STRUCTURE-ACTIVITY RELATIONSHIPS OF ANTHRACYCLINES RELATIVE TO CYTOTOXICITY AND EFFECTS ON MACROMOLECULAR SYNTHESIS IN L1210 LEUKEMIA CELLS

YASUE MATSUZAWA and TOSHIKAZU OKI

Central Research Laboratories, Sanraku-Ocean Co., Ltd.,
4-9-1 Johnan, Fujisawa, Kanagawa 251, Japan

TOMIO TAKEUCHI and HAMAO UMEZAWA

Institute of Microbial Chemistry,
3-14-23, Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

(Received for publication July 22, 1981)

The effects of 92 anthracycline compounds on the growth and DNA, RNA and protein synthesis of L1210 leukemia cells were studied in relationships to their structures and antitumor activity. The following structure-activity relationships were observed:

1) The hydroxyl groups at C-1, C-2, C-4, C-6 and C-11 on aglycones have significant relationships to the *in vitro* potency. Compounds lacking the hydroxyl group at C-6 (an anthracycline containing α_2-rhodomycinone) or containing the methoxyl group at C-6 (6-*O*-methylaclacinomycin A) are lower in their cytotoxicity and RNA and DNA synthesis-inhibiting activity than those containing the C-6 hydroxyl group. The hydroxyl group at C-2 did not influence the *in vitro* potency, but the hydroxyl group at C-1 and/or C-11 enhanced the potency. The activity which prolongs the life span of L1210-bearing mice was decreased by an hydroxyl group at C-1 or C-11, although the effective doses were lowered. In compounds which are different at C-4, *in vitro* potency increased in the following series: deoxy>hydroxy>methoxy.

2) The side chain at C-9 in aklavinone- and daunomycinone-glycosides produced slight influence on cytotoxicity and on the activity which inhibits RNA and DNA synthesis in intact cells. Ethyl, isopropyl and acetonyl derivatives at C-9 had an equal potency. The methyl derivative had the least activity.

3) A 10-carbomethoxy group in the *S*-configuration decreased the inhibitory activity on RNA synthesis. The removal of this group at C-10 resulted in a marked reduction in cytotoxicity and in the activity which inhibits nucleic acid synthesis.

4) The amino function either on the aglycone or on the sugar moiety was essential for *in vitro* activity. Compounds having an alkylamino group at C-3′ were more potent *in vitro* than those with a primary amino group, although these groups produced different effects in *in vivo* activity, depending on each anthracycline.

5) Disaccharides and trisaccharides were more potent *in vitro* than monosaccharides. This was reflected in the ratios for the IC_{50} values of DNA and RNA synthesis. The ratio of the concentration to inhibit DNA synthesis to that for RNA synthesis was $1\sim4$ in monosaccharides (group I), while in trisaccharides (group II), the ratio was $7\sim10$. In disaccharides the ratio was $5\sim6$.

The length of the sugar chain did not correlate with *in vivo* antitumor activity.

Since 1973, we have prepared about 100 anthracycline compounds; aclacinomycins[1~4], 2-hydroxyaclacinomycins[5,6], 13-methylaclacinomycins[7], 4-*O*-methylaclacinomycins[8,9], 6-*O*-methylaclacinomycin[9], 4‴-aminoaclacinomycin[10], 4‴-dehydrorhodomycin Y[11], 11-hydroxyaclacinomycins[11], 11-hydroxycinerubin A[11], rhodirubins[12], roseorubicins[13], baumycins[14,15], 4-hydroxybaumycins[16], feudomycins[17], 1-hydroxydaunorubicinol[18] and trisarubicinol[19]. We studied the effects of these various

types of anthracyclines on the growth and macromolecular biosynthesis of cultured L1210 leukemia cells. In this paper we report on the structure-activity relationships thus obtained.

Materials and Methods

L1210 Cell Growth in Culture

Mouse leukemia L1210 cells were cultured in RPMI 1640 "Nissui". Calf serum (10~20%, Chiba Serum Institute, Chiba), sodium bicarbonate (0.125%) and a mixture of penicillin (50 units/ml of medium) and streptomycin (50 μg/ml of medium) were added as supplements.

For the growth study, cells in the exponential phase (*ca.* 7×10^5 cells/ml) were diluted with fresh warm RPMI 1640 medium supplemented with 20% calf serum to a cell density of approximately 4×10^4 cells/ml. Nine tenth milliliter of the cell suspension was pipetted into each culture tube and 0.1 ml o a test compound was added to give the final concentration at 0.01 to 0.25 μg/ml. Compounds (anthra-

Table 1. Effects of AKN- and PMN-glycosides on growth and macromolecular syntheses in cultured L1210 leukemia cells.

