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Biological Characterization of Cyclothialidine, a New DNA Gyrase Inhibitor

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Cyclothialidine is a new DNA gyrase inhibitor isolated from *Streptomyces filipinensis* NR0484. Structurally, it belongs to a new class of natural products containing a unique 12-membered lactone ring that is partly integrated into a pentapeptide chain. Cyclothialidine was found to be one of the most active of all the DNA gyrase inhibitors tested in the DNA supercoiling reaction of *Escherichia coli* DNA gyrase; 50% inhibitory concentrations (in micrograms per milliliter) of 0.03 (cyclothialidine), 0.06 (novobiocin), 0.06 (coumermycin A1), 0.66 (norfloxacin), 0.88 (ciprofloxacin), and 26 (nalidixic acid) were found. In addition, DNA gyrases from gram-positive species were inhibited equally as well as DNA gyrase from *E. coli*. Cyclothialidine also inhibited the in vitro DNA replication directed from *oriC* of *E. coli*. Among the bacterial species tested, only *Eubacterium* spp. were inhibited by cyclothialidine, suggesting that it can enter the cells of *Eubacterium* and exert antibacterial activity through interference with the DNA gyrase within the cells, although its penetration into most bacterial cells appears to be poor. These results provide a basis for cyclothialidine to be a lead structure for novel antibacterial agents with DNA gyrase inhibitory activities.

DNA gyrase belongs to the DNA topoisomerase II group. Although both prokaryotic and eukaryotic cells have topoisomerase II, DNA gyrase is unique in that it has characteristics different from those of other topoisomerases and exists only in bacteria (13, 31). The enzyme from *Escherichia coli* is a tetramer consisting of two A and two B proteins. The gyrase A and B subunits are encoded by the *gyrA* and *gyrB* genes of *E. coli*, respectively.

When ATP is present, DNA gyrase catalyzes the introduction of negative supercoils into DNA; in the absence of ATP, it removes negative supercoils, albeit inefficiently. The mammalian topoisomerase, however, can only relax supercoiled DNA. DNA gyrase has proved to be essential for bacterial growth. These factors provide the basis for DNA gyrase inhibitors to exert their antibacterial activities with selective toxicities. DNA gyrase is known to be the target of two classes of antibiotics. One comprises the synthetic quinolones, typified by nalidixic acid and oxolinic acid, which target the A subunit of the enzyme and probably act by interfering with the DNA rejoining step of the gyrase-mediated DNA strand-passing reaction (14, 27, 30). The other class consists of the natural coumarin-type compounds, such as novobiocin and coumermycin A1, that inhibit gyrase probably by competing with ATP for binding to the B subunit of the enzyme (7, 8, 15, 29). Although synthetic quinolones are commercially successful antibacterial agents, there is still room for improvement in their toxicities (33) and activities against resistant strains (6, 26). The natural novobiocin derivatives are also toxic. These situations led us to initiate microbial broth screening for DNA gyrase inhibitors with the aim of identifying new lead compounds. From the screening of natural products for DNA gyrase inhibitors, we discovered a novel DNA gyrase inhibitor, cyclothialidine (Ro 09-1437) (Fig. 1) (1). It was isolated from the fermentation products of *Streptomyces filipinensis* NR0484. Cyclothialidine contains a unique 12-member lactone ring which is partly integrated into a pentapeptide chain. In this report, we describe the biological activity of cyclothialidine as a lead structure for a new class of antibacterial agents.

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MATERIALS AND METHODS

Chemicals. Cyclothialidine (molecular weight, 641; >98% pure) was isolated from the culture broth of *S. filipinensis* NR0484. Nalidixic acid, oxolinic acid, novobiocin, coumermycin A1, and adriamycin were purchased from Sigma Chemical Company. Ofloxacin (Daiichi), norfloxacin (Kyorin), ciprofloxacin (Bayer), and enoxacin (Dainippon) were >98% pure by high-pressure liquid chromatographic analysis. All other chemicals were at least analytical grade.

