# TOPOSTIN, A NOVEL INHIBITOR OF MAMMALIAN DNA TOPOISOMERASE I FROM *FLEXIBACTER TOPOSTINUS* SP. NOV.

## II. PURIFICATION AND SOME PROPERTIES OF TOPOSTIN

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We describe the isolation of inhibitors of mammalian DNA topoisomerase I, named topostins, from a culture broth of *Flexibacter topostinus* sp. nov. and some properties of the inhibitors. Topostins A1, A2 and B were isolated by differential solubility in solvents, adsorption chromatography on silica gel and gel filtration on a Sephadex LH-20 column. Topostins A1, A2 and B had specific activities of 4,700, 16,000 and 22,000 U/mg, respectively. The most active metabolite topostin B comprised two components with MW of 567 and 553 in an equimolar ratio.

DNA topoisomerases (topos) are enzymes that catalyze the concerted breakage and rejoining of the DNA backbone and are thereby presumed to be involved in various genetic processes.[1~7] Mammalian type I topos catalyze the relaxation of negatively or prositively supercoiled DNA by transiently breaking and resealing one DNA strand so that the linking number changes by steps of one. In order to elucidate the functions of topo I in various aspects of DNA metabolism attempts have been made to find specific inhibitors of topos. Antitumor agents such as epipodophilotoxins, acridins, anthracyclines and ellipticines were found to be specific inhibitors of topo II,[8~12] and the plant alkaloid camptothecin a specific inhibitor of mammalian topo I.[12~15] We have screened secondary metabolites of microbes for inhibitors of mammalian topo I with different action mechanisms and found one, named topostin, among about 2,000 culture broths which strongly inhibited topo I. In the present paper we describe the isolation and some properties of topostins.

## Materials and Methods

### Isolation of Topostins

The bacteria *Flexibacter topostinus* sp. nov. were cultivated as described in the preceding paper for 4 days. Culture broth (60 liters) was harvested and processed as follows. The broth adjusted to pH 3.0 with HCl was precipitated with 4 volumes of acetone. The supernatant was lyophilized, and dissolved in

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10 liters of water. After adjusting pH to 3.0 with HCl the solution was extracted with an equal volume of butanol. The butanol extract was evaporated to dryness. The material was suspended in 9 liters of ethyl acetate (Fraction I) and washed twice with an equal volume of 0.15 M sodium phosphate, pH 7.4 (Fraction II) followed by washing twice with equal volume of the same buffer adjusted to pH 3.0 with HCl. The solvent layer was evaporated. Subsequently the material was suspended in 150 ml of chloroform (Fraction III) and applied on a column (5 × 27 cm bed volume) of silica gel C-200 packed with chloroform. The column was developed stepwise with chloroform mixed with increasing concentrations of methanol of 0, 5, 10, 15, 20, 50 and 100%; each solvent with volume of 1,500 ml. Each fraction was assayed for inhibitory activity of topo I and the specific activity was measured. Active fractions were further analyzed by TLC with a solvent of chloroform - 2-propanol - methanol - ammonia (3 : 3 : 1 : 0.2). Materials on the TLC plate were detected by spraying a color-developing reagent (2.4% $NaMoO_4$, 1.3% phosphoric acid and 5% sulfuric acid) followed by heating at 100°C, 30 minutes. Materials recovered from TLC was purified by gel filtration through a column (5 × 25 cm bed volume) of Sephadex LH-20 equilibrated with a solvent of chloroform - methanol (1 : 1).

Purification and Assay of Topo I

Topo I was purified essentially as described[16] with some modifications. Chromatin was purified from mouse Ehrlich's ascites carcinoma, washed with 50 mM sodium phosphate buffer (containing phenylmethansulfonyl fluoride and Nonidet P-40) and extracted with 0.2 M sodium phosphate buffer. The extract containing topo I was applied to an affinity column of heparin-Sepharose 6B (Pharmacia). The activity was eluted stepwise with a range from 0.5 to 0.7 M NaCl in the elution buffer (containing 10% glycerol). The active fraction was then fractionated with a phenyl-Sepharose 4B (Pharmacia) column. The activity was eluted stepwise from 0.9 to 0.5 M ammonium sulfate in the elution buffer. Enzymatic activity was assayed by relaxation of supercoiled plasmid ColEl DNA. One U of the enzyme is defined as the minimum amount of enzyme giving complete relaxation of 0.2 $\mu$g supercoiled ColEl DNA under reaction conditions. One inhibition unit is defined as the amount of inhibitor giving 50% inhibition of 3 U of the enzyme.[17]

Characterization of Topostins

MW of topostin B was determined by FAB-MS with Jeol JMS-DX 300.

## Results

Approximately 2,000 kinds of microbial culture broth were examined for inhibitory activity of topo I from mouse Ehrlich ascites carcinoma cells. In practice the broth was treated with 4 volumes of acetone to remove macromolecules from the culture fluid, and the supernatant was evaporated to give test samples. As a result one of the samples showed activity, *i.e.* metabolites of a bacterium identified and named *Flexibacter topostinus* sp. nov.