No.	Compounds	R1	R2	MW	IC_{50}(nM)			IC_{50} ratio DNA/RNA	Inhibition rate Protein
					Growth	DNA	RNA		
1	Aclacinomycin A	H	RN-dF-C	812	12	370	47	7.9	31.3%
2	10-*epi*-Aclacinomycin A	H	RN-dF-C	812	25	3400	1200	2.8	23.7
3	Aclacinomycin B	H	RN-dF-CB	810	26	620	62	10.0	19.9
4	Aclacinomycin Y	H	RN-dF-Ac	810	12	190	12	15.8	85.5
5	MA144 M1	H	RN-dF-Am	814	14	470	43	10.9	27.4
6	MA144 N1	H	RN-dF-R	814	14	420	52	8.1	29.1
7	MA144 S1	H	RN-dF	700	17	290	57	5.1	31.7
8	MA144 T1**	H	RN	570	41	630	280	2.2	36.1
9	MA144 G1	H	RN-dF-DC	812	25	800	120	6.7	25.6
10	MA144 L1	H	mD-dF-C	798	70	1900	240	7.9	27.4
11	MA144 K1	H	D-dF-C	784	280	2600	570	4.6	31.3
12	MA144 U1	H	RN-dF-dF	830	39	1300	96	13.5	19.5
13	MA144 U5	H	dF-dF-C	785	>1000	>10000	>10000	—	1.1
14	MA144 U9	H	dF-dF	673	>1000	>10000	>10000	—	
15	MA144 A2**	OH	RN-dF-C	828	7	310	36	8.6	31.5
16	MA144 B2**	OH	RN-dF-CB	826	15	360	48	7.5	29.6
17	MA144 M2	OH	RN-dF-Am	830	8	460	48	9.6	35.4
18	MA144 N2**	OH	RN-dF-R	830	12	350	43	8.1	38.2
19	MA144 S2**	OH	RN-dF	716	7	310	59	5.3	43.0
20	MA144 T2**	OH	RN	586	37*	430	170	2.5	80.5
21	Rhodirubin B	OH	RN-R-R	814	21	370	55	6.7	39.0
22	Marcellomycin	OH	RN-dF-dF	846	7	950	50	19.0	28.4
23	Alcindoromycin	OH	mD-dF-dF	832	290	9900	1300	7.6	
24	Rudolfomycin	OH	RN-dF-Re	825	36	1600	190	8.4	
25	Mimimycin**	OH	RN-dF-dF	846	89	10000	2000	—	
26	Collinemycin**	OH	RN-dF	716	56	3400	630	5.4	

* Giant cells were observed.

** MA144 T1=Aklavin, MA144 A2=Cinerubin A, MA144 B2=Cinerubin B, MA144 N2=Rhodirubin A, MA144 S2=Musettamycin, MA144 T2=Pyrromycin, Mimimycin=10-*epi*-Marcellomycin, Collinemycin=10-*epi*-Musettamycin

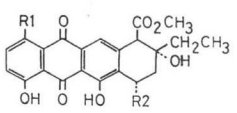


Table 2. Effects of derivatives of AKN-glycosides on growth and macromolecular syntheses in cultured L1210 leukemia cells.

No.	Compounds	R1	R2	R3	R4	R5	R6	MW	IC_{50}(nM)			IC_{50} ratio DNA/RNA	Inhibition rate Protein
									Growth	DNA	RNA		
27	2-Hydroxyaclacinomycin A	OH	H	H	$COOCH_3$	CH_2CH_3	RN-dF-C	828	42	1030	170	6.1	19.8%
28	2-Hydroxyaclacinomycin M	OH	H	H	$COOCH_3$	CH_2CH_3	RN-dF-Am	830	48	1440	120	12.0	
29	2-Hydroxyaclacinomycin N	OH	H	H	$COOCH_3$	CH_2CH_3	RN-dF-R	830	45	1400	220	6.4	
30	2-Hydroxyaclacinomycin S	OH	H	H	$COOCH_3$	CH_2CH_3	RN-dF	716	330	9500	1700	5.6	
31	2-Hydroxyaclacinomycin T	OH	H	H	$COOCH_3$	CH_2CH_3	RN	586	340	4400	1600	2.8	
32	13-Methylaclacinomycin A	H	H	H	$COOCH_3$	$CH(CH_3)_2$	RN-dF-C	826	18	560	52	10.8	27.5
33	13-Methylaclacinomycin M	H	H	H	$COOCH_3$	$CH(CH_3)_2$	RN-dF-Am	828	14	540	45	12.0	
34	13-Methylaclacinomycin N	H	H	H	$COOCH_3$	$CH(CH_3)_2$	RN-dF-R	828	19	760	52	14.6	
35	13-Methylaclacinomycin S	H	H	H	$COOCH_3$	$CH(CH_3)_2$	RN-dF	714	20	480	91	5.3	
36	13-Methylaclacinomycin T	H	H	H	$COOCH_3$	$CH(CH_3)_2$	RN	584	270	2000	530	3.8	
37	Sulfurmycin A	H	H	H	$COOCH_3$	CH_2COCH_3	RN-dF-C	840	17	480	43	11.1	32.2
38	Sulfurmycin B	H	H	H	$COOCH_3$	CH_2COCH_3	RN-dF-CB	838	17	660	60	11.0	24.6
39	Auramycin A	H	H	H	$COOCH_3$	CH_3	RN-dF-C	798	25	630	120	5.3	51.9
40	Auramycin B	H	H	H	$COOCH_3$	CH_3	RN-dF-CB	796	19	750	120	6.3	20.7
41	4-*O*-Methylaclacinomycin A	H	CH_3	H	$COOCH_3$	CH_2CH_3	RN-dF-C	826	48	580	74	7.8	33.4
42	4-*O*-Methylaclacinomycin M	H	CH_3	H	$COOCH_3$	CH_2CH_3	RN-dF-Am	828	60	660	74	8.9	
43	4-*O*-Methylaclacinomycin N	H	CH_3	H	$COOCH_3$	CH_2CH_3	RN-dF-R	828	72	720	85	8.5	
44	4-*O*-Methylaclacinomycin S	H	CH_3	H	$COOCH_3$	CH_2CH_3	RN-dF	714	120	940	220	4.3	
45	4-*O*-Methylaclacinomycin T	H	CH_3	H	$COOCH_3$	CH_2CH_3	RN	584	200	960	510	1.9	
46	6-*O*-Methylaclacinomycin A	H	H	CH_3	$COOCH_3$	CH_2CH_3	RN-dF-C	826	36	1600	110	14.5	26.8
47	10-Decarbomethoxy-aclacinomycin A	H	H	H	H	CH_2CH_3	RN-dF-C	754	66	800	450	1.8	69.5
48	7-*epi*-10-Decarbomethoxy-aclacinomycin A	H	H	H	H	CH_2CH_3	RN-dF-C	754	160	1100	730	1.5	
49	4‴-(*R*)-Aminoaclacinomycin A	H	H	H	$COOCH_3$	CH_2CH_3	RN-dF-4N(*R*)	813	15	160	16	10.0	76.1
50	4‴-(*S*)-Aminoaclacinomycin A	H	H	H	$COOCH_3$	CH_2CH_3	RN-dF-4N(*S*)	813	15	160	31	5.2	
51	4‴-(*R*)-N-Acetamide-aclacinomycin A	H	H	H	$COOCH_3$	CH_2CH_3	RN-dF-4Ac(*R*)	855	58	580	53	10.9	
52	4‴-(*S*)-Acetamide-aclacinomycin A	H	H	H	$COOCH_3$	CH_2CH_3	RN-dF-4Ac(*S*)	855	35	580	53	10.9	
53	Aclacinomycin A-oxime	H	H	H	$COOCH_3$	CH_2CH_3	RN-dF-Ox	827	36	820	63	13.0	

