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Biological effects of tolytoxin (6-hydroxy-7-O-methyl-scytophycin b), a potent bioactive metabolite from cyanobacteria

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Abstract. Tolytoxin, a macrocyclic lactone, is a potent antifungal antibiotic, exhibiting MICs in the range of 0.25 to 8 nanomolar. Tolytoxin also inhibits the growth of a variety of mammalian cells at similar doses, without specific inhibition of macromolecular synthesis. The effects in mammalian cells are primarily cytostatic, with cell death being time- and dose-dependent. Tolytoxin is highly toxic to mice, exhibiting an LD_{50} (ip) of 1.5 mg/kg. No antibacterial, antiviral, or hemolytic activities were observed.

Key words: Cyanobacteria – Secondary metabolite – Antifungal – Cytotoxin

Cyanobacteria have been shown to produce a variety of toxic and antibiotic secondary metabolites (for review see Glombitza and Koch 1989), some of which have potential for use as therapeutic or agrochemical agents.

Tolytoxin, a member of the family of macrocyclic lactones known collectively as scytophycins (Moore et al. 1986; Ishibashi et al. 1986), was originally isolated in 1977 (Moore 1981). Its chemical structure (Fig. 1) was fully elucidated only recently (Carmeli et al. 1990). Tolytoxin is produced by species of blue-green algae (cyanobacteria) belonging to the closely related genera *Scytonema* and *Tolypothrix* (Nostocales, Scytonemataceae).

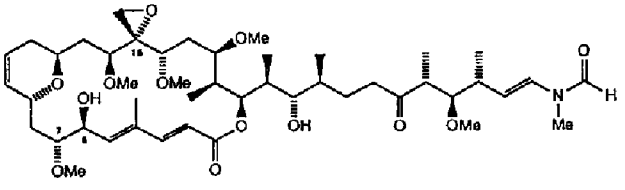

Fig. 1. Structural formula of tolytoxin

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Crude extracts of the tolytoxin-producing organisms have been shown to have good activity against P-388 lymphocytic leukemia in vivo (Moore 1981) and potent antifungal activity (Carmeli et al. 1990).

In the present report, we describe the in vitro antimicrobial spectrum, cytotoxicity to animal cells, and in vivo toxicity to mice of tolytoxin.

Materials and methods

Isolation of tolytoxin

Tolytoxin was prepared from lyophilized cells of *Scytonema ocellatum* Lyngbye ex Bornet and Flahault (UH isolate FF-66-3) as previously described (Carmeli et al. 1990). While small amounts of tolytoxin can be recovered from culture medium, most of the tolytoxin is retained with the cells.

Agar dilution assay

Antimicrobial activity was determined by the agar dilution method (Shadomy et al. 1985). Tolytoxin was dissolved in 95% ethanol for addition to cooled, molten agar. Nystatin (Sigma, St. Louis, Mo., USA) was solubilized in DMSO and diluted in distilled water. Appropriate solvent controls were also prepared. Bacterial cultures were grown on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich., USA); fungal cultures were grown on Saboraud dextrose agar. Assay plates were incubated for 48 h post-inoculation. *Candida albicans* and all bacteria were incubated at 37 °C. All other yeasts and filamentous fungi were incubated at 25 °C. MICs were estimated visually as the lowest concentration of drug causing no growth or significantly reduced growth relative to the controls.

Erythrocyte lysis

Hemolytic action was assayed using fresh blood drawn from adult Sprague-Dawley rats. The assay was performed by suspending washed blood cells in phosphate-buffered saline (123 mM NaCl, 16.4 mM Na_2HPO_4, 3.7 mM NaH_2PO_4, pH 7.4) in the presence of test agent or solvent controls. The mixture was incubated at 37 °C for 4 h. Hemolysis was estimated by pelleting cells at 1,000 × g for 3 min and measuring absorbance of the supernatant solution at 405 nm.