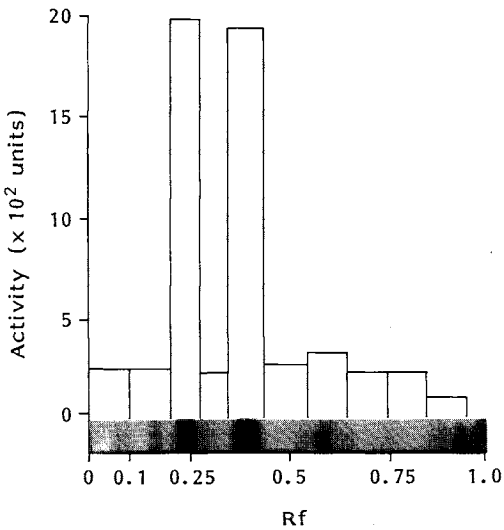
### Isolation of Topostins

Experiments were conducted to establish a simple method for isolating inhibitors in large quantities from the culture broth which showed topo I inhibition. The active fraction isolated at that stage was named topostin. The isolation procedure for topostin is outlined in Materials and Methods. In essence 6,000 ml of No. B-572 broth was precipitated with 4 volumes of acetone to remove macromolecules of various kinds, and the supernatant was lyophilized. The specific activity at this stage was 5 U/mg. This sample dissolved in 10 liters of water and adjusted to a pH value of 3.0 was extracted with butanol to transfer the acidic as well as neutral materials into a solvent layer. In the solvent layer, approximately 70% of the activity was recovered. The butanol extract was evaporated to dryness (Fraction I). The material was suspended in 5 liters of ethyl acetate. Subsequently, the solvent was washed with 0.1 M sodium phosphate

Table 1. Isolation procedure of topostins.

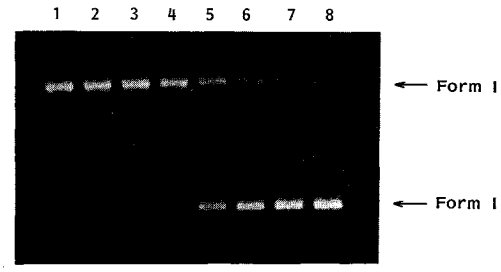
| Fraction | Total activity ($\times 10^3$ U) | Yield (%) | Specific activity (U/mg) |
|---|---|---|---|
| I | 5,000 | 100 | 70 |
| II | 2,800 | 56 | 560 |
| III | 2,700 | 54 | 800 |
| | | ↓ Silica gel column | |
| IV | | | |
| 1. Flow through | 12.5 | 0.25 | 15 |
| 2. 100% $CHCl_3$-1 | 36.0 | 0.72 | 270 |
| 3. 100% $CHCl_3$-2 | 33.0 | 0.66 | 950 |
| 4. 5% MeOH in $CHCl_3$ | 33.0 | 0.66 | 25 |
| 5. 10% MeOH in $CHCl_3$ | 500.0 | 10.00 | 1,500 |
| 6. 15% MeOH in $CHCl_3$ | 500.0 | 10.0 | 3,600 |
| 7. 20% MeOH in $CHCl_3$ | 500.0 | 10.0 | 7,100 |
| 8. 50% MeOH in $CHCl_3$ | 85.0 | 1.70 | 1,200 |
| 9. 100% MeOH | 50.0 | 1.00 | 1,700 |
| Recovery | 1,750 | 35 | |

Fig. 1. TLC profile of topostins in Fraction IV-5 (Table 1).




Fraction IV-5 materials were separated on silica gel thin layer plate with solvent and stained as described in Materials and Methods. The materials distributed along the plate were cut out from the unstained plate, eluted and assayed for topo I inhibition. Rf's are shown in the abscissa and inhibitory activities in units in the ordinate.

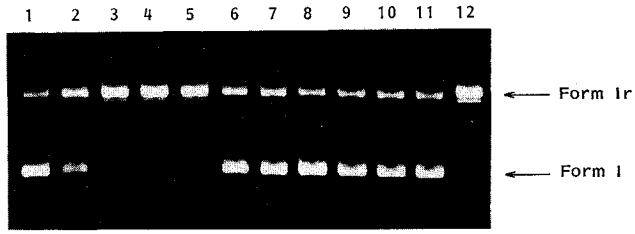
Fig. 2. Inhibition of topo I by topostin B.