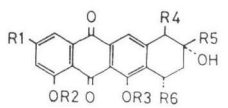
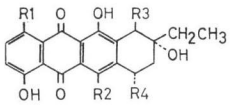


Table 3. Effects of RMN-glycosides on growth and macromolecular syntheses in cultured L1210 leukemia cells.

No.	Compounds	R1	R2	R3	R4	MW	IC_{50}(nM)			IC_{50} ratio DNA/RNA	Inhibition rate Protein
							Growth	DNA	RNA		
54	ε-RMN-A	H	OH	$COOCH_3$	RN-dF-C	828	7	420	48	8.8	35.6%
55	ε-IsoRMN-A	OH	OH	$COOCH_3$	RN-dF-C	844	4	730	66	11.1	37.1
56	β-RMN-A	H	OH	OH	RN-dF-C	786	6*	360	97	3.7	30.8
57	β-IsoRMN-A	OH	OH	OH	RN-dF-C	802	22	1500	190	7.9	29.5
58	α_2-RMN-A	OH	H	OH	RN-dF-C	786	1100	2200	1100	2.0	31.6
59	γ-RMN-A	H	OH	RN-dF-C	H	770	73	560	190	2.9	22.9
60	Roseorubicin A	H	OH	RN-RN-dF-R-R	H	1043	38	1800	190	9.5	40.1
61	Roseorubicin B	H	OH	RN-RN	H	685	88	3100	580	5.3	
62	β-Rhodomycin I	H	OH	OH	RN	544	9*	400	110	3.6	57.5

* Giant cells were observed.



cyclines) were dissolved in 0.02 M acetate buffer (pH 3.5) at a concentration of 1 mg/ml, and diluted to the desired concentration with phosphate buffered saline. In case of the hydrochloride salt, they were dissolved in distilled water instead of acetate buffer. Incubation was carried out at 37°C in a CO_2-incubator and cell numbers were periodically determined using a hemocytometer over the course of 3 days by counting viable cells stained with 0.17% trypane blue. Cytotoxicity was expressed in 50% inhibitory concentration of the control growth on day 2 (IC_{50} value). The average doubling time of L1210 leukemia cells was about 12 hours.

Incorporation of Labeled Thymidine, Uridine and Leucine into L1210 Leukemia Cells

Cells in the logarithmic phase of growth were used. L1210 cells (5×10^5 cells/ml) were incubated in RPMI 1640 medium containing 10% calf serum with gentle shaking at 37°C, preincubated with the anthracycline compound at a given concentration for 15 minutes, and thereafter 0.05 μCi/ml of [2-^{14}C]-thymidine (58.0 mCi/mmole, New England Nuclear, Boston, Mass.), 0.05 μCi/ml of [2-^{14}C]uridine (52.4 mCi/mmole, New England Nuclear) or 0.1 μCi/ml of L-[^{14}C-(U)]leucine (355.0 mCi/mmole, New England Nuclear) was added. After incubation for 60 minutes, 1 ml of cold TCA (10%) was added to 1 ml of the reaction mixture. The precipitate was collected by centrifugation at 2,000 r.p.m. for 10 minutes, washed twice with 2 ml of ice-cold 5% TCA and dissolved in 0.25 ml of 95% formic acid. The radioactivity was measured in 10 ml of BRAY's cocktail by an Aloka LSC-653 liquid scintillation spectrometer. All experiments were done in duplicate. Inhibitory effects were expressed in IC_{50} values which were obtained by plotting logarithmic anthracycline concentrations against probit percentage of the incorporation of radioactive precursors into the acid-insoluble fraction.

