNA cleavage complexes at specific sequences

Fig. 1. Structure of clerocidin.

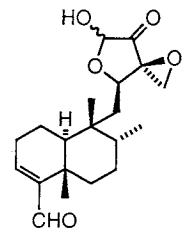
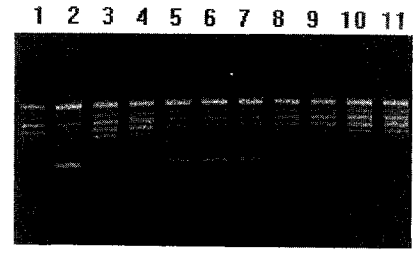
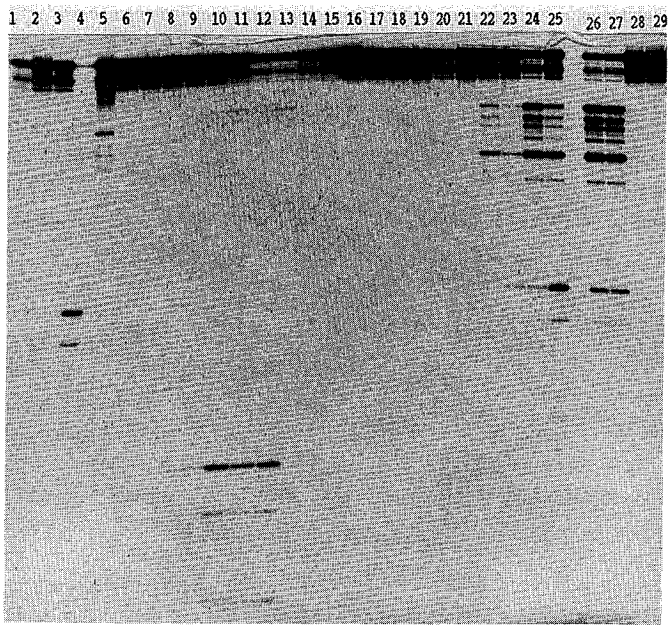


Fig. 2. Inhibition of *Escherichia coli* gyrase supercoiling by clerocidin.



Plasmid pBR322 was incubated with *E. coli* gyrase, made from overproducing strains as described previously[12], and drugs as described by REECE and MAXWELL[13]. Controls lacking inhibitor and enzyme (lane 1) and containing enzyme but no inhibitor (lane 2) are included. Ciprofloxacin (5 μg/ml) and nalidixic acid (50 μg/ml) were added to the reactions shown in lanes 3 and 4. Clerocidin concentrations (μM) in lanes 5~11 are as follows: 0.3, 1.0, 3.0, 10, 30, 100 and 300.

Fig. 3. Gyrase and topoisomerase II cleavages in the presence of clerocidin.



Cleavage reactions were performed on pOBS2A, a plasmid derived from the ICP0 promoter region of herpes simplex virus type 1. The DNA is a *Bam*H I to *Ava* I fragment of 120 bp labeled at the *Bam*H I site. Cleavages were carried out with approximately 20,000 CPM (5 ng) of DNA and 10 units of purified topoisomerase II (final volume of 20 μl) in cleavage buffer (30 mM Tris-HCl, pH 7.6, 60 mM KCl, 8 mM $MgCl_2$, 15 mM 2-mercaptoethanol, 3 mM ATP, 30 μg BSA/ml). Termination of reactions and analysis of cleavage products on DNA sequencing gels was performed as described by CHUNG *et al.*[14]. The cleavage substrate specified in the experiment was labelled at a single 5′ end using polynucleotide kinase as described elsewhere[15]. Lane 1, DNA fragment alone; lane 2, teniposide (25 μM) alone; lane 3, topoisomerase II (10 units); lane 4, topoisomerase II plus m-AMSA (50 μg/ml); lane 5, topoisomerase II plus teniposide (25 μM); lanes 6 ~ 15, topoisomerase II plus clerocidin at the following μM concentrations: 0.75, 1.5, 3.0, 15, 30, 150, 300, 600, 1,500 and 3,000; lane 16, clerocidin (3.0 mM) alone; lane 17, gyrase (40 units) alone; lane 18 gyrase plus AMSA; lane 19, gyrase plus teniposide (25 μM); lanes 20 ~ 29 gyrase plus clerocidin at the following μM concentrations: 0.75, 1.5, 3.0, 15, 30, 150, 300, 600, 1,500 and 3,000.

unique to each compound. Cleavage sites can be mapped by incubating the enzyme with a 5′ end-labeled DNA, terminating the reaction with a protein denaturant (SDS) and sequencing the cleavage sites relative to chemical sequence markers[9,10]. This analysis reveals whether a particular agent sufficiently stabilizes the topoisomerase cleavage complex to allow its detection upon addition of SDS and also reveals sequence specific cleavages unique to the test compound.