DNA supercoiling assay. Supercoiled ColE1 DNA was purified from *E. coli* Hfr H5 (ColE1) by cesium chloride density gradient centrifugation (18). Relaxed ColE1 DNA was prepared by treating the supercoiled DNA with a crude nuclear extract of rat liver containing topoisomerase I in the presence of EDTA to inhibit nucleases (4, 23). *E. coli* DNA gyrase holoenzyme was partially purified from *E. coli* D110 (*thyA end*) by novobiocin-Sepharose column chromatography by the method of Staudenbauer and Orr (28). The DNA supercoiling assay of DNA gyrase was performed by the method of Otter and Cozzarelli (23) with a slight modification. We prepared 20 μl of a DNA supercoiling assay mixture containing 50 mM Tris-HCl (pH 8.0), 20 mM KCl, 10 mM $MgCl_2$, 5 mM Spermidine · 3HCl, 50 μg of bovine serum albumin per ml, 125 μg of *E. coli* tRNA per ml, 2 mM dithiothreitol (DTT), 5 mM ATP, 0.5 μg of relaxed ColE1 DNA, and 1 U of DNA gyrase; incubation was for 30 min at 30°C, and then sodium dodecyl sulfate (SDS; final concentration, 1%) was added to stop the reaction. The production of supercoiled ColE1 DNA was monitored by agarose gel electrophoresis. The amount of product was quantitated by

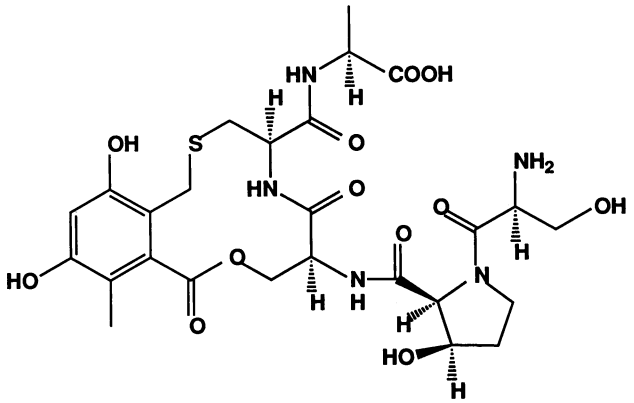

FIG. 1. Structure of cyclothialidine.

densitometry (model CS-920 densitometer; Shimadzu) of a photographic negative of gels stained with ethidium bromide. One unit of DNA gyrase was defined as the minimum amount of DNA gyrase holoenzyme that maximally supercoiled 0.5 μg of relaxed ColE1 DNA in 30 min at 30°C. The IC_{100} was defined as the MIC at which a supercoiling band of DNA completely disappeared on the agarose gel, and IC_{50} was defined as the inhibitory concentration at which the intensity of the DNA supercoiling band was half that of the control.

***oriC*-directed DNA replication assay.** The in vitro DNA replication assay was performed by using an *oriC*-containing plasmid, PTSO169, which was kindly provided by A. Kornberg, following the method of Fuller et al. (12). The standard *oriC* DNA replication assay contained, in a total volume of 25 μl, 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–KOH (pH 7.6); 2 mM ATP; 500 μM each GTP, CTP, and UTP; 50 μg of bovine serum albumin per ml; 21.6 mM creatine phosphate; 100 μM each dATP, dGTP, dCTP, and dTTP; 180 cpm of total deoxynucleotide [^{3}H]dTTP per pmol; 11 mM magnesium acetate; 100 μg of creatine kinase per ml; 3.9 μg of supercoiled PTSO169 DNA per ml; and 6% polyvinyl alcohol 24,000. The mixture was assembled at 0°C. The reactions were initiated by the addition of 200 to 280 μg of proteins (fraction II), and incubation was for 30 min at 30°C. Nucleotide incorporation was measured by determining radioactivity after trichloroacetic acid precipitation.

Susceptibility testing. A standard paper disk assay was used to study drug susceptibility (20). Diameters of inhibitory zones around the cyclothialidine-containing disks were measured after incubation of the bacteria-containing agar plate at 37°C for 18 h.

DNA topoisomerase I assay. The DNA relaxation assay of calf thymus DNA topoisomerase I, which was purchased from Bethesda Research Laboratories, was carried out by the method of Ferro et al. (9), with a slight modification. We assayed the relaxation activity of DNA topoisomerase I in a 20-μl reaction mixture containing 0.5 μg of negatively supercoiled ColE1 DNA, 35 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 72 mM KCl, 5 mM DTT, 5 mM spermidine · 3HCl, 20 μg of bovine serum albumin per ml, and calf thymus DNA topoisomerase I. After 60 min of incubation at 37°C, the reaction was stopped with 7 μl of stopping buffer (2% SDS, 20% Ficoll 400, and 800 μg of bromophenol blue per ml). Agarose gel electrophoresis was carried out in the same way as described above for the DNA gyrase assays.