Anthracycline Compounds

The following 92 compounds were tested and their structure and molecular weight are listed in Tables 1~5 and Fig. 1. Aclacinomycins, 2-hydroxyaclacinomycins, 13-methylaclacinomycins, rhodirubins, baumycins, 4-hydroxybaumycins, feudomycins and roseorubicins were prepared from the cultured broths of *Streptomyces galilaeus* MA144-M1, *S. coeruleorubidus* ME130-A4, *S.* sp. ME 505, *Actinomyces roseoviolaceus* A529, *Actinomadura* sp. D326 and their mutant strains according to the purification procedures described previously[1~18]. Various anthracycline trisaccharides having cinerulosyl-2-deoxyfucosyl-rhodosaminyl moiety were prepared by either microbial or chemical glycosidation[5,11,19,20]. Hydrochloride salts of adriamycin and daunomycin, 4-demethoxyadriamycin and 4-demethoxydaunomycin were provided by Dr. F. F. ARCAMONE, Farmitalia C. Erba, Milan; β-rhodomycin I hydrochloride was given by Dr. W. IHN, Central Institute of Microbiology and Experimental Therapy, Jena; sulfurmy-

Table 4. Effects of AMN-, DMN- and CMN-glycosides on growth and macromolecular syntheses in cultured L1210 leukemia cells.

No.	Compounds	R1	R2	R3	R4	R5	R6	MW	IC_{50}(nM)			IC_{50} ratio DNA/RNA	Inhibition rate Protein
									Growth	DNA	RNA		
63	Adriamycin	H	OCH_3	OH	H	$COCH_2OH$	D	544	37*	2600	1000	2.6	16.9%
64	*N,N*-Dimethyladriamycin	H	OCH_3	OH	H	$COCH_2OH$	RN	572	18*	340	140	2.4	60.7
65	4-Demethoxyadriamycin	H	H	OH	H	$COCH_2OH$	D	514	12*	430	560	0.8	
66	4-Demethoxy-11-deoxyadriamycin	H	H	H	H	$COCH_2OH$	D	498	60*	1300	1600	0.8	
67	Daunomycin	H	OCH_3	OH	H	$COCH_3$	D	528	38*	800	300	2.7	70.9
68	*N,N*-Dimethyldaunomycin	H	OCH_3	OH	H	$COCH_3$	RN	556	11*	290	120	2.4	43.8
69	4-Demethoxydaunomycin	H	H	OH	H	$COCH_3$	D	498	10*	160	280	0.6	
70	4-Demethoxy-11-deoxydaunomycin	H	H	H	H	$COCH_3$	D	482	21*	460	710	0.6	
71	13-Dihydrodaunomycin	H	OCH_3	OH	H	$CH(OH)CH_3$	D	530	850*	7600	3000	2.5	25.9
72	Feudomycin A	H	OCH_3	OH	H	CH_2CH_3	D	514	31*	580	580	1.0	84.8
73	Feudomycin B	H	OCH_3	OH	H	CH_2COCH_3	D	542	1500*	2800	1500	1.9	38.5
74	Feudomycin D	H	OCH_3	OH	OH	CH_3	D	516	1500	11000	15000	0.7	
75	1-Hydroxy-13-dihydrodaunomycin	OH	OCH_3	OH	H	$CH(OH)CH_3$	D	546	42	3300	1100	3.0	22.0
76	Baumycin A1	H	OCH_3	OH	H	$COCH_3$	D-X	674	22*	1700	560	3.0	25.3
77	Baumycin A2	H	OCH_3	OH	H	$COCH_3$	D-X	674	7*	1900	710	2.7	19.3
78	Daunomycinone-A	H	OCH_3	OH	H	$COCH_3$	RN-dF-C	798	4	210	21	10.0	48.6
79	Daunomycinone-S	H	OCH_3	OH	H	$COCH_3$	RN-dF	686	10	410	58	7.1	44.6
80	Daunomycinone-L	H	OCH_3	OH	H	$COCH_3$	mD-dF-C	784	29	790	83	9.5	30.2
81	Daunomycinone-K	H	OCH_3	OH	H	$COCH_3$	D-dF-C	770	120	1500	210	7.1	21.0
82	Carminomycin I	H	OH	OH	H	$COCH_3$	D	514	10*	390	490	0.8	52.2
83	4-Hydroxybaumycinol A2	H	OH	OH	H	$CH(OH)CH_3$	D-X	662	9*	1200	1000	1.2	18.5
84	4-Hydroxybaumycinol A1	H	OH	OH	H	$CH(OH)CH_3$	D-X	662	38*	1900	1400	1.4	30.7
85	4-Hydroxybaumycin A2	H	OH	OH	H	$COCH_3$	D-X	660	23*	1400	640	2.2	32.6
86	4-Hydroxybaumycin A1	H	OH	OH	H	$COCH_3$	D-X	660	8*	290	140	2.1	48.4
87	Trisarubicinol	H	OH	OH	H	$CH(OH)CH_3$	RN-dF-C	786	13	470	69	6.8	34.4

* Giant cells were observed.