Cell cultures and cytotoxicity assay

Cell lines were grown in plastic dishes (Falcon, Oxnard, Calif., USA) in Eagle's basal medium supplemented with 5% newborn calf serum (KB), Fisher's medium supplemented with 10% horse serum (L1210), Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal calf serum (LoVo), minimal essential medium supplemented with 2% heat-inactivated chicken serum (HEp-2), RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (T-47D, COLO-201) or 20% heat-inactivated fetal calf serum (HL-60, KATO-III), or McCoy's 5A supplemented with 10% heat-inactivated fetal calf serum (HBL-100). All cell culture media were supplemented with 50 μg/ml gentamycin sulfate. The cultures were incubated at 37 °C in a humidified atmosphere containing 5% carbon dioxide.

Cytotoxicity was determined by a growth inhibition assay. Cultures were exposed to graded concentrations of drug and reincubated for 72 h in the presence of the drug. Three dishes were used for each treatment. Cell numbers were determined by hemacytometer counts. IC_{50} was determined by plotting the logarithm of the drug concentration versus growth (cell number as a percentage of the control) of the treated cells (Tsuruo et al. 1979).

Macromolecular synthesis

Actively growing KB cells were seeded at 4×10^5 cells/well in 1.6 cm wells (24 well plates, Falcon) containing 2 ml media. 24 h after seeding, radiolabel ([methyl-^{3}H]-thymidine (64 Ci/mmol, ICN 24060), [5,6-^{3}H]-uridine (47 Ci/mmol, ICN 24046), or [U-^{14}C]-amino acids (50 mCi/mmol, ICN 10146) and test agent (1, 5, or 10 nM tolytoxin or vehicle control) were added to three replicate wells for each treatment. Radioisotopes were obtained from ICN. Cultures were incubated for periods ranging from 2.5 to 10 h. Following incubation, the supernatant medium was removed and the cell layer fixed with methanol. After removing the methanol, the air-dried cell layer was washed three times with ice-cold 10% trichloroacetic acid.

After a final methanol wash, the cell layer was solubilized by addition of 0.5 ml of 0.3 N NaOH containing 1% sodium dodecyl sulfate to each well. The solubilized cell layer was transferred to scintillant and radioactivity determined in a liquid scintillation counter (Freshney 1987).

Cell survival

Cell survival at various times after drug treatment was determined by a colony forming assay. KB cells (2×10^5 cells per dish) were treated with tolytoxin as described for cytotoxicity assays. After treatment, the cells were trypsinized and diluted in fresh medium. Aliquots of the treated cell suspension were added to 15 ml of fresh medium in 100-mm plates (Falcon). After 10 days incubation, colonies were stained with crystal violet. Colonies containing more than 50 cells were counted. The following calculations were made:

$$\text{Plating efficiency} = \frac{\text{number of colonies counted/dish}}{\text{number of cells seeded/dish}}$$

$$\text{Surviving fraction} = \frac{\text{PE of treated cells}}{\text{PE of control cells}}$$

Antiviral assay

Monolayers of mink lung cells (ATCC CCL 64) were cultured for periods of 1 to 3 days in 24-well plates (Costar, Cambridge, Mass., USA), then infected with approximately 100 $TCID_{50}$ of *Herpes simplex* virus (type II). Following incubation for 1 h at 37 °C, 1 ml of maintenance medium (minimal essential medium with Earle's salts supplemented with 2% heat-inactivated fetal calf serum) containing tolytoxin or vehicle, was added to the cultures. Assays were performed in duplicate. The cultures were observed daily for 3 days. Cytopathic effects and cytotoxicity were estimated visually.

Toxicity

LD_{50} was determined using 19 to 21 g random sex Swiss Webster mice. Mice (4 per group) were injected by the intraperitoneal route with the appropriate 2-fold dilution of drug, prepared by dissolving tolytoxin in ethanol and diluting the ethanol solution into a solution of 1% (v/v) Tween-20 in phosphate-buffered saline. The injection volume was 0.2 ml/mouse. Control animals were given an equal volume of solvent. The mice were observed daily for 7 days. Deaths in each group were recorded daily. The LD_{50} was calculated according to the method of Reed and Muench (1938). Blood was taken from the orbit and collected in capillary tubes for hematocrit determination and preparation of plasma. Plasma hemoglobin was determined by a modification of the method of Lijana and Williams (1979) and Standefer and Vanderjagt (1977).

