TERPENTECIN, AN INHIBITOR OF DNA SYNTHESIS

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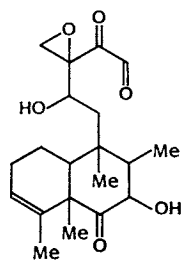
Terpentecin at a concentration of 0.78 μg/ml decreased the number of viable cells of *Escherichia coli* NIHJ to less than one thousandth the starting number in an hour when added to an exponentially growing culture in a nutrient broth. During this time, the turbidity of the cell suspension kept increasing as fast as the control. Microscopic inspection of the cells exposed to terpentecin under these conditions revealed that the cells were elongated. Terpentecin at a concentration of 6.25 μg/ml inhibited incorporation of [^{14}C]thymidine into the acid-insoluble material of cells of *E. coli* NIHJ by 70% in 30 minutes in contrast to little or no inhibition of the incorporation of [^{14}C]uridine or [^{14}C]leucine. Under similar conditions, terpentecin did not inhibit either membrane transport (uptake) of [^{14}C]thymidine into the cells or the metabolic conversion of the precursor into various cellular acid-soluble components. Terpentecin at a higher concentration (70 μg/ml) inhibited by 40% in 30 minutes the incorporation of [*methyl*-^{3}H]thymidine triphosphate into the DNA fraction of toluene-treated cells of *E. coli* JE6296 (*pol A*$^-$). Terpentecin showed stronger antibacterial activities against *Bacillus subtilis* M45T (*rec*$^-$) and *E. coli* BE1121 (*rec A*$^-$) than against their corresponding wild type strains. However, terpentecin showed no mutagenicity by the Ames test with *Salmonella typhimurium* strains TA100, TA98, TA92, TA1538, TA1537 and TA1535, and with *E. coli* WP2 (*uvr A*).

Terpentecin at a lower concentration (0.07 μg/ml) inhibited growth *in vitro* of mouse leukemia L1210 cells by 50%. With the mammalian cells again the incorporation of [^{14}C]-thymidine into the acid-insoluble cell material was inhibited more strongly than incorporation of [^{14}C]uridine and [^{14}C]leucine. There was no sign of mutagenicity by the micronucleus test using mice.

Terpentecin is a new antitumor antibiotic produced by *Kitasatosporia griseola* MF730-N6, a rare actinomycete,[1] and belongs to the diterpenoid group with the clerodane type of structure[2] (Fig. 1). It is active against various Gram-positive and Gram-negative bacteria and shows some antitumor activity, prolonging the life span of mice inoculated with either L1210, P388 or Ehrlich ascites carcinoma. There are few terpenoid antibiotics of actinomycete origin and terpentecin is most closely related in structure and in activities to clerocidin,[3,4] a product of a fungus *Oidiodendron truncatum*. The mechanism of

Fig. 1. Structure of terpentecin.



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action of this group of antibiotics has never been studied.

Materials and Methods

Chemicals

[2-^{14}C]Thymidine (56.6 mCi/mmol), [2-^{14}C]uridine (57 mCi/mmol), L-[*U*-^{14}C]leucine (342 mCi/mmol), 2,6-diamino[G-^{3}H]pimelic acid dihydrochloride (629 mCi/mmol), *N*-[^{14}C]acetyl-D-glucosamine (58.18 mCi/mmol) were the products of Radiochemical Center, Amersham, England, and [*methyl*-^{3}H]-thymidine triphosphate (dTTP) (20 mCi/mmol) was the product of ICN Radiochemicals, California, U.S.A. TCA, dimethyl sulfoxide, toluene, dithiothreitol and tris(hydroxymethyl)aminomethane (Sigma) were of the reagent grade.