DNA topoisomerase II assay. Calf thymus DNA topoisomerase II was partially purified by the procedure of Schomburg and Grosse (25) and was virtually free of topoisomerase I (as determined by an ATP-independent relaxation assay).

The DNA catenation assay of DNA topoisomerase II was carried out by the method of Barrett et al. (3) by the conversion of superhelical DNA to catenated networks. The standard reaction mixture (20 μl) contained 50 mM Tris-HCl (pH 8.0), 1 mM ATP, 4 mM $MgCl_2$, 2 mM DTT, 50 mM KCl, 25 mM NaCl, 10 μg of bovine serum albumin per ml, 16 μg of histone H1 per ml, and 0.5 μg of negatively supercoiled ColE1 DNA. For the determination of the IC_{50}, the reaction with topoisomerase II was carried out for 30 min at 37°C. The reaction was terminated by adding 4 μl of stopping buffer. Samples were electrophoresed in the same way as described above for the DNA gyrase assays.

Other methods. *E. coli* RNA polymerase assay and HeLa DNA polymerase α assay were carried out by the method of Burgess (5) and Miller et al. (19), respectively. The HeLa cell cytotoxicity assay was carried out by the method of Ohara and Terasima (22).

RESULTS

Inhibition of DNA gyrase-mediated supercoiling activity. Figure 2 shows the inhibition of the supercoiling activity of *E. coli* DNA gyrase by cyclothialidine, novobiocin, and ofloxacin. Significant inhibition of supercoiling by cyclothialidine was apparent at drug concentrations of less than 0.4 μg/ml (Fig. 2A; Table 1). The comparative IC_{50}s and IC_{100}s of cyclothialidine and other gyrase inhibitors are listed in Table 1. The B subunit inhibitor novobiocin and coumermycin A1 exhibited far more potent inhibitory activities than the A subunit inhibitor quinolones used in the assay. However, cyclothialidine (IC_{50}, 0.03 μg/ml) was two times more active than novobiocin and coumermycin A1. On the other hand, cyclothialidine did not produce the DNA gyrase-DNA-cleavable complex (Fig. 2A) (21), which was detected in the agarose gel analysis of ofloxacin inhibition (Fig. 2C). The inhibitory activities of cyclothialidine against DNA gyrases from several bacterial species were also examined (Table 1). Cyclothialidine inhibited not only the DNA gyrase from *E. coli* D110 but also those from *Micrococcus luteus*, *Staphylococcus aureus*, and *Eubacterium moniliforme*, indicating that cyclothialidine has broad-spectrum activity for the inhibition of DNA gyrase. Two B subunit inhibitors, novobiocin and coumermycin A1, were not active against the *E. moniliforme* enzyme (17), and the A subunit inhibitors displayed narrower spectra, preferentially inhibiting *E. coli* DNA gyrase. The activity ranking order among cyclothialidine, ofloxacin, novobiocin, and coumermycin A1 was consistent regardless of the DNA gyrase used, as follows: cyclothialidine ≥ novobiocin ≈ coumermycin A1 > ofloxacin.

Activity against DNA replication. DNA gyrase plays key roles in the initiation and elongation step of DNA replication (2, 10, 11, 32), and therefore, the inhibitory activity of cyclothialidine in the *oriC* DNA replication system might better predict the antibacterial activity of the compound, although cell penetrability is another factor to be considered.