Results

Antimicrobial evaluation

Tolytoxin did not show inhibitory activity against *Proteus vulgaris* ATCC 13315, *Klebsiella pneumoniae* ATCC 13883, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Clostridium sporogenes* ATCC 3584, *Bacillus subtilis*, *Neisseria mucosa*, or *Mycobacterium smegmatis* at doses of 1 μM. No specific antiviral activity was observed against *Herpes simplex* virus, at doses of up to 8 nM. Higher doses resulted in death of the host cells.

In contrast, tolytoxin exhibited potent inhibitory activity against yeasts and filamentous fungi. The in vitro MICs of tolytoxin and nystatin obtained against a panel of 2 yeasts and 12 filamentous fungi are listed in Table 1. The inhibitory spectrum did not appear to be limited to any particular group of fungi. Tolytoxin was inhibitory

Table 1. Antifungal activity of tolytoxin. Each value represents at least three replicate experiments

Organism	MIC (nM)	
	Tolytoxin	Nystatin
Alternaria alternata 1715	4	0.5
Aspergillus oryzae	0.5	0.5
Bipolaris incurvata 2118	2	0.25
Calonectria critalarae 1809	2	0.25
Candida albicans A26	8	1
Colletotrichum coccodes 1809	4	0.25
Penicillium notatum	0.25	0.125
Phyllosticta capitalensis 689-5	0.5	0.125
Phytophtora nicotianae H729	4	1
Rhizoctonia solani 1165	0.25	0.0625
Saccharomyces cerevisiae	4	1
Sclerotium rofsii 2133	1	0.125
Thielaviopsis paradoxa 1215	1	0.5
Trichophyton mentagrophytes A23	8	0.5

Table 2. IC_{50} values for cell cultures. Values were determined as described in 'Materials and methods'

Cell line	ATCC number	Tumor type	IC_{50} (nM)
L1210	CCl 219	Murine leukemia	3.9
LoVo	CCL 229	Human adenocarcinoma	8.4
KB	CCL 17	Human epidermoid carcinoma	5.3
HEp-2	CCL 23	Human epidermoid carcinoma	2.3
HL-60	CCL 240	Human promyelocytic leukemia	4.8
HBL-100	HTB 124	Human breast	2.4
T47-D	HTB 133	Human ductal carcinoma	4.9
COLO-201	CCL 224	Human colon adenocarcinoma	0.52
KATO-III	HTB 103	Human gastric carcinoma	0.78

at doses ranging from 0.25 to 8 nM, equal to or slightly less active than nystatin.

Inhibition of mammalian cell growth

Mammalian cells were tested in vitro with concentrations of tolytoxin ranging from 0.5 to 16 nM for a period of 72 h. Table 2 shows the concentrations required for 50% inhibition of cell growth relative to control cultures. Tolytoxin inhibited the proliferation of all cell lines tested. The degree of growth inhibition observed increased directly with drug concentration (Fig. 2).

Cell survival

Effects of tolytoxin on cell survival are indicated in Fig. 3. Both time- and dose-dependent cell kill were observed in KB cells.

Macromolecular synthesis

Macromolecular synthesis was measured in intact KB cells. As shown in Fig. 4, tolytoxin did not significantly inhibit macromolecular synthesis in KB cells at the range of drug concentrations which induced a significant de-

