BISUCABERIN, A NEW SIDEROPHORE, SENSITIZING TUMOR CELLS TO MACROPHAGE-MEDIATED CYTOLYSIS

I. TAXONOMY OF THE PRODUCING ORGANISM, ISOLATION AND BIOLOGICAL PROPERTIES

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Alteromonas haloplanktis strain SB-1123 isolated from deep-sea mud produced a new siderophore, bisucaberin. Bisucaberin rendered tumor cells susceptible to cytolysis mediated by murine peritoneal macrophages which were elicited by Proteose peptone and not yet activated by lymphokine. Bisucaberin exerted its sensitizing activity by both the preincubation with tumor cells and the addition to co-culture of macrophages and tumor cells. The activity of bisucaberin was specifically inhibited by ferric ion. Bisucaberin showed direct cytostasis for tumor cells but did not cause cytolysis in the absence of macrophages. Cytostasis by bisucaberin was attributable to the specific inhibition of DNA synthesis in tumor cells.

Macrophages show tumoricidal activity *in vitro* and are regarded as cytotoxic effectors against neoplastic cells *in vivo*.[1~4] Recently, several agents containing antitumor antibiotics have been found to augment cytotoxicity in effector-target systems *in vitro*.[5~8] Therefore, we have screened substances causing cytolysis of tumor cells in co-culture with macrophages which do not lyse tumor cells by themselves. These substances can be candidates for antitumor agents. In this screening system, we found a novel siderophore and designated it bisucaberin. "Siderophore" is a general term for bacterial low MW iron chelators.[9] Physico-chemical properties and structural determination of bisucaberin will be described in a accompanying paper.[10] This report describes identification of the producer and isolation and biological properties of bisucaberin.

Materials and Methods

Materials

Radiolabeled precursors, [*methyl*-^{3}H]thymidine (specific activity, 5 Ci/mmol), [6-^{3}H]uridine (specific activity, 20 Ci/mmol) and L-[4,5-^{3}H(N)]leucine (specific activity, 60 Ci/mmol), were purchased from Amersham Japan Co., Tokyo, Japan. Nocardamine was kindly donated from Dr. W. KELLER-SCHIERLEIN, Eidgenössischen Technishcen Hochschule, Zürich, Switzerland.

Lymphokine was obtained from the culture supernatant of murine spleen cells stimulated with concanavalin A according to the method of BORASCHI *et al.*[11]

The Producing Organism

The bisucaberin producing organism, strain SB-1123, was isolated from deep-sea mud collected at about 3,300 m in depth off the coast of Aomori Prefecture. This mud was kindly donated by Japan Marine Science and Technology Center, Kanagawa, Japan. Identification of this strain was performed

Fig. 1. Isolation procedure for bisucaberin.

Culture filtrate
↓ applied to Diaion HP-20 column chromatography
↓ washed with H_2O
↓ eluted with 50% aq Me_2CO
Active fraction
↓ concentrated and lyophilized
Brownish powder
↓ applied to Diaion CHP-20P column chromatography
↓ eluted with aq Me_2CO gradient (0~20%)
Active fraction
↓ concentrated and lyophilized
Brownish powder
↓ applied to silica gel column chromatography
↓ developed with $CHCl_3$ - MeOH (96 : 4)
Active fraction
↓ concentrated to dryness
White powder
↓ dissolved in hot MeOH
Colorless crystals

according to BERGEY's Manual of Systematic Bacteriology, Vol. 1.[12]

Tumor Cell

Fibrosarcoma 1023, a tumorigenic tumor cell line was kindly donated by Dr. T. TOKUNAGA, National Institute of Health, Tokyo, Japan. This cell line had been induced in a C3H/HeNicr male mouse with 3-methylcholanthrene and has been maintained in culture with DULBECCO's modified EAGLE's medium supplemented with 10% fetal bovine serum (FBS).[13]

L1210 and IMC carcinoma were cultured with EAGLE's minimum essential medium and RPMI 1640 medium, respectively, supplemented with 10% FBS.

