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Potentiation of the Cytotoxic Activity of Anti-cancer Drugs against Cultured L1210 Cells by *Bacillus thuringiensis* subsp. *israelensis* Toxin

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A 25-kilodaltons (kDa) protein was isolated from the parasporal inclusion produced from *Bacillus thuringiensis* subsp. *israelensis*. This 25-kDa protein inhibited the growth of cultured L1210 murine leukemia cells; IC_{50} of the purified 25-kDa protein was 0.9 μg/ml. This toxic protein interacted with membrane constituent lipids. When the 25-kDa protein was used in combination with anti-cancer drugs, their cytotoxicities against cultured L1210 cells were enhanced. Among the anti-cancer drugs tested, the greatest potentiating effect was found in the case of bleomycin. At a non-toxic dose (0.7 μg/ml), the 25-kDa protein potentiated the bleomycin cytotoxicity 6.4 fold. Under the conditions used, the order of potentiation among the anti-cancer drugs tested was as follows: bleomycin (6.4 fold), tegafur (4.6), vincristine (4.1), 5-fluorouracil (3.7), vinblastine (3.3), methotrexate (2.5), doxorubicin (2.3), triethylene thiophosphoramide (2.2), neocarzinostatin (2.2) and 1-(4-amino-2 methylpyridine-5-yl)methyl-3-(2-chloroethyl)-3-nitrosourea (1.3). In the case of mitomycin C, the 25-kDa protein was not effective.

Keywords——*Bacillus thuringiensis* subsp. *israerensis*; toxin; 25-kDa protein; cytotoxicity; L1210 cell; cultured cell; anti-cancer drug

The bacterium *Bacillus thuringiensis* (BT) produces parasporal proteinaceous crystal toxins (δ-endotoxin) which are highly toxic to larvae of lepidoptera, diptera and coleoptera. BT toxins show insect-specific toxicity which depends on the kind of toxins produced by various subspecies. BT subsp. *israelensis* (BTI) is known to produce a crystal toxin which is effective against dipteran larvae, particularly mosquitoes and black flies.[1] Thomas and Ellar have recently found that BTI toxin has a cytotoxic activity against mammalian cells[2] and has a strong affinity for specific phospholipids of the plasma membrane.[3] Since BTI was discovered, many papers have been published on the isolation and characterization of the toxic principles; however, almost all the studies were based on the insecticidal and hemolytic activities. In this paper, we report the cytotoxic activity of the 25-kilodaltons (kDa) protein of BTI and its potentiating effect on the cytotoxic activity of some clinically used anti-cancer drugs against cultured L1210 murine leukemia cells.

Materials and Methods

Microorganism and Culture Conditions——*Bacillus thuringiensis* subsp. *israelensis* ONR-60A cells were generously provided by Dr. T. Iizuka, Hokkaido University. Cells were inoculated into 5 ml of CSL medium (2% corn steep liquor and 1% glucose in H_2O; the pH was adjusted to 7.0 with aqueous NH_4OH) in an L-tube and aerated by agitation at 30 °C for 6 to 12 h until the OD_{570} of cell suspension became 3 to 4. One milliliter of cell suspension thus obtained was added to 50 ml of CSL medium in a 150 ml flask and aerated by agitation at 30 °C for 90 h. Then the cell suspension was centrifuged at 11000 × *g* for 10 min and the pellet obtained was washed with 50 ml of H_2O. Finally,

recrystallized from methanol to give pale yellow prisms of mp 210—212 °C; 615 mg, 52%. IR: 1725 cm^{-1}. *Anal.* Calcd for $C_{12}H_{14}N_2O_4S$: C, 51.06; H, 5.00; N, 9.95; S, 11.34. Found: C, 50.80; H, 5.01; N, 9.75; S, 11.54.

***N*-Hydroxysuccinimide Ester of *S*-[9,10-Dioxa-*syn*-(methyl, methyl)(methylene, methyl)-bimane]-thioglycolic Acid (2)**——1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (383 mg, 2.0 mmol) was added portionwise to a stirred solution of 564 mg (2.0 mmol) of **1** and 230 mg (2.0 mmol) of *N*-hydroxysuccinimde in 8 ml of anhydrous dimethylformamide (DMF). After stirring overnight at room temperature, the solution was diluted with ethyl acetate, and washed with 10% hydrochloric acid and saturated sodium chloride, then dried over anhydrous sodium sulfate. Removal of solvent left a solid, which was recrystallized from ethyl acetate–acetonitrile to give pale yellow fine needles of mp 176—178 °C; 644 mg, 85%. IR: 1800, 1770, 1740, 1720 cm^{-1}. *Anal.* Calcd for $C_{16}H_{17}N_3O_6S$: C, 50.66; H, 4.52; N, 11.08; S, 8.44. Found: C, 50.42; H, 4.50; N, 11.00; S, 8.27.