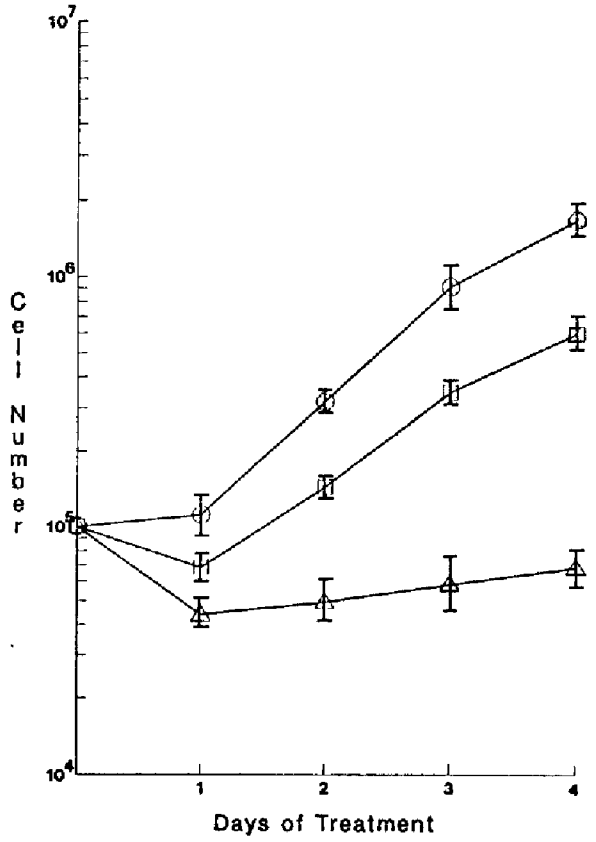

Fig. 2. The effect of tolytoxin on proliferation of KB cells. Each value represents the mean of three replicate experiments and at least two determinations per experiment. *Vertical bars* indicate standard deviations. Each dish contained tolytoxin at the indicated concentration: 0 (○); 4 nM (□); 16 nM (△)

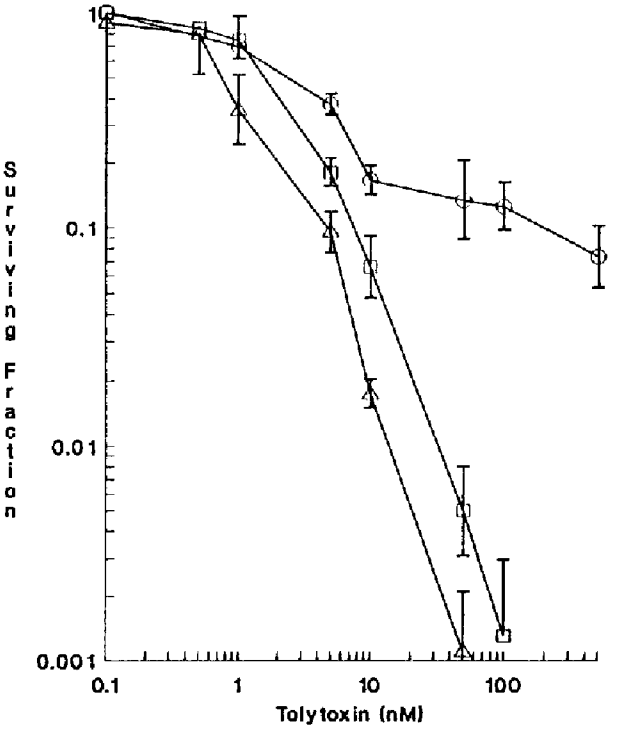

Fig. 3. The effect of tolytoxin on survival of KB cells. Each value represents the mean of three replicate experiments. Standard deviations (indicated by vertical bars) are depicted only when they are larger than the symbols. In some cases, only one half of the range of the standard deviation is shown, to improve clarity. KB cells were treated at the indicated concentrations for 6 h (○); 24 h (□); 48 h (△)