Fermentation

Fermentation medium consisted of dried sardine powder 20 g, dried cuttlefish powder 10 g, maltose 5 g and $CaCO_3$ 2 g in 1 liter of the half strength synthetic sea water (Jamarin S, Jamarin Lab., Osaka, Japan). A loopful of slant culture of strain SB-1123 was inoculated into Erlenmeyer flasks (500-ml) containing 100 ml of the fermentation medium and cultured at 27°C for 3 days on a rotary shaker (180 rpm).

Purification

Bisucaberin was isolated from fermentation broth as outlined in Fig. 1. The culture filtrate was applied to Diaion HP-20 column chromatography and the active eluate was obtained with 50% aq Me_2CO. Further purification was carried out by column chromatography on Diaion CHP-20P eluting with a linear gradient of aq Me_2CO (0~20%) followed by column chromatography on silica gel eluting with $CHCl_3$ - MeOH (96 : 4). The active substance thus obtained was crystallized from hot MeOH to yield pure bisucaberin.

Preparation of Murine Peritoneal Macrophages

Murine peritoneal macrophages were used as effector cells in cytolysis assay. Female C3H/HeNCrj mice (Charles River Japan Inc., Kanagawa, Japan) of 8 weeks-old were injected ip with 1.6 ml of 10% Proteose peptone, Difco. Peritoneal cavities were washed twice with 6 ml of Ca^{2+}, Mg^{2+}-free DULBECCO's phosphate buffered saline 3 days later. Peritoneal exudate cells thus obtained were pipetted into 96-well microtiter plates at the density of 2×10^5 cells/well. Non-adherent cells were

washed out with RPMI 1640 medium 2 hours later. RPMI 1640 medium supplemented with 100 U/ml benzylpenicillin, 100 μg/ml streptomycin, 10% FBS and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethansulfonic acid was used as the complete medium unless otherwise noted.

Cytolysis Assay

Fibrosarcoma 1023 was used as target cells in cytolysis assay. Target cell monolayer (3 to 4×10^6 cells/75 cm^2 flask in 15 ml of the complete medium) was prelabeled with 5 μCi of [^{3}H]thymidine for 18 hours. Labeled target cells were harvested by treating with trypsin-EDTA, washed with RPMI 1640 medium and then resuspended in the complete medium to 1×10^5 viable cells/ml. Cell suspension (100 μl) was added to culture wells containing the macrophage monolayer described above. Bisucaberin was added to each culture well and was present throughout the co-culture. Because bisucaberin is insoluble or hardly soluble in water and most organic solvents,[10] it was dissolved in dimethyl sulfoxide followed by dilution with RPMI 1640 medium. Dimethyl sulfoxide itself was inactive under these conditions. Release of incorporated [^{3}H]thymidine from prelabeled tumor cells was measured by liquid scintillation counting 2 days later. Maximum release was estimated by solubilizing with 0.5% sodium dodecyl sulfate. Spontaneous release was estimated by culturing target cells alone and did not exceed 10% of maximum release in the series of experiments reported here. Results were expressed as follows:

$$\%\ \text{specific lysis}=\frac{\text{Experimental release (dpm)}-\text{Spontaneous release (dpm)}}{\text{Maximum release (dpm)}-\text{Spontaneous release (dpm)}}\times100$$

Radiolabeled Precursor Incorporation into Tumor Cells

Exponentially growing tumor cells (2×10^4 cells/microtiter well) were preincubated for 4 hours with bisucaberin in the complete medium. The cells were then pulse-labeled for 1 hour with [^{3}H]-thymidine (2 μCi/ml), [^{3}H]uridine (2 μCi/ml) or [^{3}H]leucine (6 μCi/ml), separately. After washing of each well, tumor cells were harvested and the radioactivity incorporated into acid-insoluble fraction was measured by liquid scintillation counting. Results were expressed as % incorporation against that of un-preincubated cells.

Cell Growth and Viability

Exponentially growing tumor cells (2×10^4 cells/microtiter well) were cultured for 48 hours with bisucaberin. Cell growth was measured by a Coulter counter after dilution with saline.