The in vitro *oriC*-directed DNA replication was also

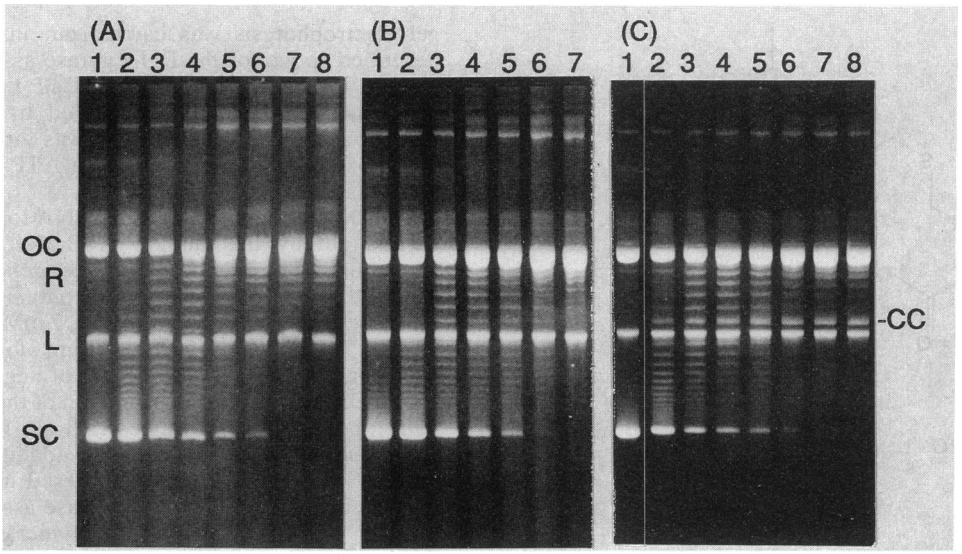

FIG. 2. Effect of cyclothialidine, novobiocin, and ofloxacin on supercoiling of relaxed ColE1 DNA by *E. coli* DNA gyrase. Experiments were performed as described in the text. (A) Cyclothialidine present during the supercoiling reaction: cyclothialidine at 0 (lane 1; control), 0.025 (lane 2), 0.05 (lane 3), 0.1 (lane 4), 0.2 (lane 5), 0.4 (lane 6), 0.8 (lane 7), and 1.6 (lane 8) μg/ml. (B) Novobiocin present during the supercoiling reaction: novobiocin at 0 (lane 1; control), 0.05 (lane 2), 0.1 (lane 3), 0.2 (lane 4), 0.4 (lane 5), 0.8 (lane 6), and 1.6 (lane 7) μg/ml. (C) Ofloxacin present during the supercoiling reaction: ofloxacin at 0 (lane 1; control), 0.8 (lane 2), 1.6 (lane 3), 3.2 (lane 4), 6.3 (lane 5), 12.5 (lane 6), 25 (lane 7), and 50 (lane 8) μg/ml. The positions of relaxed (R), supercoiled (SC), open circular (OC), and linear (L) forms of plasmid DNA and DNA gyrase-mediated cleavable complex (CC) are shown.

potently inhibited by cyclothialidine, with an IC_{50} of 0.35 μg/ml (Table 2). The inhibitory activities of ofloxacin, novobiocin, and coumermycin A1 were almost the same as that of cyclothialidine, with IC_{50}s of 0.89, 0.89, and 0.25 μg/ml, respectively. However, the inhibitory activity of cyclothialidine against DNA replication was significantly lower than that seen in the DNA supercoiling assay (IC_{50}, 0.03 μg/ml). The ratio of IC_{50}s of cyclothialidine between the replication assay and the supercoiling assay was 11.7. This ratio is similar to those of the B subunit inhibitors novobiocin (14.8) and coumermycin A1 (4.2). In contrast, the disparity between these values was not as great with the A subunit inhibitor ofloxacin (0.8). Also, if we consider the fact that the cleavable complex in the DNA gyrase assay cannot be formed, the mode of cyclothialidine inhibition against DNA gyrase must be different from those of quinolones.

Antibacterial activity. The activity of cyclothialidine against whole bacterial cells was studied by using a wide variety of bacteria including both aerobic and anaerobic species such as *E. coli*, *Bacillus subtilis*, *S. aureus*, *Micrococcus flavus*, *E. moniliforme*, and *Bacteroides fragilis*. Cyclothialidine exhibited antibacterial activity only against

TABLE 1. In vitro activity against DNA gyrase supercoiling activity[a]