Bacterial Strains

Escherichia coli NIHJ, *E. coli* BEM11 (*tol C*, F$^-$, *thr*, *leu*, *pro*, *his*, *thi*, *arg*, *lac*, *gal*, *ara*, *xyl*, *mtl*, *tsx*, *str*, *sup*), *E. coli* BE1121 (*rec A(1)*, *his*$^+$, otherwise the same as BEM11), *E. coli* JE6269 (*pol A1*, *pur E*, *trp*, *lys*, *pro C*, *leu*, *thi*, *lac Z*, *xyl*, *ara*, *ton A*, *tsx*, *str*, *sup*), *Bacillus subtilis* M45T (*rec*$^-$, *arg*$^-$, *try*$^+$) and *B. subtilis* H17A (*rec*$^+$, *arg*$^+$, *try*$^-$) were used. For the Ames test, *Salmonella typhimurium* TA100 (*his G*, *rfa*, *Δuvr B*, +*R*), TA98 (*his D*, otherwise the same as TA100), TA92 (*his G*, +*R*), TA1538 (*his D*, *rfa*, *Δuvr B*), TA1537 (*his C*, otherwise the same as TA1538) and TA1535 (*his G*, otherwise the same as TA1538) and *E. coli* WP2 (*uvr A*, *trp*$^-$) were used.

Determination of Inhibition by Terpentecin of Cell Growth

E. coli NIHJ was shake-cultured at 37°C in nutrient broth. Cell growth was followed by reading the turbidity ($A_{660\,nm}$) of the cultures. At a growth stage of $A_{660\,nm}$ 0.1†, terpentecin (dissolved in DMSO) was added to give required concentrations and $A_{660\,nm}$ of the cultures was followed with time.

Determination of Effect of Terpentecin on Cell-viability (Colony-forming Ability)

E. coli NIHJ was grown as above. At a growth stage of $A_{660\,nm}$ 0.1, a 4.75-ml portion was transferred into an L-shaped culture tube, mixed with 0.25 ml of terpentecin solution (a desired concentration of terpentecin in 5% DMSO), and incubated further for 3 hours. During this time, 0.2 ml samples were withdrawn at 30 minute intervals; 0.1 ml portions of a 10-fold dilution series in water prepared from each sample were spread on nutrient agar plates, incubated at 37°C for 18 hours and the number of colonies was counted.

Inspection of Cell Morphology of *E. coli* NIHJ

From a culture of *E. coli* NIHJ ($A_{660\,nm}$ 0.1), a 990-μl portion was transferred into a small test tube, mixed with 10 μl of a terpentecin solution (prepared by dissolving 0.25 μg terpentecin in 5% DMSO; for the control, 10 μl of 5% DMSO was used) and incubated further at 37°C for 2 hours with shaking. A sample of the cell suspension was smeared on a slide glass, dried, fixed over a flame, stained with gentian violet solution and submitted to microscopic inspection.

Incorporation of Labeled Precursors into Acid-insoluble Cell Material of *E. coli* NIHJ

The procedures were as reported.[5] *E. coli* NIHJ was grown in an L-shaped tube with shaking in a nutrient broth which consisted of 1% (w/v) Polypepton (Daigo-Eiyo Co.), meat extract (Kyokuto Co.) 1.0% (w/v), NaCl 0.2% (w/v), and water to volume. The pH was adjusted to 7.0. At an early exponential growth phase ($A_{660\,nm}$ 0.1), 80 μl portions were transferred into microtubes; each was mixed with 10 μl of terpentecin solution (at an appropriate concentration in 5% DMSO), kept at 37°C for 3 minutes, mixed with a 10-μl solution containing 50 mμCi of either [2-^{14}C]thymidine, [2-^{14}C]-uridine, or L-[*U*-^{14}C]leucine, and kept at 37°C for 30 minutes with occasional shaking. From each of the labeled cell suspensions, a 90-μl portion was taken and placed on a Whatman 3 MM filter disk (2.5 cm diameter), which was processed to determine the radioactivity in acid-insoluble cell material.[6]

† Optical density at 660 nm is 0.1 (1 cm light path).