Cell viability was assessed by exclusion of 0.2% trypan blue.[14]

Results

Identification of the Producer

Strain SB-1123 was a typical marine bacteria requiring a seawater base for growth. It was Gram-negative aerobic rod and motile with polar-flagella. The DNA G+C content was 44.1 mol%. These characteristics were identical to those of the genus *Alteromonas*. Strain SB-1123 did not grow at 4°C. It utilized D-mannose, sucrose, *N*-acetylglucosamine, succinate, fumarate and citrate, and did not utilize erythritol, glycerol, D-sorbitol, DL-malate and α-ketoglutarate. It did not produce soluble pigment in all media tested. These characteristics were identical to those of *Alteromonas haloplanktis*, distinguishing them from other species of *Alteromonas*. Other cultural and physiological characteristics also conformed except for the utilization of aconitate. Therefore, strain SB-1123 was identified as *Alteromonas haloplanktis*. This strain was deposited in Fermentation Research Institute of the Agency of Industrial Science and Technology, Japan with accession number of FERM-P8803.

Fermentation and Purification of Bisucaberin

Strain SB-1123 grew well in the fermentation medium and reached stationary phase after 2 days

culture. Bisucaberin-production was proportional to growth. Two marine products in the fermentation medium, sardine and cuttlefish, were essential to bisucaberin production.

Seven liters of culture filtrate was subjected to the purification process and 2 g of crude crystals was obtained.

Induction of Macrophage-mediated Tumor Cell Lysis by Bisucaberin

The addition of bisucaberin to macrophage-tumor cell co-culture induced strong tumor cell lysis (Fig. 2). Macrophages alone did not lyse tumor cells in the absence of bisucaberin. Induced lysis was dependent on bisucaberin concentration and reached the maximum at 18 μg/ml of bisucaberin. Bisucaberin itself induced little cytolysis of tumor cells without macrophages at this concentration. At higher than 18 μg/ml, macrophage-mediated cytolysis was diminished, while some direct cytotoxicity to tumor cells was observed.

Bisucaberin bound to ferric ion by the addition of $FeCl_3$ and, consequently, turned red and became quite soluble in water. Ferric-bisucaberin induced only slight cytolysis in macrophage-tumor cell co-culture even at 54 μg/ml. Nocardamine, a trihydroxamate siderophore desferrioxamine E, induced no cytolysis in co-culture in spite of structural similarity to bisucaberin.

Dependence of Bisucaberin-induced Cytolysis on E/T Ratio

The effects of varying the ratio of effector to target cells (E/T ratio) was examined. Bisucaberin-induced cytolysis of tumor cells was proportional to E/T ratio of macrophage-tumor cell co-culture and reached the plateau at the ratio of ten or more (Fig. 3). Even at low E/T ratio (1/1), bisucaberin induced macrophage-mediated cytolysis of tumor cells to some extent.

Effects of Treatment Condition on Cytolysis

Macrophages and tumor cells were separately preincubated with 18 μg/ml of bisucaberin and co-cultured with the untreated counterpart cells in the absence of bisucaberin. When ^{3}H-labeled tumor cells were preincubated with bisucaberin for 18 hours, they were strongly lysed by the co-culture

Fig. 2. Induction of macrophage-mediated tumor cell lysis by bisucaberin and related compounds.

Fibrosarcoma 1023 was cultured with macrophages (open symbols) or without macrophages (closed symbols) in the presence of indicated amount of bisucaberin (○, ●), ferric bisucaberin (△) or nocardamine (□).

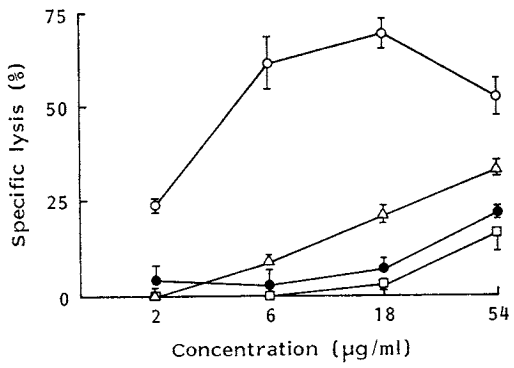

Fig. 3. Dependence of bisucaberin-induced cytolysis on E/T ratio.

Fibrosarcoma 1023 (1×10^4 cells/well) was cultured with macrophage ranging from 1×10^4 to 2×10^5 cells/well in the presence of 18 μg/ml of bisucaberin.