Fig. 3 shows specific cleavage fragments formed when eukaryotic topoisomerase II and gyrase are incubated with clerocidin, teniposide, and m-AMSA. The fragments formed when clerocidin is incubated with gyrase are seen in lanes 21 ~ 27 of Fig. 3. Thus, clerocidin causes sequence-specific DNA cleavage when incubated with bacterial DNA gyrase in addition to inhibiting supercoiling.

Consistent with the results reported by KAWADA *et al.*[8], cleavage bands are also observed when clerocidin is incubated in the presence of topoisomerase II (Fig. 3 lanes 8 ~ 13). In this particular assay at equivalent concentrations of clerocidin there is a higher yield of cleavage fragments from the DNA gyrase containing reactions than from the topoisomerase II containing reactions (compare Fig. 3 lanes 21 ~ 27 with lanes 7 ~ 13). At the highest concentrations of clerocidin DNA cleavage by both topoisomerase II and DNA gyrase is inhibited (lanes 13 ~ 15, 28, 29).

Cleavage fragments are distinct for each combination of agent and enzyme (compare Fig. 3 lanes 4, 5, 9, 18, 19 and 24). In the absence of inhibitors, gyrase cleavages were not detected, in keeping with the idea that the cleavage complex is difficult to detect in the absence of inhibitors that extend the

half life of the cleaved intermediate.

Comparison of the activities of clerocidin and the quinolone *anti*-bacterial agents, ciprofloxacin and oxolinic acid, in this kind of assay show that clerocidin is as active as ciprofloxacin and more active than oxolinic acid at equivalent concentrations of each compound (data not shown). The oxolinic acid and ciprofloxacin induced cleavage sites are different from each other and the clerocidin induced cleavage sites (data not shown).

A physiological comparison of the action of clerocidin and the topoisomerase II inhibitor, teniposide, was made by measuring rates of DNA synthesis *in vivo* in the presence and absence of the compounds. Vero cells were grown in the presence or absence of the compounds and DNA replication was monitored by incorporation of [^{3}H]-TdR (Table 1). Teniposide strongly inhibited DNA synthesis after 1 hour (60% reduction in incorporation) or after 6 hours (90% reduction) of exposure. In contrast, clerocidin was less inhibitory (30% inhibition at 1 hour and 6 hours) suggesting that this agent could be less cytotoxic to animal cells than teniposide.

Table 1. Inhibition of DNA synthesis in vero cells by teniposide and clerocidin.

Time after drug addition	^{3}H (CPM)		
	No drug	Teniposide	Clerocidin
1 hour	18,772	6,400	12,000
6 hours	22,866	2,406	16,112

Vero cells were seeded in 60 mm petri dishes in 10% fetal bovine serum (in EAGLE's minimal medium) and allowed to settle down and attach for 30 minutes. Cultures were treated with either 2 μM clerocidin, 2 μM teniposide or untreated (control). At 1 hour or 6 hours after addition of drugs, rates of DNA synthesis were measured by a 60 minutes pulse with [^{3}H]-TdR (25 μCi/dish). Thymidine incorporation rates were then determined by TCA precipitation.

A collection of bacterial species for which mutants resistant to nalidixic acid or ciprofloxacin were available was used to assess the level of cross resistance of clerocidin. The data in Table 2 show that in all cases except *Staphylococcus aureus* both the ciprofloxacin-resistant mutants and the nalidixic acid-resistant mutants are also resistant to clerocidin. Since these nalidixic acid-resistant mutants and ciprofloxacin-resistant mutants are in the structural gene for the gyrase A protein, the cross resistance indicates that the mode of action of clerocidin also involves DNA gyrase.

To confirm that the observed interaction of clerocidin with gyrase is its primary mode of action, a mutation (*cle^{R}1*) causing resistance to clerocidin was selected in *E. coli* strain HB101. This was done by growth in L broth containing inhibiting levels of

Table 2. Cross resistance between clerocidin and the quinolones.