Preparation of the Acid-soluble and Acid-insoluble Fractions from [^{14}C]Thymidine-labeled Cells

The method of fractionation was as reported[7] with minor modifications. From a culture of *E. coli* NIHJ ($A_{660\,nm}$ 0.1), a 900-μl portion was transferred into a small test tube, mixed with 80 μl of terpentecin solution (prepared by dissolving 10 μg terpentecin in 5% DMSO), incubated at 37°C for 10 minutes with shaking, mixed with 20 μl of [2-^{14}C]thymidine solution (1 μCi), incubated again at 37°C with shaking, and chilled on an ice bath. The labeled cells were collected by centrifugation (8,000×*g*, 10 minutes, at 0°C), washed twice with 1 ml of cold nutrient broth by centrifugation, and suspended in 1 ml of cold nutrient broth. They were then mixed with cold 0.25 ml of 0.5 M sodium phosphate - 0.5% NaCl buffered solution (PBS, pH 7.4) and with cold 0.25 ml of 20% TCA in this order, kept at 0°C for 2 hours and filtered through a Millipore membrane filter (HA 0.45 μm, 2.4 cm diameter). The acid-insoluble material on the filter (referred to as acid-insoluble fraction) was washed with 1 ml of cold PBS, while the washing was combined with the first filtrate (referred to as acid-soluble fraction). The radioactivity in both fractions was determined.

DNA Synthesis in Toluene-treated Cells of *E. coli*

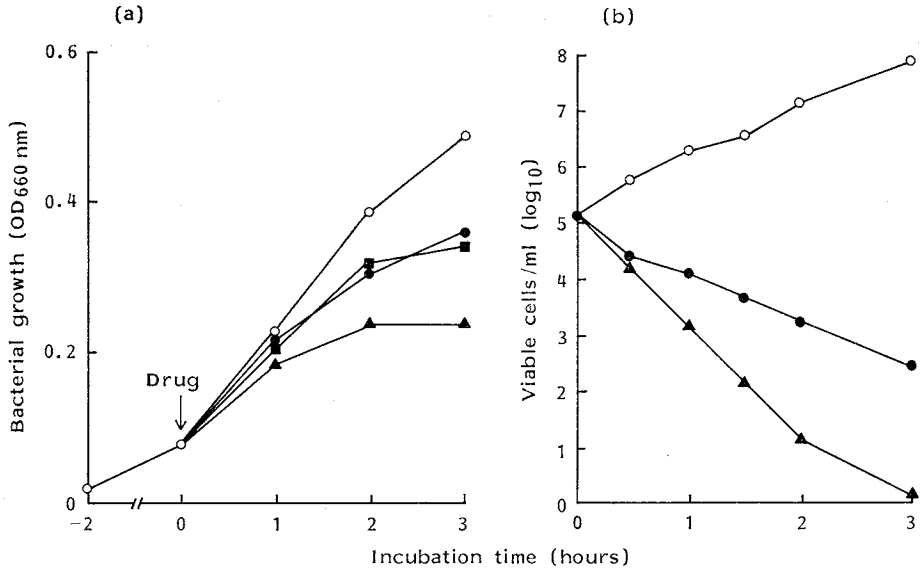
Experiments on replicative DNA synthesis were performed as reported[8] with minor modifications. In brief, growth of *E. coli* JE6269 was terminated at $A_{660\,nm}$ 0.4 by rapid chilling and the cells were collected by centrifugation, treated with 1% toluene, suspended in 50 mM phosphate buffer (pH 7.4) and used for determination of DNA synthesis. An assay mixture contained, in 100 μl, 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 25 μM each of dNTP's except dTTP, 0.5 μCi of [^{3}H]dTTP (20 Ci/mmol), 150 mM KCl, 1.5 mM ATP, a test compound at the desired concentration, 6×10^8 toluene-treated cells (added last), and water to volume. After incubation at 30°C for 30 minutes, a 100-μl portion of a mixture was collected on a Whatman 3 MM filter disc (2.4 cm diameter), washed immediately with cold 5% TCA and washed further with cold 5% TCA and absolute EtOH. After the discs had been dried at room temp for 30 minutes, their radioactivity was determined.