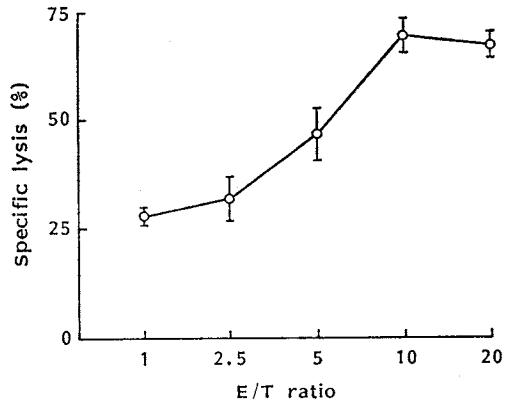

Fig. 4. Effects of treatment conditions on cytolysis.

Cytolysis was measured under the following conditions: (A) Bisucaberin (BSC) or lymphokine (LK) was added to macrophage-tumor cell co-culture at the beginning of co-culture; (B) macrophages were preincubated for 4 hours with BSC or LK, and tumor cells were preincubated for 18 hours with BSC followed by the co-culture with the untreated counterpart cells; (C) BSC or LK was added to macrophage-tumor cell co-culture 1 day after the beginning of co-culture.

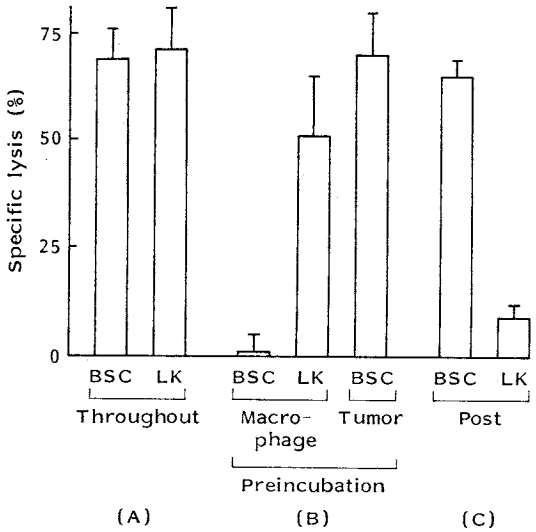

Fig. 5. Inhibition of macromolecular synthesis by bisucaberin.

Fibrosarcoma 1023 cultured with indicated amount of bisucaberin was pulse-labeled with [^{3}H]-thymidine (○), [^{3}H]uridine (□) or [^{3}H]leucine (△).

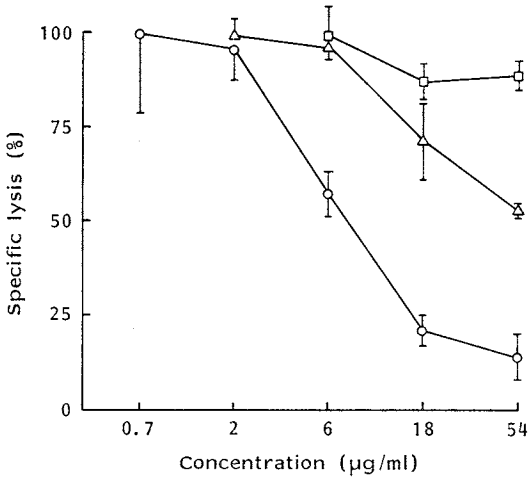

Table 1. Growth inhibition of tumors by bisucaberin.

Bisucaberin (μg/ml)	Inhibition (%)		
	L1210	IMC carcinoma	Fibro-sarcoma 1023
100	85	86	85
25	85	86	85
6.3	69	56	71
1.6	16	14	24
0.4	6	8	11

with macrophages (Fig. 4(B)). On the contrary, preincubation of macrophages with bisucaberin for 4 hours did not induce tumor cell lysis in the co-culture. These macrophages were able to be activated by lymphokine under the same condition. Prolonged preincubation of macrophages was also ineffective. When ^{3}H-labeled tumor target cells were mixed with equal amount of cold tumor cells preincubated with bisucaberin, incorporated radiolable was not released by the co-culture with macrophages (data not shown). The cytolysis was also induced by the addition of bisucaberin 1 day after the beginning of the co-culture (Fig. 4(C)). Macrophages at this stage no longer primed for lymphokine activation.

