DYNEMICINS, NEW ANTIBIOTICS WITH THE 1,5-DIYN-3-ENE AND ANTHRAQUINONE SUBUNIT

II. ANTITUMOR ACTIVITY OF DYNEMICIN A AND ITS TRIACETYL DERIVATIVE

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Dynemicin A showed extremely potent *in vitro* cytotoxicity against a variety of murine and human tumor cells. In the experimental animal tumor models implanted ip with P388, L1210 leukemias and B16 melanoma cells, dynemicin A administered ip significantly prolonged life-span of tumor-bearing mice with the wide range of activity. This antibiotic administered iv was also active against iv implanted P388 and L1210 leukemias. In the macromolecule biosynthesis of B16 melanoma cells, dynemicin A inhibited DNA synthesis specifically. The triacetyl derivative exhibited similar *in vitro* and *in vivo* antitumor activities to those of the parent antibiotic.

Dynemicin A is a new antibiotic isolated from the culture broth of *Micromonospora chersina* sp. nov. M956-1 (ATCC 53710), which contains the 1,5-diyn-3-ene and anthraquinone subunit in the molecule. The isolation, chemical characterization, structure and some biological activities of dynemicin A have been reported previously[1~3]. Dynemicin A and its triacetyl derivative have been found to possess broad antimicrobial activity, especially potent against Gram-positive bacteria. On the whole, the latter was 2 to 8 times more active than the former[1].

This paper describes the details of the *in vitro* cytotoxicity against murine and human tumor cells, *in vivo* antitumor activity in experimental animal tumor models and inhibition of macromolecule biosynthesis of dynemicin A and its triacetyl derivative.

Materials and Methods

Drug Preparation

Dynemicin A, triacetyldynemicin A and esperamicin A_1 were prepared at Bristol-Myers Squibb Research Institute according to the procedures described in detail elsewhere[4,5]. For the *in vitro* and *in vivo* tests, they were dissolved in DMSO followed by dilution with sterile 0.9% NaCl or water. Doxorubicin (Kyowa Hakko), mitomycin C (Kyowa Hakko) and vincristine sulfate (Sigma) were used as a reference compound.

Animals

Female CDF_1 (Balb/c × DBA/2, 6-week old) and male BDF_1 (C57BL/6 × DBA/2, 5-week old) mice purchased from Japan SLC Inc. (Hamamatsu) were used for *in vivo* antitumor evaluations. Feed and water were provided *ad libitum* through experiments.

Tumors

In vitro tumor cell lines, B16-F10 (murine melanoma), HCT-116 (human colon carcinoma) and Moser (human colon carcinoma) were obtained from Pharmaceutical Research and Development

Division, Bristol-Myers Squibb Co. (Conn., U.S.A.). P388 (murine leukemia), P388/VCR (a resistant subline of P388 to vincristine), P388/ADM (a resistant subline to doxorubicin), K562 (human myelogenous leukemia) and K562/ADM (a resistant subline to doxorubicin) were kindly provided by Cancer Institute of the Japanese Foundation for Cancer Research (Tokyo). Lymphocytic leukemia P388, lymphoid leukemia L1210 and melanotic melanoma B16 which were used for *in vivo* experiments were generously provided by Kitasato Institute (Tokyo).

In Vitro Cytotoxicity

B16-F10 and Moser cells were grown in EAGLE's minimum essential medium (Nissui), which contains kanamycin (60 μg/ml), supplemented with 10% heat-inactivated fetal calf serum (FCS, Nisseizai) and 2 mM L-glutamine, and HCT-116 cells were grown in McCOY's 5A medium (Gibco) supplemented with 10% FCS, 1.2 mM L-glutamine, essential and non-essential amino acids (Gibco), vitamins (Gibco) and antibiotics (100 μg/ml streptomycin and 100 U/ml benzylpenicillin) at 37°C under humidified atmosphere in a 5% CO_2 incubator. For P388, P388/VCR, P388/ADM, K562 and K562/ADM, RPMI-1640 medium (Nissui) supplemented with 10% FCS, 2 mM L-glutamine and antibiotics (100 μg/ml streptomycin and 100 U/ml benzylpenicillin) was used as a growing medium.