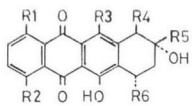
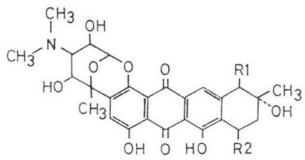
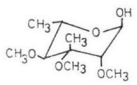


Table 5. Effects of nogalamycins and steffimycins on growth and macromolecular syntheses in cultured L1210 leukemia cells.

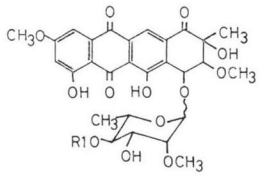
No.	Compounds	R1	R2	MW	IC_{50}(nM)			IC_{50} ratio DNA/RNA	Inhibition rate Protein
					Growth	DNA	RNA		
88	Nogalamycin	$COOCH_3$	NL	788	48	2200	320	6.9	17.9
89	Nogamycin	H	NL	730	120	3200	440	7.3	17.5
90	7-*con*-*O*-Methylnogarol	H	OCH_3	542	76	4600	8100	0.6	43.2
91	Steffimycin	H		575	73	>10000	>10000	—	12.1
92	Steffimycin B	CH_3		589	>5000	>10000	>10000	—	



Nogalamycins



NL: Nogalose



Steffimycins

cins and auramycins were supplied by Dr. A. FUJIWARA, Nippon Roche Research Center, Kamakura; nogalamycin, nogamycin, 7-*con*-*O*-methylnogarol, steffimycin and steffimycin B were obtained from the Upjohn Co., Kalamazoo; musettamycin, marcellomycin, collinemycin, mimimycin, alcindoromycin, rudolphomycin and carminomycin I were obtained from Bristol Laboratories, Syracuse, and the hydrochloride salts of *N*,*N*-dimethyladriamycin and *N*,*N*-dimethyldaunomycin were provided by Dr. E. M. ACTON, SRI International, Menlo Park.

Results

Inhibitory Effect on the Growth of L1210 Leukemia Cells

Among the 92 compounds tested, aklavinone (AKN)-, modified AKN-, ε-pyrromycinone (PMN)-, rhodomycinone (RMN)-, adriamycinone (AMN)-, daunomycinone (DMN)-, carminomycinone (CMN)-glycosides and nogalamycin and its derivatives markedly inhibited the growth of L1210 cells as shown in Tables 1~5, whereas the compounds containing no amino sugar such as MA144 U5 and U9, and steffimycin B did not show any cytotoxicity. The IC_{50} values expressed as the compound concentrations required for a 50% inhibition of the control growth were 12, 26, 17, 41, 70 and 281 nM for aclacinomycin A (ACM), aclacinomycin B (ACM-B), MA144 S1, T1 (=aklavin), L1 and K1, respectively. PMN-glycosides such as cinerubin A (=MA144 A2), cinerubin B (=MA144 B2), musettamycin (=MA144 S2), pyrromycin (PM, =MA144 T2), MA144 M2, N2 were generally more cytotoxic than their corresponding AKN-glycosides. 13-Methylaclacinomycins, sulfurmycins and auramycins having isopropyl, acetonyl and methyl group at the C-9 position of AKN showed almost the same cytotoxicity as aclacinomycins. On the other hand, 2-hydroxy-, 4-*O*- and 6-*O*-methyl-, 10-*epi*-, 10-decarbomethoxy-aclacinomycins were less active than ACM. Among the cinerulosyl-2-deoxyfucosyl-rhodosaminyl-rhodomycinone glycosides, ε-isoRMN-A was most potent, followed by β-RMN-A, ε-RMN-A, β-isoRMN-A and γ-RMN-A. α_2-RMN-A had the least cytotoxicity. The IC_{50} values for adriamycin (AM) and daunomycin (DM) were 37 and 38 nM, and those for *N*,*N*-dimethyl and 4-demethoxy derivatives of AM and DM were more cytotoxic (10~18) than parent compounds. The cytotoxicity of feudomycin (FM) A,

Fig. 1. Structures of sugar moieties.