Three units of topo I was used for the assay. Topostin B was added to the reaction mixture at 0.2, 0.5, 1.0, 2.0, 3.0 and 4.0 $\mu$M in lanes 2 to 7, respectively, as shown. Lane 1, no drug, lane 8, no enzyme. Form I and Ir indicate the positions of supercoiled form and the relaxed form of the substrate ColE1 DNA, respectively.

buffer (pH 7.5) and neutral and acidic materials were separated into solvent (Fraction II) and aqueous layers, respectively. The solvent layer was washed twice again with 0.15 M sodium phosphate at pH 3.0. As a result, a crude extract (Fraction III) having a total weight of 3,280 mg, total units of $2,700 \times 10^3$, a specific activity of 823 U/mg, and a purification degree of approximately 150-fold was obtained from the solvent layer containing neutral materials which showed a recovery of 55% of the starting activity. Following adsorption chromatography on silica gel, 86% of the activity was recovered in the 10~20% methanol - chloroform eluate fractions (Table 1). TLC of the 10% methanol - chloroform fraction developed as described revealed the activity to be in the Rf values of 0.2 and 0.4 (Fig. 1). After recovery from TLC

Fig. 3. Mechanism of inhibition of topo I by topostin B.




Inhibition pattern was examined changing either the enzyme concentrations (lanes 1 to 5) or the substrate DNA concentrations (lanes 6 to 10). 1.0, 1.5, 2.0, 3.0 and 4.0 U of the enzyme were added to the reactions in lanes 1 to 5, respectively. 0.3, 0.6, 1.0, 1.5 and 2.0 $\mu$g of DNA were added to the reactions in lanes 6 to 10, respectively. All reaction tubes except 11 and 12 contained 2 U (or 6 $\mu$M) of topostin B. Lane 11, DNA only, lane 12, complete system with DNA and enzyme.

with 50% methanol in chloroform and gel filtration on Sephadex LH-20 column, topostin A1 was obtained from Rf 0.2 and topostin B from Rf 0.4 regions, respectively. In the same way topostin A2 (Rf 0.2 in TLC) was isolated from the 15% methanol-chloroform eluate.

### Some Properties of Topostins

Topostins are soluble in methanol, methanol-chloroform (1 : 1) and DMSO, slightly soluble in ethanol, and chloroform and insoluble in ethyl acetate, acetone and water. FAB-MS spectra of topostin B, the most active metabolite exhibited molecular ion peak at $m/z$ 576 and 590 $(M+Na)^+$ in positive ion measurement and $m/z$ 552 and 566 $(M-H)^-$ in negative ion measurement, respectively. As shown in Fig. 2, inhibition of topo I by topostin was evident as the substrate form I remaining increased with an increase in amount of topostin B in the reaction mixture. The $IC_{50}$, concentrations of topostin giving 50% inhibition of 3 U of the enzyme, were 5.4, 1.5 and 1.0 $\mu$g/ml, respectively, for topostins A1, A2 and B. Topostins A1, A2 and B, thus, possessed specific activities of 4,700, 16,000 and 22,000 U/mg, respectively. Purification for topostin B with the highest specific activity was 3,200-fold.

### Mechanism of Inhibition of Topo I by Topostins

An investigation was conducted to see if topostin B inhibited topo I activity by interacting with the enzyme or with the substrate DNA. Changes in inhibition pattern were examined when either the substrate DNA or the enzyme was increased in the reaction mixture containing constant amount of topostin B, as seen in Fig. 3. When the substrate was increased (lanes 6~10), no reversal of inhibition was observed. In contrast, however, when the enzyme was increased (lanes 1~5), the inhibition was released, *i.e.* excess of the enzyme titrated out the inhibitor. Consequently, it was concluded that topostin interacted primarily with the enzyme and not the substrate DNA.

## Discussion

To clarify the relationship between DNA conformation and DNA metabolism we attempted to search for specific inhibitors of mammalian topo I, one of the key enzymes involved in various aspects of DNA metabolism by changing the higher order structure of DNA.[1~7] Topos have recently been found to be the targets of several kinds of antitumor drugs.[8~15] Thus, as a part of an anticancer agents program, we have screened approximately 2,000 microbial culture broth for inhibitor of mammalian topo I. We found inhibitory activity in culture broth of *F. topostinus* sp. nov. Active metabolites were isolated and purified according to the differences in physical properties in partition between solvents and in adsorption to silica

gel column. We have isolated 3 kinds of topostins, A1, A2, and B, with varied specific activities of 4,700, 16,000, and 22,000 U/mg, respectively. MW of topostin B with the highest specific activity was 553 (molecular formula $C_{33}H_{63}NO_5$; data not shown) and 567 (molecular formula $C_{34}H_{65}NO_5$; data not shown). In view of their intermediate polarity they are presumed to penetrate relatively easily through plasma membranes of mammalian cells to exert their biological effects. Topostin B was shown to inhibit catalytic activity by interacting not with DNA, but with enzyme. Although the major *in vivo* target of the topostins is yet to be established, topostins are presumed to be useful for dissection of biological functions of topo I. Furthermore, understanding the mechanism of action of topostins may also be important in the establishment of topo I as a useful therapeutic target for cancer chemotherapy.

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