STUDIES ON ANTINEOPLASTIC ACTIVITY OF NAPHTHOMYCIN, A NAPHTHALENIC ANSAMYCIN, AND ITS MODE OF ACTION

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An antibiotic, identical with naphthomycin, was isolated from a soil *Streptomyces*. The antibiotic displayed significant therapeutic activity by ip administration against murine tumors: Ehrlich carcinoma and IMC carcinoma implanted ip. The maximum increase of life-span was more than 169% in Ehrlich carcinoma, and 128% in IMC carcinoma. The antibiotic exhibited a potent cytotoxicity against murine leukemic cells: P388, L1210, and L5178Y. IC_{50} was $0.4 \sim 1.3$ μg/ml in culture. The activity of naphthomycin was reversed by SH compounds: 2-mercaptoethanol, dithiothreitol, and glutathione. DNA and RNA syntheses were more markedly inhibited by naphthomycin than protein synthesis in L5178Y cells. Approximately 50% inhibition of nucleic acid syntheses was observed at an antibiotic concentration of 2 μg/ml. Naphthomycin blocked alkaline phosphodiesterase obtained from L5178Y cells: IC_{50} was *ca.* 7.6 μg/ml. The antibiotic neither caused metaphase arrest nor prevented tubulin polymerization. The results suggest that the mechanism of cytotoxicity of naphthomycin is the inhibition of various SH enzymes, particularly those involved in nucleic acid biosynthesis. The mode of action is unique in the ansamycin group of antibiotics.

In a screening program for tumor-inhibitory antibiotics, we have isolated an antibiotic, identical with naphthomycin[1~4], from a strain of *Streptomyces*, which was obtained from a soil sample collected in Iwo Jima, Japan. Since naphthomycin was discovered as a vitamin K antagonist[1], and the anti-tumor activity and mode of action have not appeared in the literature, we have studied these and our results are presented in this publication.

Materials and Methods

Naphthomycin

We isolated naphthomycin from culture filtrate of a *Streptomyces*. Antibiotic concentrations in fermentation and extraction samples were assayed by inhibition of [³H]thymidine uptake into the TCA-insoluble fraction of murine T-lymphoma L5178Y cells. The antibiotic obtained was identified as naphthomycin by elemental analysis, UV, IR, FD-MS, ¹H NMR and ¹³C NMR, and by comparison with a sample of naphthomycin[2], which Dr. J. P. Scannell, Research Division, Hoffmann-La Roche Inc., Nutley, New Jersey, generously gave us.

Chemotherapy Studies

The animals used were ICR (Crj: CD-1) female mice, 6 weeks of age, for Ehrlich carcinoma and sarcoma 180; CDF_1 (BALB/cAnNCrj × DBA/2NCrj) female mice, 8 weeks of age, for L1210 and P388 leukemia and IMC carcinoma; and C57BL/6NCrj for Lewis lung carcinoma. Ehrlich carcinoma and sarcoma 180 were maintained by successive ip passage at 1 week intervals in ICR female mice; IMC carcinoma and P388 leukemia in CDF_1 female mice; L1210 leukemia in DBA/2 female mice; and Lewis lung carcinoma by continuous sc passage at 2-week intervals in the axillary region of C57BL/6

female mice.

Ehrlich and IMC carcinomas, and P388 leukemia were transplanted ip with 1×10^6 cells/mouse, and L1210 leukemia with 1×10^5 cells/mouse. Sarcoma 180 was inoculated sc 2×10^6 cells/mouse, and Lewis lung carcinoma sc with 1 mm³ block of solid tumor. Each group consisted of 10 animals in the experiments with solid neoplasms using a control group of 17 mice, and of 6 animals with the ascitic tumors.

Cell Culture

Murine leukemia P388 and L1210 were grown in RPMI1640 medium, supplemented with 10% fetal calf serum, benzylpenicillin 100 units/ml and streptomycin 100 μg/ml, pH 7.2, at 37°C in a humidified atmosphere at 5% CO_2. Mouse lymphoblastoma L5178Y cells were cultured in the above medium, in which fetal calf serum was replaced by horse serum. The cells (2×10^4/ml) were inoculated into the medium with various concentrations of naphthomycin and grown at 37°C for 3 days. The cell number was determined using a Coulter counter.