Compound	*E. coli* D110		*M. luteus*[b]		IC_{100} (μg/ml) for *S. aureus* ATCC 25923[c]	*E. moniliforme* ATCC 25546[d]	
	IC_{50} (μg/ml)	IC_{100} (μg/ml)	IC_{50} (μg/ml)	IC_{100} (μg/ml)		IC_{50} (μg/ml)	IC_{100} (μg/ml)
Cyclothialidine	0.03	0.8	0.005	0.05	0.4	0.12	0.4
Gyrase A subunit inhibitors							
Nalidixic acid	26	>1,500	—[e]	—	—	—	—
Oxolinic acid	7.7	>250	—	—	—	—	—
Ofloxacin	1.1	50	>100	>100	400	10.8	800
Norfloxacin	0.66	25	—	—	>400	—	—
Ciprofloxacin	0.88	25	—	—	400	—	—
Enoxacin	2.6	>50	—	—	—	—	—
Gyrase B subunit inhibitors							
Novobiocin	0.06	1.6	0.013	0.2	0.4	16.8	100
Coumermycin A1	0.06	1.6	0.41	1.6	0.8	3.9	50

[a] One unit of each DNA gyrase was used for the DNA supercoiling assay.
[b] Purchased from Bethesda Research Laboratories.
[c] A 50% ammonium sulfate precipitate.
[d] Purified by heparin-Sepharose column chromatography and novobiocin-Sepharose column chromatography.
[e] —, not tested.

TABLE 2. Activity in in vitro DNA replication system

Compound	IC_{50} (μg/ml) for:		IC_{50} ratio (replication/gyrase)
	Replication	Gyrase	
Cyclothialidine	0.35	0.03	11.7
Ofloxacin	0.89	1.1	0.8
Novobiocin	0.89	0.06	14.8
Coumermycin A1	0.25	0.06	4.2

Eubacterium species among the bacterial species tested. In the paper disk assay (paper disk diameter, 8 mm), 40 μg of cyclothialidine per disk displayed an inhibitory zone diameter of 17.5 mm against *E. moniliforme*. Two other *Eubacterium* species, *E. limosum* and *E. lentum*, were also inhibited by the compound to a similar degree. However, against the other strains, it was not active even when a cell wall mutant of *E. coli*, strain DC2, and an L-form variant of *Proteus vulgaris* were used as indicator strains (data not shown). Minimum synthetic medium did not improve the antibacterial activity of cyclothialidine.

Selectivities to gyrase and topoisomerases. Next, we examined the selectivities of DNA gyrase inhibitors against DNA topoisomerases I and II of calf thymus (Table 3). Ofloxacin was found to have the weakest activity against topoisomerase I; this was followed by cyclothialidine and ciprofloxacin. In the evaluation with topoisomerase II, cyclothialidine exhibited the least inhibitory activity of all the compounds tested. In contrast, coumermycin A1 was the most potent inhibitor of both mammalian topoisomerases. When selectivity ratios (IC_{50} for calf thymus topoisomerases/IC_{50} for *E. coli* DNA gyrase) were calculated, cyclothialidine gave the highest selectivity ratios in the evaluation with calf thymus DNA topoisomerases I and II.

Activity against enzymes by using DNA as a substrate. To evaluate selectivity in more detail, cyclothialidine was tested for its inhibitory activity against enzymes by using DNA as a substrate (Table 4), namely, *E. coli* RNA polymerase and HeLa cell DNA polymerase α. Cyclothialidine inhibited none of these enzymes, showing IC_{50}s of greater than 1,000 μg/ml. Coumermycin A1 was the most potent inhibitor of *E. coli* RNA polymerase and HeLa DNA polymerase α; this was followed by novobiocin and ofloxacin. *E. coli* RNA polymerase and HeLa DNA polymerase α exhibited the same profiles of susceptibility to these four DNA gyrase inhibitors. Table 4 also shows the cytotoxicities of these compounds in HeLa cells. Again, cyclothialidine was by far the least toxic compound.

TABLE 4. In vitro activity against enzymes by using DNA as a substrate and cytotoxicity in HeLa cells

Compound	IC_{50} (μg/ml) for:			
	E. coli		HeLa DNA polymerase α	Cytotoxicity in HeLa cells
	DNA gyrase	RNA polymerase		
Cyclothialidine	0.03	>1,000	>1,000	>800
Novobiocin	0.06	440	320	46
Coumermycin A1	0.06	18	39	—[a]
Ofloxacin	1.1	>1,000	545	150

[a] —, not tested.