***S*-[9,10-Dioxa-*syn*-(methyl, methyl)(methylene, methyl)-bimane]-thioglycolyl-L-tryptophan (3)**——A solution of 76 mg (0.2 mmol) of **2** in 5 ml of acetonitrile was added to a stirred solution of 23 mg (0.22 mmol) of L-tryptophan and 37 mg (0.44 mmol) of sodium bicarbonate in 10 ml of water. Stirring was continued overnight at room temperature, and the solution was concentrated to about 2/3 of the original volume under reduced pressure, diluted with 30 ml of water, and then washed once with ethyl acetate. The aqueous solution was acidified with concentrated hydrochloric acid, then extracted with ethyl acetate. The extract was washed with water, and then dried over anhydrous sodium sulfate. After removal of the solvent, the residue was recrystallized from methanol to give pale yellow fine prisms of mp 215—217 °C; 67 mg, 72%. $[\alpha]_D^{22} = +16°$ ($c = 0.40$, DMF). IR: 1720, 1615 cm^{-1}. *Anal.* Calcd for $C_{23}H_{24}N_4O_5S \cdot 1/2\ H_2O$: C, 57.85; H, 5.28; N, 11.74; S, 6.70. Found: C, 58.11; H, 5.07; N, 11.69; S, 6.52.

***S*-[9,10-Dioxa-*syn*-(methyl, methyl)(methylene, methyl)-bimane]-thioglycolyl-glycyl-L-tryptophan (4)**——Preparation of **4** was carried out as described for **3** from 57 mg (0.22 mmol) of glycyl-L-tryptophan and 76 mg (0.2 mmol) of **2**. **4** was obtained as a pale yellow powder of mp 216—220 °C (dec.) by precipitation from ethanol–ethyl acetate; 53 mg, 50%. $[\alpha]_D^{22} = +7.0°$ ($c = 0.82$, DMF). IR: 1730, 1655 cm^{-1}. *Anal.* Calcd for $C_{25}H_{27}N_5O_6S \cdot C_2H_5OH$: C, 56.73; H, 5.82; N, 12.25; S, 5.60. Found: C, 56.52; H, 5.53; N, 11.99; S, 5.44.

***tert*-Butyl Ester of *S*-[9,10-Dioxa-*syn*-(methyl, methyl)(methylene, methyl)-bimane]-thioglycolyl-glycine (5)**——EDC (43 mg, 0.25 mmol) was added to a stirred solution of **1** (74 mg, 0.25 mmol), glycine *tert*-butylester hydrochloride (42 mg, 0.25 mmol) and triethylamine (25 mg, 0.25 mmol) in 2 ml of anhydrous DMF. Stirring was continued overnight at room temperature, and the solution was diluted with ethyl acetate. The extract was washed with saturated sodium bicarbonate, saturated sodium chloride, 10% citric acid and saturated sodium chloride, and then dried over anhydrous sodium sulfate. Recrystallization from ethyl acetate gave pale yellow leaflets of mp 147—148 °C; 48 mg, 49%. IR: 3380, 1755, 1680 cm^{-1}. *Anal.* Calcd for $C_{18}H_{25}N_3O_5S$: C, 54.67; H, 6.37; N, 10. 63; S, 8.09. Found: C, 54.75; H, 6.37; N, 10.57; S, 7.94.

***S*-[9,10-Dioxa-*syn*-(methyl, methyl) (methylene, methyl)-bimane]-thioglycolyl-glycine (6)**——Compound **5** (40 mg, 0.1 mmol) was treated with 0.5 ml of 5 N hydrogen chloride in dioxane for 2 h at room temperature. Excess reagent and solvent were removed under reduced pressure. The residue was treated twice with methylene chloride. Recrystallization from water afforded pale yellow fine needles of mp 265—268 °C; 27 mg, 79%. IR: 3310, 1720, 1655 cm^{-1}. *Anal.* Calcd for $C_{14}H_{17}N_3O_5S$: C, 49.55; H, 5.05; N, 12.39; S, 9.43. Found: C, 49.82; H, 5.24; N, 12.09; S, 9.15.