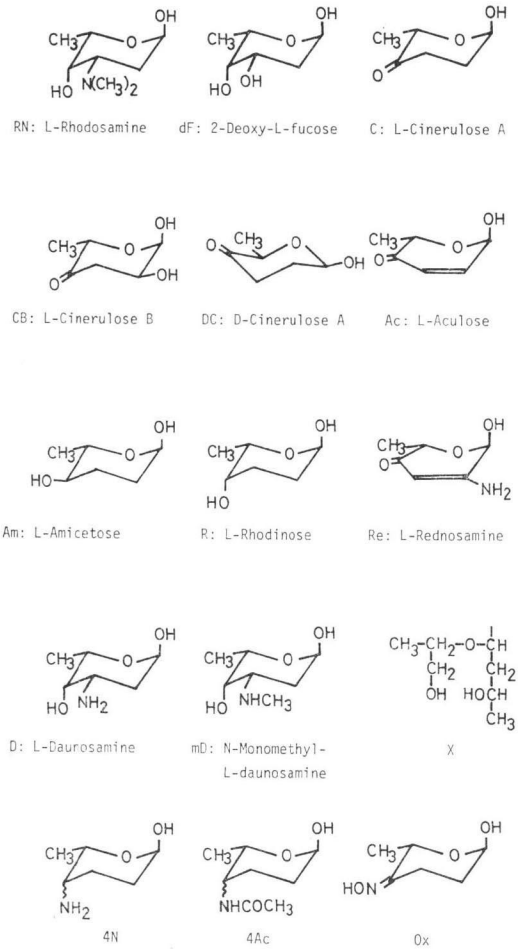
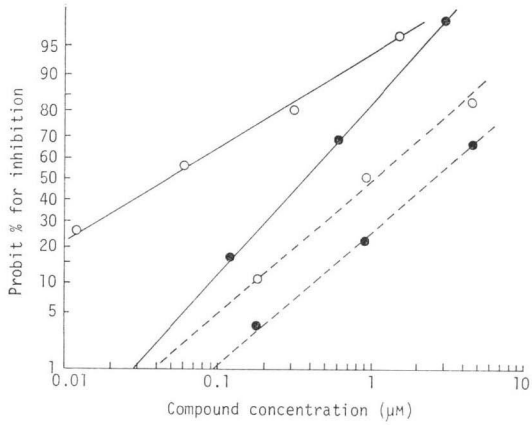


Fig. 2. Assay for inhibition on DNA and RNA synthesis of L1210 leukemia cells in culture by aclacinomycin A and adriamycin.

——: Aclacinomycin A, ------: Adriamycin
○: RNA synthesis, ●: DNA synthesis.



which was modified at the C-9 of DM, was almost the same as that of DM, but FM-B (acetonyl at C-9) and FM-D (methyl at C-9) were less cytotoxic. Baumycins (BM) A1 and A2 and 4-hydroxybaumycin A1, which has an unique residue at C-4′ of DM, were significantly more cytotoxic than DM and carminomycin I (CM). Among nogalamycin (NLM) analogs, NLM was the most cytotoxic followed by 7-*con*-*O*-methylnogarol (7-OMEN) and nogamycin (NM).

Inhibitory Effects on Macromolecular Synthesis

The effects on DNA and RNA synthesis in cultured L1210 leukemia cells were expressed in IC_{50} values (nM) which were obtained by plotting the logarithmic compound concentrations against probit percentage of inhibition on nucleic acid synthesis, as illustrated in Fig. 2. On the other hand, the effects in inhibiting protein synthesis were expressed in inhibition percentage of the incorporation of ^{14}C-leucine at a concentration of 10 μg/ml. As shown in Tables 1～5, the results indicated that ACM, ACM-B, MA144 M1, N1, S1, Y1, A2, B2, M2, N2, S2, rhodirubin B, marcellomycin, 13-methylaclacinomycins, sulfurmycins, auramycins, ε-RMN-A, ε-isoRMN-A, β-RMN-A, and trisarubicinol showed much greater inhibitory effect on RNA synthesis than AM, DM, CM and their analogs. In general, AKN-, PMN- and RMN-disaccharides and trisaccharides inhibited RNA synthesis more than 5～19 times that of DNA synthesis. Monosaccharides such as AM, DM, CM and their analogs, PM, aklavin and β-rhodomycin I inhibited RNA and DNA synthesis at approximately equal concentrations compared with ACM. 10-Decarbomethoxy ACM (10-DACM), 10-*epi*-ACM, β-RMN-A and γ-RMN-A had a weaker activity in inhibiting RNA synthesis, and inhibited almost equally RNA and DNA synthesis as did AM and DM. With respect to *N*-methylation of daunosamine, *N*,*N*-dimethyl-DM and -AM inhibited RNA and DNA