Incorporation of Radioactive Precursors into TCA-insoluble Fraction of L5178Y Cells

The cells (4×10^5/ml) in the logarithmic phase of growth, in 180 μl, were distributed into wells of Nunclon plates, to which 20 μl of naphthomycin solution and 20 μl of [³H]thymidine, [³H]uridine or [³H]alanine were added. The final concentration of labeled precursors was 2.3 μCi/ml. The reaction mixtures were incubated for 30 minutes at 37°C in a CO_2 incubator. The cells were harvested on glass fiber filters, and washed once with phosphate-buffered saline and twice with 5% TCA (trichloroacetic acid). The radioactivity, collected on glass fiber filters, was determined in a scintillation counter.

Effect on Mitosis

L1210 cells (5×10^4/ml) were grown in 10 ml of RPMI1640 medium, supplemented with 10% fetal calf serum, for 48 hours at 37°C in an atmosphere of 5% CO_2 and 95% air. The antibiotic solution of 0.1 ml was added to the cell culture, and the number of mitotic cells was counted after 2-hour incubation at 37°C.

Alkaline Phosphodiesterase

The enzyme was assayed in a 96-well microplate (Nunc-Immuno Plate I), using thymidine-5′-monophosphate-p-nitrophenylester as a substrate. The reaction mixture, in 200 μl, contained: 50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM the substrate, extract of 1×10^6 L5178Y cells, and various concentrations of naphthomycin, pH 9.0. The cells were ruptured in a Dounce homogenizer with a tightly fitting pestle (type B). The reaction was started by addition of the substrate, and increase in optical density at 405 nm, after incubation at 37°C for 45 minutes, was measured, using ELISA-Analyzer ETY-96 (Oriental Instruments Co., Ltd., Tokyo).

Tubulin Polymerization

Tubulin was prepared from porcine brain by the polymerization-depolymerization method of SHELANSKI et al.[5] Tubulin was mixed with various concentrations of naphthomycin and vincristine, and kept at 0°C for 1 hour in a buffer of 100 mM MES [2-(N-morpholino)ethanesulfonic acid], 1 mM EGTA [ethyleneglycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid], 0.5 mM $MgCl_2$ and 1 mM GTP, pH 6.4. Then the temperature was raised to 37°C, and the turbidity change was observed for 30 minutes by absorbance at 460 nm.

Radioactive Compounds

[Methyl-³H]thymidine (25 Ci/mmol) was purchased from Amersham Japan, Tokyo. [5,6-³H]-Uridine (38.4 Ci/mmol) and L-[3-³H]alanine (72.4 Ci/mmol) were products of New England Nuclear, Boston, Mass.

Results

Chemotherapeutic Activity

Naphthomycin displayed a significant therapeutic activity against Ehrlich ascitic carcinoma in

Table 1. Effect of ip administration of naphthomycin on the life-span of mice inoculated ip with Ehrlich carcinoma.

Dose (mg/kg/day)	Schedule (days)	Mean survival time (days)	T/C (%)	Survivors on 60 days	
Control		15.0± 2.4		0/6	
16	1, 3, 5	22.2± 3.1	148	0/6	$P<0.01$
8	1, 3, 5	>40.3±15.0	>269	2/6	$P<0.01$
4	1, 3, 5	28.3± 4.9	189	0/6	$P<0.01$

Table 2. Effect of ip administration of naphthomycin on the life-span of CDF_1 mice inoculated ip with IMC carcinoma.

Dose (mg/kg/day)	Schedule (days)	Mean survival time (days)	T/C (%)	
Control		12.2±1.9		
8	1, 5, 9	27.8±5.8	228	$P<0.01$
4	1, 5, 9	26.5±6.1	217	$P<0.01$
2	1, 5, 9	19.2±7.4	157	$P<0.05$
1	1~10	16.5±9.6	135	
0.5	1~10	11.7±0.5	96	

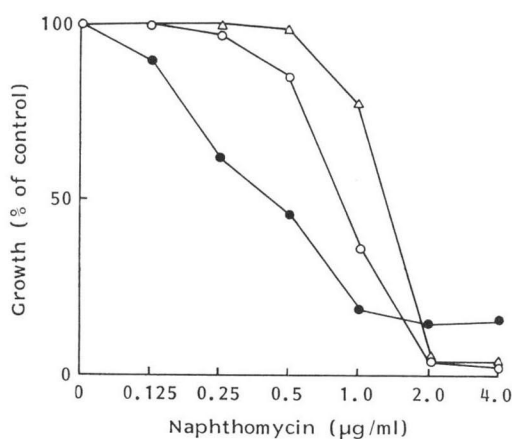Fig. 1. Effect of naphthomycin on growth of L5178Y (○), L1210 (△), and P388 (●) leukemia cells in culture.