Organisms			MIC (μg/ml)				
			Clerocidin	Ofloxacin	Ciprofloxacin	Novobiocin	Nalidixic acid
Escherichia coli	SC8294	Parent	12.5	0.1	<0.05	>100	6.3
	14,990	R to C[a]	50	6.3	3.1	>100	>100
	14,991	R to N[b]	100	1.6	0.8	>100	>100
Serratia sp.	SC9782	Parent	12.5	0.4	0.1	>100	1.6
	14,992	R to C	100	12.5	3.1	>100	>100
	14,993	R to N	100	50	25	>100	>100
Proteus sp.	SC9574	Parent	25	0.4	0.1	50	12.5
	14,994	R to C	25	6.3	3.1	50	>100
	14,995	R to N	50	12.5	12.5	50	100
Pseudomonas sp.	SC8329	Parent	12.5	0.8	0.2	>100	>100
	14,998	R to N	50	12.5	6.3	>100	>100
Klebsiella sp.	SC10440	Parent	12.5	0.1	<0.05	100	6.3
	14,999	R to N	50	1.6	0.4	>100	100
Staphylococcus aureus	SC2400	Parent	0.1	0.4	0.4	0.2	>100
	R to C	R to C	0.1	50	>100	0.2	>100
	R to N	R to N	0.1	6.3	25	0.2	>100
S. aureus	SC1276	Parent	0.2	0.4	0.4	0.2	>100
	SC2960	R to Nov[c]	0.2	0.8	0.8	>100	>100

[a] Ciprofloxacin.
[b] Nalidixic acid.
[c] Novobiocin.

clerocidin. The MIC for clerocidin of the resistant mutant, *E. coli* strain SGB864, is 22-fold higher than that of HB101. As shown in Table 3 the strain containing *cle*^{R}*1* is less sensitive to a number of quinolone antibacterials in addition to clerocidin. Again, cross resistance between quinolones and clerocidin is observed.

If this mutation is in the *gyrA* gene as most mutations causing quinolone resistance are, then it probably alters the *gyrA* component of gyrase resulting in a loss of interaction with the inhibitor. Such a resistant allele should be recessive to wild

Table 3. Sensitivity of *Escherichia coli* HB101 and clerocidin-resistant mutant, SGB864, to anti-bacterial agents.

Compound	(μg/ml)	Zone diameters (mm)	
		HB101	SGB864
Clerocidin	25	20	0
Nalidixic acid	50	30	21
Norfloxacin	30	32	16
Ciprofloxacin	10	25	12
Enoxacin	14	35	19
Ampicillin	10	14	13
Cycloserine	10	15	13
Gentamicin	10	14	12
Tetracycline	30	29	22
Chloramphenicol	30	23	18
Novobiocin	30	13	14
Rifamycin	50	12	10

Table 4. Clerocidin zone of inhibition diameters on resistant mutant, parent, and *gyrA* containing transformants.

Clerocidin (μg/disk)	Zone diameters (mm)			
	HB101 *cle*$^{+}$	SGB955 *cle*$^{+}$/*gyrA*	SGB864 *cle*^{R}*1*	SGB954 *cle*^{R}*1*/*gyrA*
50	22	20	0	13
100	30	24	0	16
150	28	27	0	18

Table 5. *In vitro* activity of clerocidin, nalidixic acid, and ciprofloxacin against bacteria.

Organism (strain No.)	MIC (μg/ml)		
	Clerocidin	Nalidixic acid	Ciprofloxacin
Escherichia coli 8294	12.5	1.6	<0.05
Klebsiella pneumoniae 9527	3.1	0.8	<0.05
Proteus mirabilis 3855	3.1	6.3	<0.05
Providencia rettgeri 8479	1.6	3.1	<0.05
Proteus vulgaris 9416	0.8	1.6	<0.05
Salmonella typhi 1195	6.3	1.6	<0.05
Shigella sonnei 8449	6.3	1.6	<0.05
Enterobactor cloacae 8236	25	6.3	<0.05
E. aerogenes 10078	25	6.3	0.1
Citrobacter freundii 9518	3.1	1.6	<0.05
Serratia marcescens 9783	3.1	1.6	0.2
Pseudomonas aeruginosa 9545	0.8	25	0.4
P. cepacia 14164	25	100	25
Acinetobacter anitritus 14207	3.1	3.1	0.4
Staphylococcus aureus 1276	0.1	>100	0.2
Streptococcus faecalis 9011	0.1	>100	1.6
S. agalactiae 9287	0.1	>100	0.8
Micrococcus luteus 2495	<0.05	>100	1.6
Bacillus subtilis 3777	0.1	1.6	<0.05
Streptococcus pneumoniae 8900	0.8	ND	3.1
S. viridans 11382	0.8	ND	3.1
Bacteroides fragilis 9844	<0.05	ND	3.1
Clostridium perfringens 11256	0.2	ND	0.4
C. sporogenes 2372	0.1	ND	0.2
C. difficile 11251	0.2	ND	12.5
Bifidobacterium dentium 11260	0.4	ND	1.6
Peptococcus variabilis 11264	<0.05	ND	0.2
Propionibacterium acnes 4020	<0.05	ND	1.6
Nocardia autotrophica	0.4	>100	100
Mycobacterium fortuitum	0.4	12.5	0.4

type, and introduction of the wild type allele into the resistant strain should cause it to become sensitive to the inhibitor. When SGB864 (*cle*^{R}*1*) was transformed with a plasmid (pKC16) containing *gyrA*[11] it regained sensitivity to clerocidin (Table 4). Thus, the *cle*^{R}*1* mutation is located within the *gyrA* gene.