Exponentially growing cells were harvested, counted and suspended in the culture medium at 1.5×10^4 (B16-F10), 2.5×10^4 (Moser), 3.0×10^4 (HCT-116), 1.3×10^4 (P388, P388/VCR and P388/ADM), 8.0×10^4 (K562 and K562/ADM) cells/ml, respectively. After planting into wells of a 96-well or 24-well tissue culture plate with test materials, they were incubated for 72 hours (B16-F10, Moser, HCT-116, P388, P388/VCR and P388/ADM) or 48 hours (K562 and K562/ADM). The cytotoxic activities against B16-F10, HCT-116 and Moser cells were colorimetrically determined at 540 nm after staining viable cells with 0.008% neutral red solution while those against P388 and K562 cell lines were determined by directly counting the number of viable cells in a cell counter (Sysmex).

In Vivo Antitumor Activity

Murine leukemias P388 and L1210 were implanted ip with 10^6 and 10^5 cells per mouse or implanted iv with 5×10^5 and 10^4 cells per mouse, respectively. Murine melanoma B16 was implanted ip with 0.5 ml of 10% tumor brei (on day 0). The graded doses of test materials were administered once a day on days 1 to 3 (Q1D × 3) ip or iv, or on days 1, 5 and 9 (Q4D × 3) ip after tumor implantation. Death or survival of the drug- and vehicle-treated animals was recorded daily during the observation period of 45 days and median survival time (MST) was calculated for each of the test (T) and control (C) groups. A T/C value over 125% is considered significant antitumor effect.

Inhibition of Macromolecule Biosynthesis

B16-F10 cells harvested and washed with the culture medium described above were planted into wells of a 24-well tissue culture plate as 1.0 ml aliquot of 5×10^5 (for DNA and RNA synthesis) or 1×10^5 (for protein synthesis) cells/ml. The plates were pre-incubated for 16 hours at 37°C in a 5% CO_2 incubator. After that, the cells were exposed to test materials for 45 minutes at 37°C and then incubated with 7.4 kBq [*methyl*-^{3}H]thymidine, 7.4 kBq [2-^{14}C]uridine or 29.6 kBq L-[4,5-^{3}H]leucine for 30 minutes. After gently washing with cold 5% TCA solution, the radio-activity incorporated into the acid-insoluble fraction was determined in a liquid scintillation counter (Aloka, LSC-701).

Acute Toxicity in Mice

Acute toxicity of dynemicin A was determined in male *dd*Y mice weighing 20 to 24 g after a single ip administration of graded doses of the antibiotic to groups of 5 to 8 mice. LD_{50} value was calculated according to the method of VAN DER WAERDEN[6] on day 10.

Results

In Vitro Cytotoxicity

Both dynemicin A and its triacetyl derivative demonstrated extremely potent cytotoxicity against

Table 1. *In vitro* cytotoxicity against murine and human tumor cells.

Compound	IC_{50}(ng/ml)							
	B16-F10	P388	P388/VCR	P388/ADM	HCT-116	Moser	K562	K562/ADM
Dynemicin A	4.1	0.021	0.019	0.022	0.28	0.0027	0.024	0.026
Triacetyldynemicin A	2.7	0.021	0.020	0.022	0.18	0.0040	0.019	NT
Esperamicin A_1	1.8	0.052	0.079	0.18	0.30	0.016	0.24	0.49
Doxorubicin	30	16	52	230	130	500	50	7,500
Mitomycin C	730	8.6	9.9	12	41	82	520	3,400
Vincristine sulfate	13	13	95	170	NT	NT	NT	NT

P388/VCR: Vincristine-resistant P388 subline. P388/ADM: Doxorubicin-resistant P388 subline. K562/ADM: Doxorubicin-resistant K562 subline. NT: Not tested.

Table 2. Antitumor activity against P388 leukemia (ip) in mice.

Compound	Dose[a] (mg/kg/day)	MST[b] (day)	T/C (%)	Average weight change on day 4 (g)
Dynemicin A	1.0	9.5	95	−1.3
	0.5	15.5	**155**	−1.0
	0.25	15.0	**150**	−1.3
	0.13	14.0	**140**	−0.5
	0.063	14.0	**140**	+0.8
	0.031	14.0	**140**	+0.3
	0.016	13.5	**135**	+0.8
	0.008	12.0	120	+1.0
	0.004	11.0	110	+0.5
Triacetyldynemicin A	1.0	11.0	110	−0.8
	0.5	13.5	**135**	−1.5
	0.25	13.5	**135**	−1.0
	0.13	12.5	**125**	0.0
	0.063	12.5	**125**	+0.5
	0.031	13.0	**130**	+1.5
Mitomycin C	4.0	22.5	**225**	−1.0
	2.0	15.5	**155**	+0.5
	1.0	14.5	**145**	−0.5
	0.5	15.0	**150**	+0.3
	0.25	14.0	**140**	+0.8
	0.13	11.5	115	+1.0
Vehicle control	—	10.0	—	+2.4

[a] QID × 3, ip.
[b] Median survival time.