DISCUSSION

Cyclothialidine, with its novel structure, was characterized to be one of the most potent inhibitors of DNA gyrase. The compound inhibited *E. coli* DNA gyrase with an IC_{50} of 0.03 μg/ml (Table 1). This potency was twofold greater than that of the B subunit inhibitors novobiocin and coumermycin A1.

The other feature was its high selectivity for DNA gyrase. Since DNA gyrase is a DNA topoisomerase, calf thymus DNA topoisomerases I and II were used for the selectivity evaluation. Among the six compounds evaluated, cyclothialidine showed the least activity toward mammalian DNA topoisomerase II, with an IC_{50} of 1,900 μg/ml (Table 3). Against DNA topoisomerase I from calf thymus, however, ofloxacin was less active than cyclothialidine. This highly selective activity of cyclothialidine was confirmed by the evaluation with two nucleic acid polymerases, *E. coli* RNA polymerase and HeLa DNA polymerase α. Cyclothialidine did not inhibit these two enzymes by 50% when it was used up to a concentration of 1,000 μg/ml.

The *oriC* DNA replication assay provided a more precise evaluation system for DNA gyrase inhibitors, since DNA gyrase is known to play key roles in DNA replication. Cyclothialidine retained its high level of activity, being more active than ofloxacin and novobiocin. Therefore, DNA replication was likely inhibited by cyclothialidine as a result of its DNA gyrase inhibition. However, the activity of cyclothialidine in the replication assay was weaker than expected from its anti-DNA gyrase activity, which was more than 30 times greater than the activities of quinolones, which inhibit the A subunit. A similar disparity in activity was observed with the B subunit inhibitors but not with the A subunit inhibitor ofloxacin. Although the inhibitory activities

TABLE 3. In vitro activity against mammalian DNA topoisomerases

Compound	IC_{50} (μg/ml) for:			Selectivity ratio	
	Calf thymus topoisomerase		*E. coli* D110 DNA gyrase	Topoisomerase I/gyrase	Topoisomerase II/gyrase
	Topoisomerase I	Topoisomerase II			
Cyclothialidine	1,700	1,900	0.03	57,000	63,000
Novobiocin	760	400	0.06	13,000	6,700
Coumermycin A1	39	75	0.06	700	1,250
Ofloxacin	3,600	800	1.1	3,300	730
Norfloxacin	750	520	0.66	1,100	780
Ciprofloxacin	1,000	65	0.88	1,100	70
Adriamycin[a]	—[b]	0.65	—	—	—

[a] Positive control for topoisomerase II.
[b] —, not tested.

of cyclothialidine and coumarin-type compounds against DNA gyrase were affected by ATP (data not shown) (21), this disparity cannot be attributed to the effect of ATP, since the ATP concentration in the replication assay (2 mM) was lower than that in the supercoiling assay (5 mM). The results strongly suggest that cyclothialidine is different from the quinolones, including its mode of action. This was supported by our finding that cyclothialidine did not produce the DNA gyrase-DNA-cleavable complex as quinolones do (Fig. 2).

Against whole cells of bacteria, cyclothialidine was active only against *Eubacterium* spp. In view of the report that *Eubacterium* spp. are known to be highly susceptible to antibiotics (34), together with the result of the present study that its DNA gyrase is strongly inhibited by cyclothialidine, it would be reasonable to speculate that *Eubacterium* spp. have a lower penetration barrier, and their growth is thereby inhibited by small molecules such as cyclothialidine. The fact that the compound did not exhibit activity against the outer membrane mutant *E. coli* DC2 (24), in which alteration of its outer membrane structure confers a high degree of susceptibility to antibacterial agents, suggests that it penetrates poorly through the cytoplasmic membrane of *E. coli*.

The cytotoxicity of cyclothialidine was weaker than those of novobiocin and ofloxacin. However, this apparently higher level of safety of cyclothialidine with regard to cytotoxicity could be explained in two ways: (i) cyclothialidine is extremely selective, showing no cytotoxicity, or (ii) cyclothialidine merely does not enter the cells. However, more evidence is required to draw a firm conclusion on the cytotoxicity of cyclothialidine.

All the results obtained so far indicate that cyclothialidine is a promising lead compound for chemical modification aiming at a new class of antibacterial agents (16).

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