Table 3. Effect of sulfhydryl compounds on the growth-inhibitory activity of naphthomycin.

Addition	μM	Growth inhibition (%)
Naphthomycin	1.4	64
+ 2-Mercaptoethanol	10	13
	20	3
+ Dithiothreitol	10	12
	20	2
+ Glutathione	10	26
	20	0

L5178Y cells of 1.0×10^4/ml were inoculated to RPMI1640 medium with 10% horse serum, and naphthomycin was added to the cells at a concentration of 1 μg/ml or 1.4 μM with or without SH compounds. The cell growth was observed by counting the cell number after 68 hours incubation at 37°C. The cell number in control medium was 6.6×10^5/ml.

ICR mice at doses of 4, 8 or 16 mg/kg/day, when administered ip on days 1, 3 and 5 (Table 1). A maximum ILS (increase of life-span) of more than 169% was observed at a dose of 8 mg/kg/day×3 days. Some animals survived as long as 60 days after tumor transplantation. As summarized in Table 2, the antibiotic showed a similar tumor-inhibitory activity against IMC ascitic carcinoma in CDF_1 mice by the ip route. A maximum ILS of 128% was demonstrated at a dose of 8 mg/kg/day × 3 days.

No significant activity was observed in mice, bearing L1210 or P388 leukemia by the ip-ip route over a dose range of 1~8 mg/kg/day×2 or 3 days (data are not shown). Naphthomycin did not inhibit growth of sc solid tumor of sarcoma 180 by the ip injection at a dose range of 2~16 mg/kg/day,

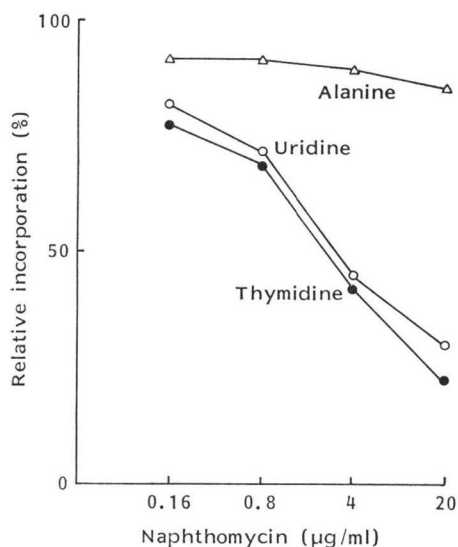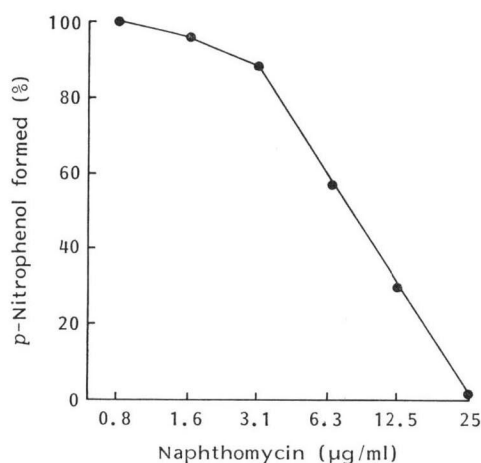Fig. 2. Effect of naphthomycin on macromolecular synthesis in L5178Y cells.

Fig. 3. Effect of naphthomycin on alkaline phosphodiesterase, derived from murine T-lymphoma L5178Y cells.

when given on day 1, 3, 5, 7 and 9; but rather stimulated the growth (data are not shown). The tumor growth and pulmonary metastasis of Lewis lung carcinoma in C57BL/6 mice were not significantly affected by the antibiotic at a dose range of $1 \sim 8$ mg/kg/day, when administered on days 1, 3, 5, 7 and 9 (data are not shown).

Cytotoxicity in Culture

In vitro studies, using murine leukemic cells of P388, L5178Y and L1210, revealed that naphthomycin exhibits a potent cytotoxicity. Approximately 50% growth inhibition was observed at an antibiotic concentration of 0.44 µg/ml with P388 cells, at 0.83 µg/ml with L5178Y and 1.31 µg/ml with L1210 (Fig. 1). As presented in Table 3, the growth-inhibitory activity of naphthomycin was markedly reversed by SH compounds (2-mercaptoethanol, dithiothreitol and glutathione), suggesting an interaction with the SH group.