***O*-{*S*-[9,10-Dioxa-*syn*-(methyl, methyl)(methylene, methyl)-bimane]-thioglycolyl}-DL-3-indolelactic Acid (7)**——A suspension of 70 mg (0.25 mmol) of **1** in 4 ml of thionyl chloride was stirred for 2 h at room temperature. Excess reagent was removed under reduced pressure at room temperature, and the residue was treated twice with absolute benzene to remove traces of reagent. To a stirred, ice-water-cooled solution of the residual oil in 4 ml of anhydrous tetrahydrofuran, a mixture of 51 mg (0.25 mmol) of DL-3-indolelactic acid and 51 mg (0.5 mmol) of triethylamine in 1 ml of anhydrous tetrahydrofuran was added dropwise. The whole was stirred for a further 1 h at 0 °C then overnight at room temperature. The solution was diluted with ethyl acetate, washed with 10% hydrochloric acid, water and then dried over anhydrous sodium sulfate. Recrystallization from ethyl acetate gave pale yellow fine needles of mp 195—196 °C (dec.); 48 mg, 40%. IR: 3340, 1730 cm^{-1}. *Anal.* Calcd for $C_{23}H_{23}N_3O_6S \cdot 1/2\ H_2O$: C, 57.73; H, 5.06; N, 8.78; S, 6.69. Found: C, 57.72; H, 4.93; N, 8.62; S, 6.89.

Hydrolysis of 3, 4 and 7 by Carboxypeptidase A——a) Linear Relation of the Fluorescence Intensity *vs.* Enzyme Concentration: A solution (10—200 μl) of 2.65×10^{-8}—2.65×10^{-7} M carboxypeptidase A (Sigma Chem. Comp.; activity, 50 units per mg protein) in 10% aqueous lithium chloride was added to a solution of 10 μl of **4** (4.28×10^{-3} M, 41% dimethyl sulfoxide (DMSO)) and 2.0 ml of 0.025 M Tris–HCl buffer containing 0.5 M sodium chloride (pH 7.5) at 25 °C (final concentration of DMSO: 1.9%), and the increase in emission at 483 nm (appearance of **6**) was recorded (excitation at 399 nm). The rate of hydrolysis was established by comparing the rate of increase of fluorescence intensity with the fluorescence intensity of a standard solution of **6**.

b) Kinetic Parameter (K_m and k_{cat}) Measurement: A solution (20 μl) of 2.65×10^{-4} M carboxypeptidase A in 10% aqueous lithium chloride was added to a solution of 10 μl of **3** (0.646×10^{-4}—2.15×10^{-4} M), **4** (0.606×10^{-4}—2.02×10^{-4} M) or **7** (0.676×10^{-4}—2.25×10^{-4} M) in 0.025 M Tris–HCl buffer containing 0.5 M sodium chloride (pH 7.5, final concentration of DMSO, 1.9%) at 25 °C, and measurement was carried out in the same manner as described for a).

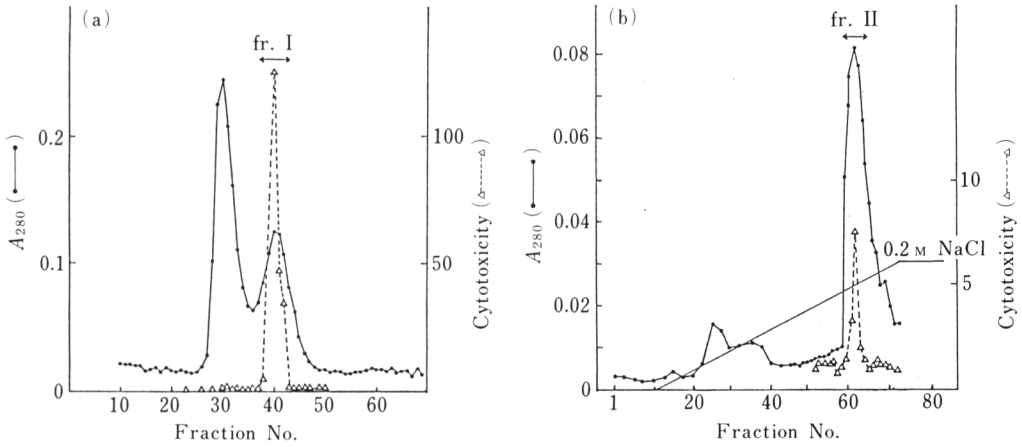

Fig. 1. Purification of Crude BTI Protein by (a) Sephadex G-75 and (b) DEAE-Cellulose Column Chromatography

●—●, absorption at 280 nm; △—△, cytotoxicity. The cytotoxic activity was expressed as the value of [number of untreated control cells]/[number of treated cells].