Table 3. Antitumor activity against L1210 leukemia (ip) in mice.

Compound	Dose[a] (mg/kg/day)	MST[b] (day)	T/C (%)	Average weight change on day 4 (g)
Dynemicin A	1.0	9.0	113	−1.5
	0.5	9.5	119	−1.0
	0.25	10.0	**125**	0.0
	0.13	12.0	**150**	−0.3
	0.063	10.0	**125**	−0.8
	0.031	10.0	**125**	+0.8
	0.016	10.5	**131**	+1.8
	0.008	8.5	106	+1.8
Mitomycin C	4.0	14.0	**175**	−1.3
	2.0	12.5	**156**	−0.1
	1.0	11.0	**138**	−0.8
	0.5	10.5	**131**	+0.3
	0.25	9.5	119	+0.8
	0.013	9.0	113	+1.5
Vehicle control	—	8.0	—	+1.3

[a] QID × 3, ip.
[b] Median survival time.

murine and human tumor cells, having IC_{50} values ranging from 0.0027 to 4.1 ng/ml, which indicate that they have similar or stronger cytotoxic potential than esperamicin A_1 (Table 1). Against human tumors such as Moser and K562 cells, their activities were 10,000 ~ 60,000 times superior to those of mitomycin C in terms of IC_{50} values. Dynemicin A was interestingly as active against drug-resistant sublines P388/VCR, P388/ADM and K562/ADM as against the corresponding sensitive cells.

In Vivo Antitumor Activity

As shown in Tables 2 and 3, dynemicin A administered ip gave significant chemotherapeutic activity against ip implanted P388 and L1210 leukemias. Although the maximum T/C values of dynemicin A were

Table 4. Antitumor activity against B16 melanoma (ip) in mice.

Compound	Dose[a] (mg/kg/day)	MST[b] (day)	T/C (%)	Average weight change on day 4 (g)
Experiment 1:				
Dynemicin A	1.0	12.5	100	−1.3
	0.5	16.0	**128**	+0.5
	0.25	16.0	**128**	+0.5
	0.13	18.0	**144**	+0.8
	0.063	13.5	108	+1.0
	0.031	15.0	120	+1.0
Mitomycin C	2.0	23.0	**184**	+0.8
	1.0	19.0	**152**	+0.8
	0.5	15.5	124	+1.0
	0.25	14.0	112	+1.5
Vehicle control	—	12.5	—	+1.3
Experiment 2:				
Triacetyl-dynemicin A	1.0	16.5	122	−0.3
	0.5	21.0	**156**	+0.5
	0.25	18.5	**137**	+0.5
	0.13	21.0	**156**	+0.3
	0.063	15.5	115	+1.0
Mitomycin C	2.0	30.0	**222**	+0.5
	1.0	20.5	**152**	+1.0
	0.5	18.0	**133**	0.0
	0.25	15.0	111	+0.8
Vehicle control	—	13.5	—	+1.0

[a] Q4D × 3, ip.
[b] Median survival time.

Table 5. Antitumor activity against iv implanted P388 and L1210 leukemias in mice.

Compound	Dose[a] (mg/kg/day)	MST[b] (day)	T/C (%)	Average weight change on day 4 (g)
P388 leukemia (iv):				
Dynemicin A	1.0	16.0	**178**	−1.3
	0.5	14.0	**156**	−0.8
	0.25	12.0	**133**	0.0
	0.13	10.0	111	+0.3
Doxorubicin	8.0	16.0	**178**	−2.3
	4.0	14.0	**156**	−0.5
	2.0	10.5	117	+0.3
	1.0	9.5	106	+0.5
Vehicle control	—	9.0	—	+0.8
L1210 leukemia (iv):				
Dynemicin A	1.0	7.0	100	−2.0
	0.5	12.0	**171**	−0.3
	0.25	10.0	**143**	−0.8
	0.13	9.5	**136**	−0.5
Doxorubicin	8.0	12.0	**171**	−0.5
	4.0	9.0	**129**	0.0
	2.0	9.0	**129**	+0.3
	1.0	8.0	114	+0.8
Mitomycin C	8.0	8.0	114	−2.0
	4.0	9.0	**129**	−1.5
	2.0	9.0	**129**	−0.5
	1.0	8.0	114	0.0
Vehicle control	—	7.0	—	+0.6