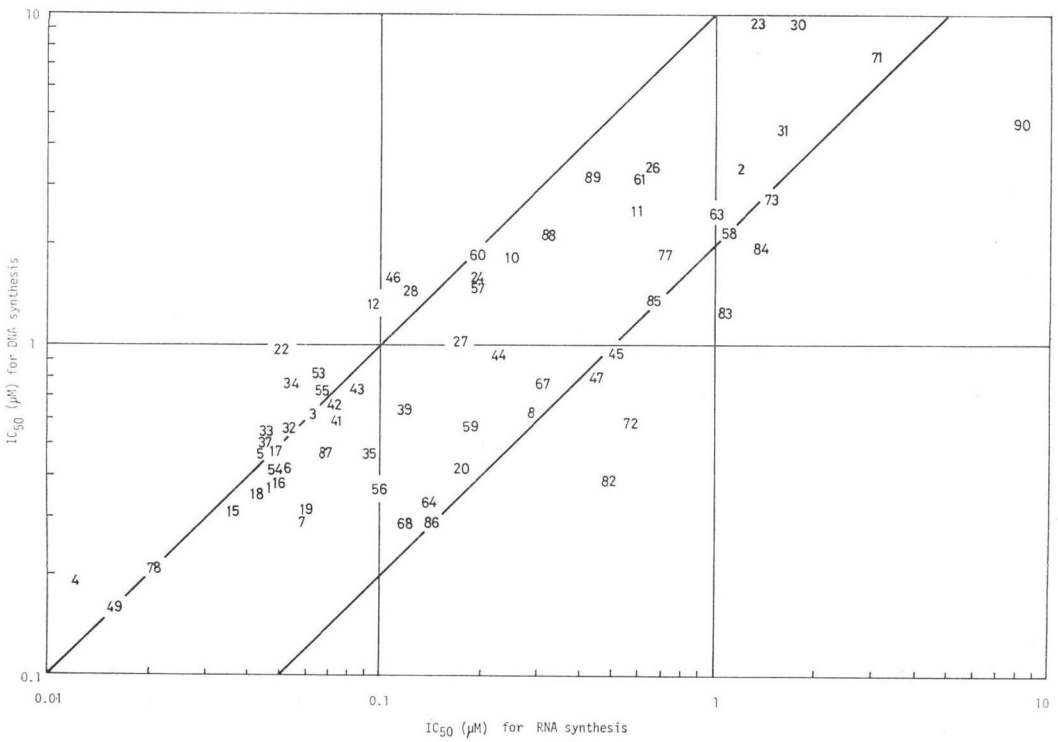
syntheses 3 ~ 7-times more strongly than DM and AM, retaining the same IC_{50} ratio of 2.4 ~ 2.6. Similarly, the *N*-demethylation of PMN- and AKN-glycosides caused a marked reduction of inhibitory activity on DNA (7 ~ 10-fold loss) and RNA (12 ~ 26-fold loss) synthesis. Thus, the IC_{50} ratios significantly decreased from 8 to 4.6 with *N,N*-didemethylation of ACM and 19 to 7.5 with *N*-monodemethylation of marcellomycin.

Discussion

We have found first that ACM and its related oligosaccharides inhibit RNA synthesis to a greater degree than DNA synthesis[1,21]. This is one characteristic of this class of anthracyclines. Subsequently, CROOKE *et al.*[22] have studied and confirmed that anthracycline glycosides may be divided into two classes on the basis of their effects on DNA and RNA synthesis. Class I anthracyclines, *i.e.* AM, DM, PM *etc.* inhibited DNA, whole cellular RNA and nucleolar RNA synthesis at approximately comparable concentrations. Class II anthracyclines, *i.e.* ACM and related oligosaccharides inhibited whole cellular RNA synthesis at 6 to 7-fold lower concentrations than those required to inhibit DNA synthesis, and nucleolar RNA synthesis was inhibited to an even greater degree than whole cell RNA synthesis (170 to 1250-fold lower). BACHUR *et al.*[23] and DASKAL *et al.*[24] also reported that there were major differences in the intracellular disposition of class I and II anthracyclines and in the rate and extent of nucleolar ultrastructural aberration caused by them. ACM located primarily in the cytoplasm, whereas DM was found almost exclusively in nucleus.

We compared the inhibitory effects of 92 compounds on the growth and nucleic acid and protein syntheses of L1210 leukemia cells. For comparison, the ratios of the concentrations necessary to inhibit

Fig. 3. Correlation between inhibition of RNA and DNA synthesis by various anthracycline glycosides. The numbers are shown compounds as in Tables 1 ~ 5.



by 50% (IC_{50}, nM) of whole cell DNA and RNA synthesis are listed in Tables 1~5. The effects of anthracyclines on nucleic acid synthesis were classified into three types when the concentrations required for 50% inhibition of DNA and RNA were plotted as shown in Fig. 3. IC_{50} values of group I anthracyclines (monosaccharides) such as DM, AM, CM, aklavin inhibited DNA and RNA synthesis at approximately an equal concentration (IC_{50} ratios of 1~4), while IC_{50} values of group II anthracyclines such as aclacinomycins, cinerubins and related trisaccharides to inhibit RNA synthesis was one-tenth that of DNA synthesis (IC_{50} ratios were over 7). The third group included mainly disaccharides, showing IC_{50} ratios of 5~6. The correlation coefficients (r) between IC_{50} for DNA synthesis and that for RNA synthesis were as follows: overall, 0.79; in group I, 0.87; in group II, 0.89; in group III, 0.99.

From the results based on the cytotoxicity and inhibitory effects on macromolecular synthesis in cultured L1210 leukemia cells, structure-activity relationship of anthracycline glycosides can be discerned both in respect of the number and types of sugar moieties and in respect of the substitution pattern in the aglycone portion of the molecules.