Effects of Bisucaberin on the Macromolecular Synthesis of Tumor Cells

After 4 hours preincubation of tumor cells with bisucaberin, the incorporation of [^{3}H]thymidine was specifically inhibited in a dose-dependent manner (Fig. 5). The inhibition reached nearly 80% at 18 μg/ml of bisucaberin. Leucine incorporation was also inhibited but its inhibition was lower by about one order of magnitude than that of thymidine. Uridine incorporation was not affected by bisucaberin in concentration ranging from 0.7 to 54 μg/ml.

Cytostatic Effects of Bisucaberin on Tumor Cell Growth

Tumor cell growth for 48 hours was measured in the presence of bisucaberin (Table 1). Bisuca-

berin inhibited the growth of fibrosarcoma 1023. The inhibition reached 85% at 18 μg/ml of bisucaberin. This was also the case of growth of other two tumors, L1210 and IMC carcinoma. IC_{50} of bisucaberin for these tumor cells were 3.9 μg/ml and 5.1 μg/ml, respectively. On the other hand, cell viability was not affected by bisucaberin. More than 98% of fibrosarcoma 1023 was viable with up to 18 μg/ml of bisucaberin. At 54 μg/ml, nonviable cells increased to 10 to 15%.

Discussion

Peptone elicited macrophages used in this study were not tumoricidal unless they had been activated by macrophage activating factors such as lymphokines, lipopolysaccharides, *etc.* Bisucaberin induced strong lysis of tumor cells co-cultured with these nonactivated macrophages. Induced cytolysis was mediated by macrophages because tumor cells cultured with bisucaberin alone remained their viability and did not show significant lysis except at an excess of bisucaberin. High concentration of bisucaberin showed some direct cytotoxicity to tumor cells, but also seemed to be toxic for macrophages to decrease net lysis of tumor cells. Macrophage mediation was supported by the dependence of the cytolysis on E/T ratio.

Bisucaberin exerted its effect by sensitizing tumor cells to lysis by nonactivated macrophages and not by activating macrophages to be tumoricidal: Preincubation of tumor cells with bisucaberin was substitutive for the addition of bisucaberin to macrophage-tumor cell co-culture, but preincubation of macrophages was not. Furthermore, activation of macrophages or stabilization of macrophage-cytotoxic factor by bisucaberin which would leak out of preincubated tumor cells can be ruled out by that no cytolysis was occurred when ^{3}H-labeled and un-preincubated tumor cells were mixed with non-labeled and bisucaberin-preincubated ones and then co-cultured with macrophages. Bisucaberin may substitute for one of tumoricidal mechanisms which are present in activated macrophages but not in nonactivated ones. HIBBS *et al.* reported release of intracellular iron content from tumor cells co-cultured with activated macrophages and consequent inhibition of mitochondrial respiration.[15,16] This may be correlated with the bisucaberin-induced sensitization of tumor cells to macrophage-mediated cytolysis.

That the sensitization of tumor cells to cytolysis is due to iron-chelation by bisucaberin seems apparent because ferric-bisucaberin was inactive. Nocardamine was also inactive although its structure closely resembles that of bisucaberin except for increase of hydroxamate residues from two to three. This suggests that extracellular chelation of iron is not responsible for the induction of cytolysis. BERGERON *et al.* suggested that the rate of transmembrane movement is important to the activity of spermidine catecholamine iron chelators.[17] Nocardamine may move slowly across the cellular membrane because of its larger molecular weight than that of bisucaberin and therefore does not induce cytolysis. This may be the case of ferric-bisucaberin. Binding of ferric ion made bisucaberin quite soluble in water and possibly resulted in the formation of a large molecular complex. These changes may diminish the rate of trans-membrane movement of bisucaberin. The participation of intracellular iron-chelation in sensitizing tumor cells to macrophage-mediated cytolysis needs further investigation.

Bisucaberin seems to be a candidate for antitumor activity because 1) toxicity of bisucaberin for macrophages is relatively low; 2) sensitization of tumor cells by bisucaberin is so effective that a small number of macrophages can lyse these cells; 3) cytolysis can be induced even if bisucaberin is later added to macrophage-tumor cell co-culture. These features lead to an expectation that bisucaberin renders tumors susceptible *in vivo* to killing by tumor-associated macrophages.[18]

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