[a] Q1D × 3, iv.
[b] Median survival time.

not high in both leukemias, the wide range of activity was seen and the antitumor activity was approximately 16~32 times more potent than that of mitomycin C in terms of minimum effective dose. Similar anti-P388 leukemia activity was determined for the triacetyl derivative of dynemicin A. Both dynemicin A and its triacetyl derivative were also active against ip implanted B16 melanoma with maximum T/C values of 144~156% (Table 4).

When administered iv, dynemicin A demonstrated quite promising anti-leukemic activities against both iv implanted P388 and L1210 leukemias with maximum T/C values of 178 and 171%, respectively, the potency being approximately 8~16 times stronger than that of doxorubicin (Table 5).

Inhibition of Macromolecule Biosynthesis

Dynemicin A strongly inhibited the incorporation of [*methyl*-^{3}H]thymidine into the acid-insoluble fraction of B16-F10 cells with IC_{50} value of 2.2 ng/ml while the IC_{50} values for the incorporation of [2-^{14}C]uridine and L-[4,5-^{3}H]leucine were 8.5 and 14 μg/ml, respectively. These results indicate that the inhibitory effect of dynemicin A on DNA synthesis was approximately 4,000 and 6,000 times stronger than that on RNA and protein synthesis, respectively.

Acute Toxicity in Mice

When administered ip, dynemicin A demonstrated delayed-type toxicity in non-tumor-bearing mice.

The first death of mice received 10 mg/kg of dynemicin A, which was the highest dose tested, was seen at 3 days post-administration. The LD_{50} value was determined to be 0.58 mg/kg ip. Therefore, the acute toxicity of dynemicin A in mice was approximately 20 times less than that of esperamicin A_1 (LD_{50} value: 0.026 mg/kg ip).

Discussion

Dynemicin A is a novel antitumor antibiotic produced by *M. chersina* sp. nov. M956-1 (ATCC 53710) isolated from a soil sample collected in Gujarat State, India[1]. According to the structural studies[2,3], dynemicin A is described to be an entry in the family of diynene antitumor antibiotics such as esperamicins[5,7] and calicheamicin[8]. Since these antibiotics are receiving increasing attention due to their extremely potent antitumor activity[9,10], the *in vitro* and *in vivo* antitumor activities of dynemicin A were determined in comparison with its triacetyl derivative. Both dynemicin A and the triacetyl derivative achieved extremely potent *in vitro* cytotoxicity against murine and human tumor cell lines representing a variety of histological types. Dynemicin A administered ip also showed significant *in vivo* antitumor activity in mice against ip implanted P388 and L1210 leukemias and B16 melanoma with the wide range of activity. Similar antitumor results were obtained for the triacetyl derivative against both P388 leukemia and B16 melanoma. In the present experiments, one of the important characteristics for dynemicin A is antileukemic activity against iv implanted murine leukemias. Dynemicin A administered iv was 8~16 times more active against iv implanted P388 and L1210 leukemias than doxorubicin. Although dynemicin A gave more potent *in vitro* cytotoxicity than esperamicin A_1 in all cell lines tested except in B16-F10, the acute toxicity in mice was approximately 20 times less than that of esperamicin A_1.

LONG *et al.*[11] revealed in their alkaline elution studies that esperamicins in very low concentrations was capable of producing both single and double strand DNA breaks, and ZEIN *et al.*[12] reported that calicheamicin interacted with double-helical DNA in the minor groove and caused site-specific double-stranded cleavage. Recently, SUGIURA *et al.*[13] described a possible mechanism of dynemicin A for DNA intercalation and cleavage. In the present experiments, dynemicin A specifically inhibited the incorporation of radio-labelled thymidine into the acid-insoluble fraction of B16-F10 cells. This result indicates that dynemicin A is a potent inhibitor of DNA synthesis. The above evidence suggest that dynemicin A has specific and unique interaction with DNA molecule and this may cause strong *in vitro* cytotoxicity and *in vivo* antitumor activity.