Results and Discussion

In the presence of 0.78 μg/ml of terpentecin, the turbidity of a culture of *E. coli* NIHJ kept increasing at a rate comparable with that of the control for the first hour and reached a plateau 3 hours after addition of the antibiotic (Fig. 2a). There was no sign of cytolysis (indicated by a decrease in turbidity) until 5 hours later. Although the turbidity of the culture kept increasing for some time after addition of terpentecin, the colony-forming ability of the cells was rapidly lost. It decreased to 10% of the control 30 minutes after addition of the antibiotic and kept decreasing exponentially thereafter (Fig. 2b). Microscopic inspection of cells that had been exposed to 0.25 μg/ml terpentecin for 2 hours revealed that they were abnormally elongated (Fig. 3). The morphological alteration seems to explain the inconsistency between the turbidity of the cultures and the cell viability. Elongation of bacterial cells is often caused by antibiotics interfering with either DNA synthesis or cell-wall synthesis.[5,9] The lack of cytolysis suggested that DNA synthesis rather than cell-wall synthesis was inhibited. To confirm this possibility, the effect of terpentecin on incorporation of radioactive precursors of various cellular macromolecules into the acid-insoluble material of cells was determined. As shown in Fig. 4, terpentecin inhibited incorporation of [^{14}C]thymidine (IC_{50} 3.2 μg/ml) but not of [^{14}C]uridine and [^{14}C]leucine. In a separate experiment, incorporation of *N*-[^{14}C]acetylglucosamine and 2,6-[^{3}H]-diaminopimelic acid (both are cell-wall precursors) was not inhibited (data not shown).

Incorporation of exogenous [^{14}C]thymidine into cellular DNA includes the following steps; (1) permeation into the cells, (2) the stepwise phosphorylation to dTTP and (3) polymerization into DNA together with endogenous dXTP's. We asked which of these processes was inhibited by terpentecin. Membrane transport (influx) of thymidine was determined as reported[5] in the presence (2.5 μg/ml)

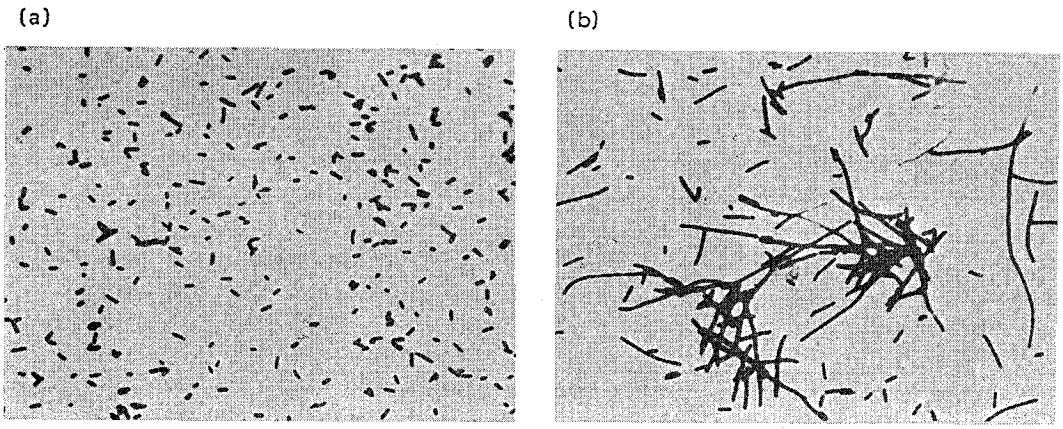
Fig. 2. Cell-growth inhibition by terpentecin of *Escherichia coli* NIHJ.