Effect on Macromolecular Biosynthesis

The effect of naphthomycin on nucleic acid and protein syntheses was examined by incorporation of [³H]thymidine, [³H]uridine and [³H]alanine into TCA-insoluble fraction of L5178Y cells. As illustrated in Fig. 2, the antibiotic prevented the incorporation of thymidine and uridine, but did not significantly affect that of alanine. Approximately 50% inhibition of nucleic acid syntheses was demonstrated at a concentration of 2 µg/ml. The results suggested that naphthomycin inhibits DNA and RNA syntheses more profoundly than protein synthesis.

Effect on Mitosis

Since the structure of naphthomycin is related to that of maytansine[2], an inducer of metaphase arrest, the effect on mitosis of L1210 cells was investigated (see Materials and Methods). Naphthomycin did not significantly affect the number of mitotic cells in a concentration range of $0.25 \sim 8$ µg/ml; whereas Colcemid markedly enhanced the number of mitotic figures in a simultaneous experiment (data are not shown). The cells, treated with 0.2 µg/ml of Colcemid showed 20.5% mitotic figures,

while control cells had 2.2% mitosis. The results suggest that naphthomycin does not cause meta-phase arrest.

Effect on RNA Polymerase Reaction

RNA polymerase reaction was performed at 37°C for 10 minutes by the method of BURGESS[12], using *Escherichia coli* enzyme (Boehringer Mannheim GmbH, West Germany) and calf thymus DNA (Sigma Chemical Co., St. Louis, Mo.). Naphthomycin did not significantly affect the reaction at a concentration range of $0.16 \sim 20$ μg/ml (data are not shown). The reaction mixture contained 0.1 mM dithiothreitol, which inhibited the activity of naphthomycin (see Table 3).

Effect on Alkaline Phosphodiesterase, an SH Enzyme

We studied the effect of naphthomycin on alkaline phosphodiesterase, derived from L5178Y cells, as an example of SH enzyme[13]. The antibiotic prevented enzymic activity of alkaline phosphodies-terase in a reaction mixture, free of SH compounds (Fig. 3). Approximately 50% inhibition was observed at an antibiotic concentration of 7.6 μg/ml. The results suggest that naphthomycin is able to block SH enzymes.

Effect on Tubulin Polymerization

The effect of naphthomycin on tubulin polymerization was studied using porcine tubulin, that polymerized progressively at 37°C for 30 minutes and reached a maximum in 30 to 60 minutes. Naphthomycin did not significantly prevent tubulin polymerization in a concentration range of $0.7 \sim 23$ μg/ml, whereas vincristine blocked the polymerization at a concentration of $0.9 \sim 3.6$ μg/ml (data are not shown). The results, showing that naphthomycin does not inhibit tubulin polymerization, seem to be in accord with the results that the antibiotic does not induce metaphase arrest.

Discussion

The current studies reveal that naphthomycin, an antibiotic of the naphthalenic ansamycin group, exhibits a significant activity against a certain murine tumors, and the mode of tumoricidal action seems to be the inhibition of various SH enzymes, especially those participating in nucleic acid biosynthesis. However, the studies with DNA and RNA polymerases were difficult, because the reaction media con-tained dithiothreitol, an SH compound, which reversed the activity of naphthomycin.

The activity of naphthomycin is prevented by SH compounds, such as 2-mercaptoethanol, di-thiothreitol and glutathione. The results are in accord with the report by BALERNA *et al.*[1] that the antibiotic reacts with cysteine. They described that naphthomycin is a vitamin K antagonist, and reaction with SH compound such as cysteine may not be connected with antibiotic action but simply lead to inactivation. However, the present studies, particularly those on alkaline phosphodiesterase, suggest that naphthomycin inhibit the activity of SH enzymes by interacting with enzyme SH group. Alkaline phosphodiesterase, derived from L5178Y cells, is an SH enzyme[13].

Rifamycins and related ansamycins act on RNA polymerase and/or reverse transcriptase[5], while maytansine, ansamitocins and other ansamycins interact with tubulin[9~11]. Of the ansamycin group of antibiotics, naphthomycin shows a novel mechanism of action: the inhibition of SH enzymes, par-ticularly those involved in nucleic acid biosynthesis.

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