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References

1) KONISHI, M.; H. OHKUMA, K. MATSUMOTO, T. TSUNO, H. KAMEI, T. MIYAKI, T. OKI, H. KAWAGUCHI, G. D. VANDUYNE & J. CLARDY: Dynemicin A, a novel antibiotic with the anthraquinone and 1,5-diyn-3-ene subunit. J. Antibiotics 42: 1449~1452, 1989
2) KONISHI, M.; H. OHKUMA, K. MATSUMOTO, T. TSUNO, H. KAMEI, T. MIYAKI, T. OKI, H. KAWAGUCHI & J. CLARDY: Dynemicin A, a novel antibiotic with potent antimicrobial and antitumor activity. Program and Abstracts of the 29th Intersci. Conf. on Antimicrob. Agents Chemother., No. 427, p. 172, Houston, Sept. 17~20, 1989
3) KONISHI, M.; H. OHKUMA, T. TSUNO, T. OKI, G. D. VANDUYNE & J. CLARDY: Crystal and molecular structure of dynemicin A: A novel 1,5-diyn-3-ene antitumor antibiotic. J. Am. Chem. Soc. 112: 3715~3716, 1990
4) KONISHI, M.; H. OHKUMA, K. MATSUMOTO, K. SAITOH, T. MIYAKI, T. OKI & H. KAWAGUCHI: Dynemicins, new antitumor antibiotics with the 1,5-diyn-3-ene and anthraquinone subunit. I. Production, isolation and physico-chemical properties. J. Antibiotics 44: 1300~1305, 1991
5) KONISHI, M.; H. OHKUMA, K. SAITOH, H. KAWAGUCHI, J. GOLIK, G. DUBAY, G. GROENEWOLD, B. KRISHNAN & T. W. DOYLE: Esperamicins, a novel class of potent antitumor antibiotics. I. Physico-chemical data and partial structure. J. Antibiotics 38: 1605~1609, 1985

6) VAN DER WAERDEN, B. L.: Wirksamkeits- und Konzertrationsbestimmung durch Tierversuche. Arch. Exp. Path. Pharmak. 195: 389~412, 1940
7) GOLIK, J.; G. DUBAY, G. GROENEWOLD, H. KAWAGUCHI, M. KONISHI, B. KRISHNAN, H. OHKUMA, K. SAITOH & T. W. DOYLE: Esperamicins, a novel class of potent antitumor antibiotics. 3. Structures of esperamicins A_1, A_2, and A_{1b}. J. Am. Chem. Soc. 109: 3462~3464, 1987
8) LEE, M. D.; T. S. DUNNE, C. C. CHANG, G. A. ELLESTAD, M. M. SIEGEL, G. O. MORTON, W. J. MCGAHREN & D. B. BORDERS: Calichemicins, a novel family of antitumor antibiotics. 2. Chemistry and structure of calichemicin $\gamma 1^I$. J. Am. Chem. Soc. 109: 3466~3468, 1987
9) SCHURIG, J. E.; W. C. ROSE, H. KAMEI, Y. NISHIYAMA, W. T. BRADNER & D. A. STRINGFELLOW: Experimental antitumor activity of BMY-28175 a new fermentation derived antitumor agent. Invest. New Drug 8: 7~15, 1990
10) THOMAS, J. P.; S. G. CARVAJAL, H. L. LINDSAY, R. V. CITARELLA, R. E. WALLACE, M. D. LEE & F. E. DURR: LL-E33288 antibiotics. A complex of novel potent antitumor agents: Antitumor activity and tolerance in mice. Program and Abstracts of the 26th Intersci. Conf. on Antimicrob. Agents Chemother., No. 229, p. 138, New Orleans, Sept. 28~Oct. 1, 1986
11) LONG, B. H.; J. GOLIK, S. FORENZA, B. WARD, R. REHFUSS, J. C. DABROWIAK, J. J. CATINO, S. T. MUSIAL, K. W. BROOKSHIRE & T. W. DOYLE: Esperamicins, a class of potent antitumor antibiotics: mechanism of action. Proc. Natl. Acad. Sci. U.S.A. 86: 2~6, 1989
12) ZEIN, N.; A. M. SINHA, W. J. MCGAHREN & G. A. ELLESTAD: Calicheamicin γ_1: an antitumor antibiotics that cleaves double-stranded DNA site specifically. Science 240: 1198~1201, 1988
13) SUGIURA, Y.; T. SHIRAKI, M. KONISHI & T. OKI: DNA intercalation and cleavage of an antitumor antibiotic dynemicin that contains anthracycline and enediyne cores. Proc. Natl. Acad. Sci. U.S.A. 87: 3831~3835, 1990