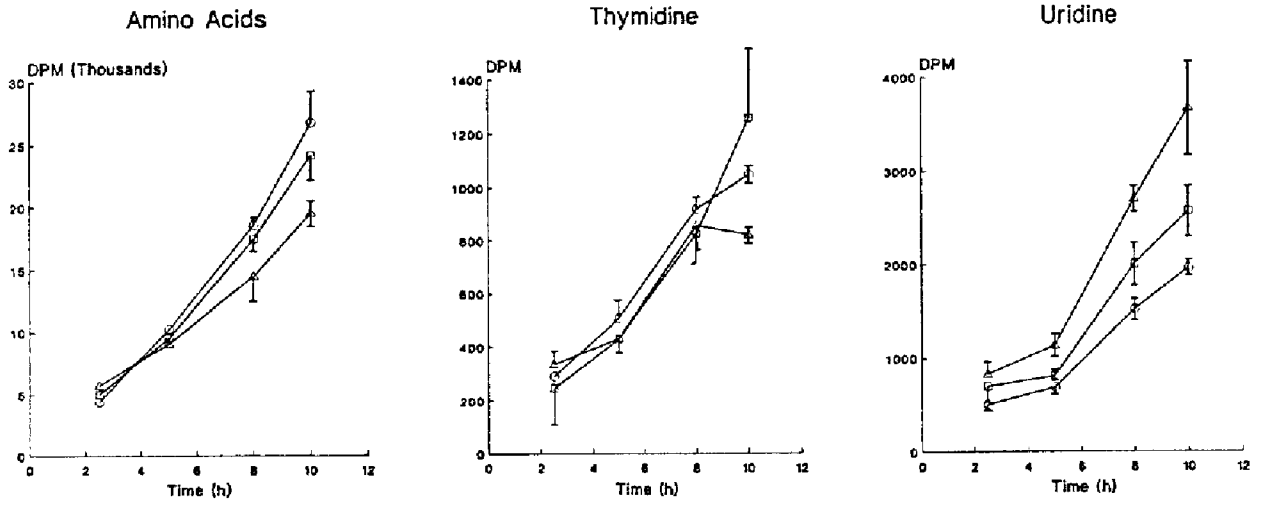

Fig. 4. The effect of tolytoxin on macromolecular synthesis in KB cells. Replicates and standard deviations were as described in the legend to Figure 3. Each well contained tolytoxin at the indicated concentration: 0 (○); 5 nM (□); 10 nM (△)

crease in cell proliferation. Stimulation of RNA synthesis was observed in the presence of tolytoxin.

Erythrocyte lysis

Suspensions of rat erythrocytes in phosphate-buffered saline treated with tolytoxin at concentrations up to 1 μM showed no differences to the untreated control.

Acute toxicity

Tolytoxin was toxic to mice when administered in a single bolus dose by the intraperitoneal route. After administration of the toxin, mice became ataxic and drowsy. Cyanosis and coma followed. Animals receiving fatal doses died within 18 hours. The LD_{50} was determined to be 1.5 mg/kg. Post mortem examination indicated accumulation of fluid in the peritoneal cavity. A concomitant decrease in plasma volume, as estimated by hematocrit, was observed. Hematocrit values increased approximately 10% in moribund animals (data not shown).

Discussion

Tolytoxin is a broad-spectrum antifungal and cytostatic agent, effective at extremely low doses, which has no apparent effect on prokaryotic cells. The cytostatic effects observed at low doses of tolytoxin could be reversed if tolytoxin-treated cells were immediately resuspended in drug-free medium, indicating that tolytoxin does not form covalent linkages with its cellular target.

The possibility that tolytoxin acts through a mechanism similar to that of the polyene antibiotics was examined by testing for erythrocyte lysis, which proved negative both in vitro and in vivo. Little inhibition of macromolecular synthesis was observed during the course of a 10-h incubation. Erythromycin (Taubman et al. 1963) and cycloheximide (Pestka 1971), known specific inhibitors or protein synthesis, suppress protein synthesis within 1 h at cytostatic doses (approximately 1 μM). Since little inhibition of macromolecular synthesis was observed for the first 8 h following exposure to tolytoxin, we conclude that tolytoxin does not specifically inhibit these processes. Inhibition observed after 10 h exposure may be due to a general decline in vigor.

The LD_{50} of tolytoxin administered by intraperitoneal injection was 1.5 mg/kg, indicating high susceptibility to this toxin. Most animals died in coma within hours of receiving a lethal dose. Accumulation of fluid in the peritoneum, accompanied by decrease in plasma volume, suggest that tolytoxin causes vascular damage. Death may be due to shock resulting from the effusion of fluid.

The broad antifungal and cytostatic spectrum, as well as the lack of effect on bacterial growth, lead us to hypothesize that tolytoxin inhibits some fundamental cell process, peculiar to eukaryotes.

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