Interest in clerocidin and the structurally related antibiotic, terpentecin, has focused mainly on their cytotoxic and antitumor actions. However, Table 5 shows that clerocidin has broad spectrum antibacterial activity. Its activity against the Gram-positive bacteria tested is equal to or greater than that of ciprofloxacin. Clerocidin has very good activity against anaerobic bacteria. It is more active than ciprofloxacin against these organisms.

References

1) WANG, J. C.: DNA topoisomerases. Annu. Rev. Biochem. 54: 665~697, 1985
2) SUGINO, A.; C. L. PEEBLES, K. N. KREUZER & N. R. COZZARELLI: Mechanism of action of nalidixic acid: purification of *E. coli nalA* gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. Proc. Natl. Acad. Sci. U.S.A. 74: 4767~4771, 1977
3) GELLERT, M.; K. MIZUUCHI, M. H. O'DEA, T. ITOH & J. TOMIZAWA: Nalidixic acid resistance: a second genetic character involved in gyrase activity. Proc. Natl. Acad. Sci. U.S.A. 74: 4772~4776, 1977
4) OSBURNE, M. S.; W. M. MAIESE & M. GREENSTEIN: In vitro inhibition of bacterial DNA gyrase by cinodine, a glycocinnamoylspermidine antibiotic. Antimicrob. Agents Chemother. 34: 1450~1452, 1990
5) ROSEN, T.; P. B. FERNANDES, M. A. MAROVICH, L. SHEN, J. MEO & A. G. PERNET: Aromatic dienoyl tetramic acids. Novel antibacterial agents with activity against anaerobes and staphylococci. J. Med. Chem. 32: 1062~1069, 1989
6) ANDERSEN, N. R.; H. O. B. LORCK & P. R. RASMUSSEN: Fermentation, isolation and characterization of antibiotic, PR-1350. J. Antibiotics 36: 753~760, 1983
7) ANDERSEN, N. R. & P. R. RASMUSSEN: The constitution of clerocidin: a new antibiotic isolated from *Oidiodendron truncatum*. Tetrahedron Lett. 25: 465~468, 1984
8) KAWADA, S.; Y. YAMASHITA, N. FUJII & H. NAKANO: Induction of a heat-stable topoisomerase II-DNA cleavable complex by nonintercalative terpenoids, terpentecin and clerocidin. Cancer Res. 51: 2922~2925, 1991
9) SPITZNER, J. R.; I. K. CHUNG & M. T. MULLER: Eukaryotic topoisomerase II preferentially cleaves alternating purine-pyrimidine repeats. Nucleic Acids Res. 18: 1~11, 1990
10) SPITZNER, J. R. & M. T. MULLER: Application of a degenerate consensus sequence to quantify recognition sites by vertebrate DNA topoisomerase II. J. Mol. Recog. 2: 63~74, 1989
11) MIZUUCHI, K.; M. MIZUUCHI, M. H. O'DEA & M. GELLERT: Cloning and simplified purification of *Escherichia coli* DNA gyrase A and B proteins. J. Biol. Chem. 259: 9199~9201, 1984
12) HALLETT, P.; A. J. GRIMSHAW, D. B. WIGLEY & A. MAXWELL: Cloning of the DNA gyrase genes under *tac* promoter control: overproduction of the gyrase A and B proteins. Gene 93: 139~142, 1990
13) REECE, R. J. & A. MAXWELL: Tryptic fragments of the *Escherichia coli* DNA gyrase A protein. J. Biol. Chem. 264: 19648~19653, 1989
14) CHUNG, I. K.; V. B. MEHTA, J. R. SPITZNER & M. T. MULLER: Eukaryotic topoisomerase II cleavage of parallel stranded DNA tetraplexes. Nucleic Acids Res. 20: 1973~1977, 1992
15) CHUNG, I. K. & M. T. MULLER: Aggregates of oligo (dG) bind and inhibit topoisomerase II activity and induce formation of large networks. J. Biol. Chem. 266: 9508~9514, 1991