(a) Effects on turbidity of the cultures. The arrow indicates the time of terpentecin addition. Open circles represent the culture which received no terpentecin (control) while solid symbols represent the ones which received terpentecin at 0.1 μg/ml (●), 0.2 μg/ml (■) and 0.78 μg/ml (▲).

(b) Effect on cell viability (colony-forming ability). Open circles represent the culture which received no terpentecin (control) while solid symbols represent the ones which received terpentecin at 0.1 μg/ml (●) and 0.78 μg/ml (▲).

Fig. 3. Morphological changes of *Escherichia coli* NIHJ induced by terpentecin.
Control (a), and 0.25 μg/ml terpentecin-treated (b).



or absence of terpentecin. Negligible inhibition of this process was observed (data not shown). We next divided the total intracellular radioactivity into acid-soluble and acid-insoluble fractions to know whether the blockade was at the polymerization step or somewhere before. As shown in Table 1, the radioactivity in the acid-soluble fraction from terpentecin-treated cells was rather larger than that from control cells (1,524 vs. 1,347 dpm). In marked contrast were the reverse results with the acid-insoluble fractions (2,376 vs. 8,465 dpm). These strongly suggest that terpentecin inhibits the polymerization step. ATP-dependent DNA synthesis in toluene-treated cells with 4 dXTP's as the sub-

strates is believed to represent replicative DNA synthesis in cells.[8] Terpentecin inhibited this process (Table 2), either with *E. coli* JE6269 (DNA-repair defective) or with *E. coli* NIHJ, although at higher concentrations of the antibiotic than required for inhibition of DNA synthesis in living cells (Fig. 4 and Table 1).

The difference in sensitivity to terpentecin between DNA synthesis in living cells and in toluene-treated cells could be caused by metabolic conversion of terpentecin to a stronger inhibitor of DNA synthesis in living cells. How does terpentecin (or its metabolites) inhibit DNA synthesis? Covalent modification of DNA, such as strand scission, adduct formation, *etc.* are the most likely mechanisms explaining both bactericidal effects and inhibition of DNA synthesis. Antibiotics with these activities generally show stronger antibacterial activities against bacterial strains defective in DNA recombination and/or DNA repair than against proficient strains.[10,11] Antibacterial activities of terpentecin against *rec*$^+$ and *rec*$^-$ strains were compared by the agar dilution method of YOSHIMOTO *et al.*[12] using *B. subtilis* M45T (*rec*$^-$) and H17A (*rec*$^+$) and *E. coli* BE1121 (*rec*$^-$) and BEM11 (*rec*$^+$). In both cases, the *rec*$^-$ strains

Fig. 4. Effects of terpentecin on DNA, RNA and protein biosynthesis in *Escherichia coli* NIHJ.

○ [2-^{14}C]Thymidine, □ [2-^{14}C]uridine, △ L-[*U*-^{14}C]-leucine.

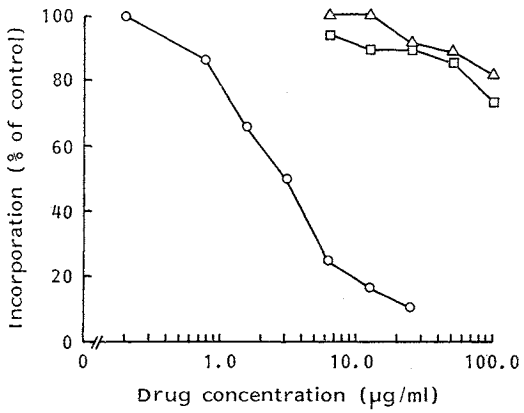


Table 1. Effect of terpentecin on [^{14}C]thymidine incorporation into acid-soluble and -insoluble material of *Escherichia coli* NIHJ.