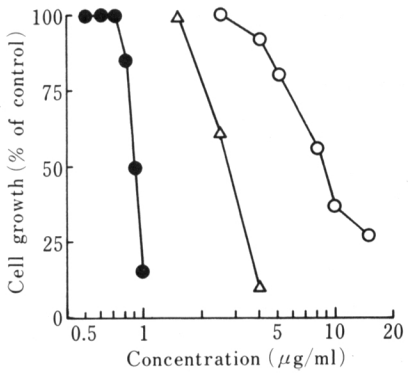

Fig. 2. Cytotoxicity of Crude BTI Protein (○), Fr. I (△) and Fr. II (Purified 25-kDa Protein) (●) against Cultured L1210 Cells

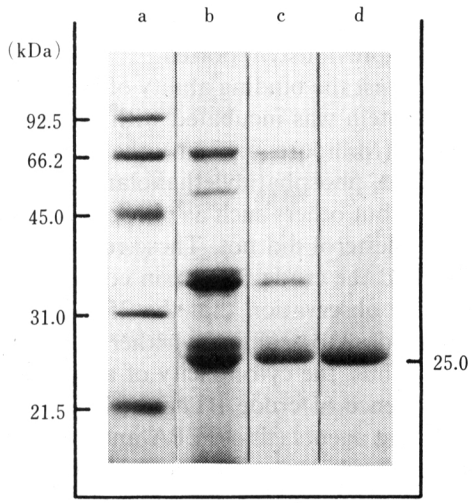

Fig. 3. SDS–PAGE Analysis of BTI Toxin at each of the Separation Steps

Lane: (a) protein standards, (b) crude BTI protein, (c) Fr. I, and (d) Fr. II (purified 25-kDa protein).

TABLE I. Purification and Cytotoxic Activity of 25-kDa Protein

Protein fraction	Total protein recovery (%)	25-kDa protein[a] recovery (%)	Cytotoxicity IC_{50} (μg/ml)	Purification[b] (fold)
Crude protein	100	25.6	6.2	1
Fr. I	51.9	25.0	2.88	2.2
Fr. II	16.9	16.5	0.90	6.9

a) The amount of 25-kDa protein was estimated from SDS–PAGE analysis and is expressed as % of the value of crude protein (assumed to be 100). *b*) Calculated from IC_{50} values.

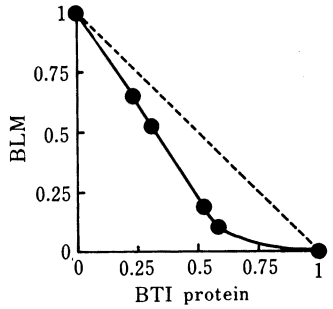

Fig. 4. Isobologram of BLM Combined with Crude BTI Protein

IC_{50}s of BLM and crude BTI protein were 23 μg/ml and 6.2 μg/ml, respectively and each IC_{50} was assumed to be unity. The fractions of doses used in combination resulting in 50% cell growth inhibition were plotted on appropriate axes.

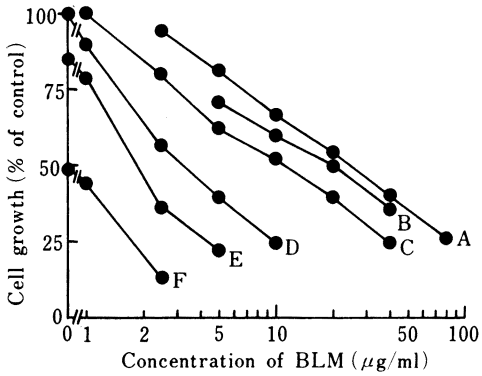

Fig. 5. Dose–Response Curves of BLM against Cultured L1210 Cells Treated with Several Concentrations of Purified 25-kDa Protein

The concentrations (μg/ml) of the purified 25-kDa protein used were A; 0, B; 0.3, C; 0.5, D; 0.7, E; 0.8, and F; 0.9.

protein revealed that its sequence is the same as those of the 25-kDa moiety of 27- and 28-kDa proteins previously reported.[8,9]

To test the binding ability of the 25-kDa protein to membrane constituent lipids, the 25-kDa protein was incubated with a test lipid in H_2O at 37 °C for 2 h. After filtration of the mixture (Millipore, 0.45 μm), the cytotoxicity of the filtrate was examined. Among the lipids examined, phosphatidylethanolamine, phosphatidylcholine and sphingomyelin bound to the protein, but others such as phosphatidylserine, phosphatidylinositol, cerebroside, cardiolipin and cholesterol did not. These results are similar to those reported by Thomas and Ellar,[3] who used the crude BTI toxin containing mainly the 28-kDa protein.