	Acid-soluble (dpm)	Acid-insoluble (dpm)	Total (dpm)
Control	1,347	8,465	9,812
Terpentecin (10 μg/ml)	1,524	2,376	3,900

Table 2. DNA synthesis in toluene-treated *Escherichia coli*.

E. coli JE6269				
Drug	Conc (μg/ml)	dpm		
		10 minutes	20 minutes	30 minutes
None	—	1,162	1,523	1,825
Terpentecin	70	1,244	1,332	1,084
Ditrisarubicin A[a]	10	NT	68	NT
E. coli NIHJ				
Drug	Conc (μg/ml)	dpm		
		10 minutes	20 minutes	30 minutes
None	—	814	1,429	2,029
Terpentecin	70	553	987	698
Ditrisarubicin A[a]	10	130	NT	NT

[a] Positive control[14].

[^{3}H]Thymidine, when added instead of [^{3}H]dTTP, was poorly incorporated under these conditions indicating the effectiveness of the toluene treatment.

NT: Not tested.

were over 8 times more sensitive to terpentecin than were the corresponding *rec*$^+$ strains.

Possible DNA lesion(s) induced by terpentecin should be repaired at least in part by a recombination mechanism. An interesting finding in this connection was that terpentecin has no mutagenecity, either with strains of *S. typhimurium* sensitive to point mutations (TA100, TA1535) or with ones sensitive to frame-shift mutations (TA98, TA1537, TA1538) in the presence or absence of S9-mixture. Characterization of DNA lesion(s) requires additional studies. Attempts were made to determine direct effect of terpentecin on isolated DNA, if any; these include possible alteration of Tm and/or of the UV absorption profile of DNA, possible strand scission of DNA, and possible effects on characteristic cleavages of DNA with some restriction endonucleases. None was positive, however (data not shown). It is unlikely that the target of terpentecin is DNA polymerase or some other proteins important for DNA synthesis because of the following reason. Inactivation of such proteins, even though irreversible, does not explain the bactericidal effect of terpentecin which was revealed by the colony counting method (Fig. 2b). In this method, the cell suspensions including terpentecin were greatly diluted and plated on the agar medium to allow viable cells to form colonies. The logic is that once the concentrations of terpentecin are greatly reduced, the newly synthesized proteins (DNA polymerase or others) can replace the inactivated proteins and support synthesis of DNA and in turn the cells can start growing to form colonies.

Terpentecin was originally isolated because of its effectiveness in elongating the life span in tumor-inoculated mice. Terpentecin at 0.07 μg/ml inhibited growth *in vitro* of mouse leukemia L1210 cells by 50% in 2 days. With the mammalian cells as with *E. coli*, incorporation of [^{14}C]thymidine in 60 minutes was inhibited more strongly by terpentecin (1.6 μg/ml) than was the incorporation of [^{14}C]-uridine or [^{14}C]leucine; $IC_{40\%}$[†] values for incorporation of thymidine, uridine and leucine into cultured L1210 leukemia cells were 1.6, 7.2 and 28 μg/ml, respectively. The target of terpentecin, therefore, seems to be a structure, most likely the DNA template, which is common to bacterial and mammalian cells. Possible mutagenicity to mammalian cells was also determined by the micronucleus test.[13] Terpentecin dissolved in 5% dimethyl sulfoxide solution was injected intraperitoneally into 7-week-old male ICR mice and 24 hours later marrow specimens were taken from femurs and the number of cells with micronuclei among 1,000 polychromatic erythrocytes of each specimen was counted. The marrow specimens taken from the mice treated with 25 mg/kg and 50 mg/kg terpentecin showed 4 and 6 cells, respectively, with micronuclei as opposed to 3 in the control and 63 in the specimen derived from a mouse treated with 2 mg/kg mitomycin C. Thus here again little or no mutagenicity was apparent. The lack of mutagenicity should be advantageous if terpentecin is used as an antitumor agent.

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† Concentration of a drug required for 40% inhibition.

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