The observation that the 25-kDa protein interacted with membrane constituent lipids prompted us to examine whether the 25-kDa protein affected the cytotoxicity of anti-cancer drugs. Thus, the cytotoxicity of anti-cancer drugs against cultured L1210 cells was tested in the presence of crude BTI protein or purified 25-kDa protein. The drugs tested were two alkylating agents (thio-TEPA and ACNU), three anti-metabolites (5-FU, TGF and MTX), two vinca alkaloids (VLB and VCR) and four antibiotics (MMC, BLM, NCS and ADM). First, the IC_{50} of each anti-cancer drug against cultured L1210 cells was determined and the evaluation of cytotoxicity of an anti-cancer drug in combination with BTI toxin was performed by testing seven concentrations of each test compound. These concentrations, expressed as a percentage of the IC_{50}, were 0, 10, 25, 50, 75, 100 and 150%. Each concentration of an anti-cancer drug was tested in combination with each concentration of BTI protein. The concentrations of an anti-cancer drug and BTI protein in the combination that resulted in 50% growth inhibition of cultured L1210 cells were determined and an isobologram[10] was constructed. The isobologram of BLM combined with crude BTI protein is shown in Fig. 4, where the IC_{50} dose of each compound was assumed to be unity. The line connecting the points for doses in the combinations was below the dotted line, showing that the drug combination was synergistic. Except for MMC, such synergism was also observed with other anti-cancer drugs tested. However, when the same combination experiment was carried out using purified 25-kDa protein, an isobologram similar to that obtained with crude BTI toxin was not observed. This may be caused by the steep dose–response curve of 25-kDa protein compared to those of the anti-cancer drugs. Such a limitation in the usefulness of isobolograms has been reported.[10] Therefore the concentration of purified 25-kDa protein

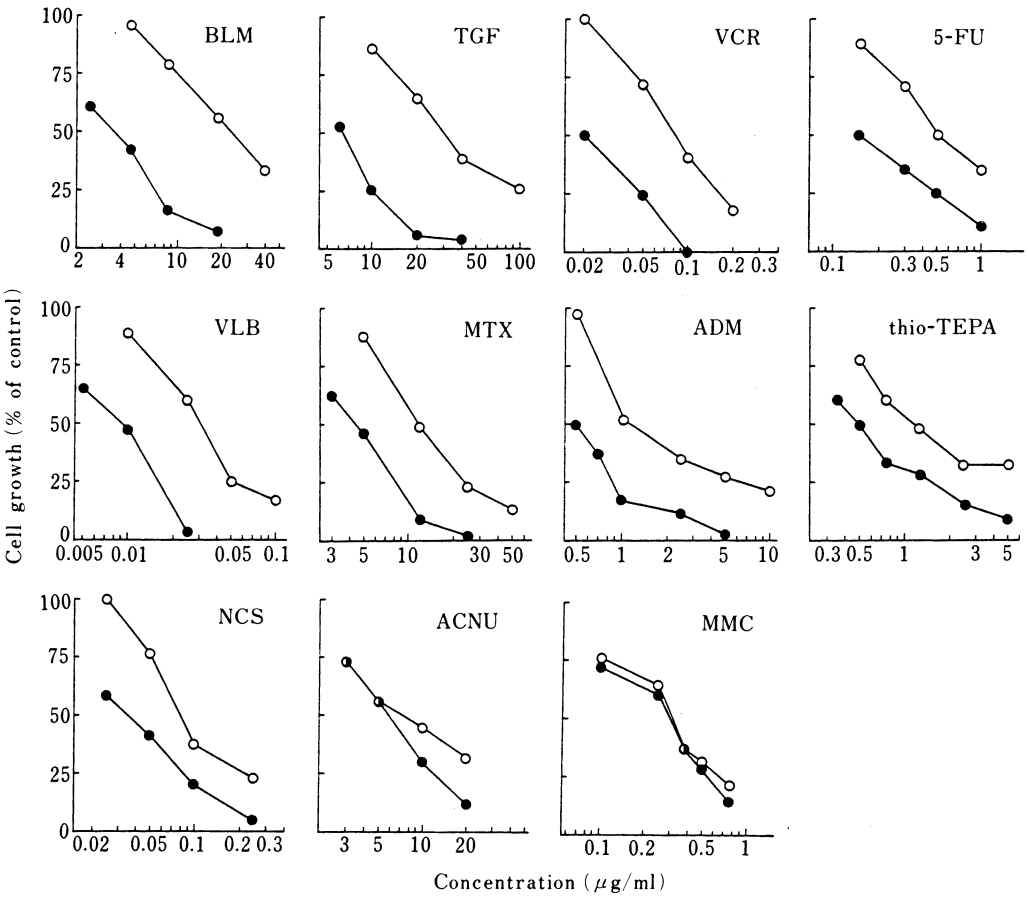

Fig. 6. Effect of BTI-25-kDa Protein on the Cytotoxicity of Anti-cancer Drugs against L1210 Cells

Cells were treated with (●) or without (○) 0.7 μg/ml of purified 25-kDa protein.

that affected the cytotoxicity of anti-cancer drugs was tested in detail using BLM. The concentrations of 25-kDa protein tested were 0.3, 0.5, 0.7, 0.8 and 0.9 μg/ml. Since the dose–response curve of purified 25-kDa protein was so steep, careful preparation and treatment of the solutions of purified 25-kDa protein were required. At doses of 0.3, 0.5 and 0.7 μg/ml, the 25-kDa protein was not toxic by itself but at doses of 0.8 and 0.9 μg/ml, the values of the surviving fraction of cells were about 85 and 50%, respectively (Fig. 5). The results of combination experiments with BLM and purified 25-kDa protein are shown in Fig. 5. At the dose of 0.5 μg/ml, the cytotoxic effect of 25-kDa protein in combination was small, but at doses of 0.7, 0.8, and 0.9 μg/ml, a great enhancing effect was observed. Accordingly the concentration of 0.7 μg/ml of 25-kDa protein, which is not toxic by itself, but showed a great potentiating effect on the cytotoxicity of BLM, was adopted and the combination experiments with other anti-cancer drugs were carried out. Dose–response curves of anti-cancer drugs with or without 0.7 μg/ml of purified 25-kDa protein are shown in Fig. 6, and the values of IC_{50} of each anti-cancer drug with or without 25-kDa protein and the ratios of [IC_{50} of anti-cancer drug] *versus* [IC_{50} of anti-cancer drug with 0.7 μg/ml of 25-kDa protein] are listed in Table II. The greatest potentiating effect was found in the case of BLM (6.4 fold), followed by TGF (4.6), VCR (4.1), 5FU (3.7), VLB (3.3), MTX (2.5), ADM (2.3), thio-TEPA (2.2), NCS (2.2),

TABLE II. Values of IC_{50} of Anti-cancer Drugs with or without 25-kDa Protein

Anti-cancer drug	IC_{50} (μg/ml)[a]		Ratio of IC_{50}[b] (fold)
	Without	With (0.7 μg/ml of 25-kDa protein)	
Bleomycin	23	3.6	6.4
Tegafur	30	6.5	4.6
Vincristine	0.082	0.020	4.1
5-Fluorouracil	0.52	0.14	3.7
Vinblastine	0.030	0.0090	3.3
Methotrexate	11	4.4	2.5
Doxorubicin	1.2	0.52	2.3
Thio-TEPA	1.1	0.50	2.2
Neocarzinostatin	0.078	0.036	2.2
ACNU	7.4	5.9	1.3
Mitomycin C	0.30	0.29	1.0

a) IC_{50} values were determined from the dose–response curves in Fig. 6. *b*) Ratio of [IC_{50} of anti-cancer drug] to [IC_{50} of anti-cancer drug with 25-kDa protein].

ACNU (1.3) and MMC (1.0). In the case of MMC, the 25-kDa protein was not effective. As MMC is an alkylating antibiotic, it might be considered that the 25-kDa protein is not effective in potentiating the cytotoxicity of alkylating anti-cancer drugs, including thio-TEPA and ACNU. The mechanism of potentiation is not clear yet, but the interaction between the 25-kDa protein and cellular membrane lipids may play an important role.

The cytotoxic potentiation of anti-cancer drugs by some other compounds might be an important means to overcome the drug resistance of cancer cells. One of the causes of drug resistance is known to be the efflux of drugs from cells. Recently, several compounds which influence cytotoxic activity of anti-cancer drugs have been reported.[11,12] In this connection, the 25-kDa protein of BTI might be worth investigating further. Studies along this line are in progress.

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