With respect to the AKN-, 13-methyl AKN-, 2-hydroxy AKN-, 4-*O*-methyl AKN-, PMN- and DMN-glycosides and roseorubicins, there was a gradual increase in cytotoxicity when the oligosaccharide chain length was increased from one to five sugar residues. This was reflected in the ratios for the IC_{50} values of DNA and RNA synthesis. Monosaccharides having only rhodosamine (aklavin, 13-methylaklavin, 2-hydroxyaklavin, 4-*O*-methylaklavin and PM) behaved as did AM, DM and CM (representative of group I anthracycline) in respect to their IC_{50} ratios (about 1~4) on DNA and RNA synthesis. In this connection, disaccharides (MA144 S1, 4-*O*-methyl S1, 2-hydroxy S1, musettamycin, collinemycin and roseorubicin B) having 2-deoxyfucosyl-rhodosaminide or rhodosaminyl-rhodosaminide moiety were between groups I and II in respect to their IC_{50} ratios (5~6). On the other hand, 4′-substituted derivatives of group I anthracyclines, 4′-*O*-tetrahydropyranyladriamycin and 4′-*O*-tetrahydropyranyldaunomycin[25], BM A1 and A2, 4-hydroxy BM A1 and A2 and 4-hydroxybaumycinol A1 and A2 were more cytotoxic than the corresponding parent compounds, but 4′-substituted moieties had no influence on the IC_{50} ratio for DNA and RNA synthesis, having the ratio of 1~2, similar to the group I anthracyclines. However, when dihydrocarminomycinone and daunomycinone were glycosidated with the trisaccharide moiety of cinerulosyl-2-deoxyfucosyl-rhodosaminide by microbial and chemical processes, the resulting anthracycline trisaccharides (trisarubicinol and DMN-A) showed the characters of group II anthracyclines in respect to their IC_{50} ratios (6.8~10) and a markedly increased potency against cell growth and nucleic acid synthesis.

Except for the nogalamycins and steffimycins, all of the potent anthracyclines have rhodosamine, *N*-monomethyldaunosamine or daunosamine as the first sugar. MA144 U5 was an inactive analog of ACM having only a neutral sugar; 2-deoxyfucose in place of rhodosamine. Thus, it was suggested that the amino sugar was necessary for activity. Nogalamycins which did not possess an amino sugar, but had a *N,N*-dimethylamino function in their aglycone moiety, were active as shown in Table 5. ACM having a *N,N*-dimethyl group on its amino sugar was more potent than its *N*-monodemethyl and *N,N*-didemethyl analogs (MA144 L1 and K1). A similar relationship was observed between the following compounds; marcellomycin and alcindoromycin, AM with *N,N*-dimethyl AM, DM and *N,N*-dimethyl DM. It has been reported that the *N,N*-dimethylamino function in anthracyclines is related to their mutagenicity[26,27]. ACM, cinerubin A, rhodirubin B, NLM and steffimycin were not mutagenic, while DM, AM and BM were highly mutagenic in the AMES′ and *rec*$^-$ tests. Furthermore, it has been found by UMEZAWA *et al.*[26] that *N*-demethylation of ACM correlates with its mutagenic activity, and the *N*-methyl derivatives of AM and DM are devoid of mutagenicity. These effects suggests that the modification of the amino group may change the modes of the binding or the intercalation of the aglycone moiety to DNA.

Substitutions of the terminal sugar in ACM by aculose, which is an α,β-unsaturated hexulose, and 4-aminohexose resulted in increased activity in inhibiting nucleic acid synthesis, but not in antitumor activity *in vivo* (Table 6).

Anthracycline oligosaccharides possessing a cinerulosyl-2-deoxyfucosyl-rhodosaminide moiety were prepared from the fermentation broth of several *Streptomyces*, by microbial glycosidation using a mutant strain KE303 of *S. galilaeus* MA144-M1[5,11], or by chemical modification[8,9]. The position of

the hydroxyl group on the aromatic ring of these trisaccharides influenced the degree of cytotoxicity and the inhibitory effect on macromolecular biosynthesis. α_2-RMN-A which has hydroxyl groups at C-1, C-4 and C-11 was less potent than ACM with the hydroxyl at C-4 and C-6, suggesting that the hydroxyl group at C-6 had an important role for potency. Cinerubin A, ε-RMN-A and ε-isoRMN-A which have hydroxyl groups at C-1 and/or C-11 of ACM, and also at C-6 were more potent in their cytotoxicity and inhibitory effects on RNA and DNA synthesis, and they have a strong toxicity *in vivo*[28,29], as shown in Table 6. 2-Hydroxy ACM had marked antitumor activity *in vivo*, in spite of its relatively low potency *in vitro*. Derivatives of ACM such as 4-*O*-methyl ACM and 6-*O*-methyl ACM were 2~5-fold lower in potency than ACM in the *in vitro* system. Among these oligosaccharides, the replacement of ethyl at C-9 of ACM with a methyl group as in auramycin A resulted about a 50% decrease of potency *in vitro*. 13-Methyl ACM which has the isopropyl group on C-9 and sulfurmycin A having the acetonyl group on C-9 showed almost the same potency as ACM. This suggests that there may be an optimum length of C-9 side chain for potency. The removal of the 10-carbomethoxy group from ACM resulted in a marked reduction (1/10) of the activity which inhibits RNA synthesis and a 2~5-fold reduction in the cytotoxicity and DNA inhibitory actions. The replacement of the 10-carbomethoxy group with the hydroxyl group (β-RMN-A) produced a high antitumor activity *in vivo* (T/C: 200%) at a broad range of doses (2~6 mg/kg/day), but its inhibitory potency on RNA synthesis was lowered to one-tenth of ACM (the IC_{50} ratio of 3.7). Thus, it is suggested that the 10-carbomethoxy group closely correlates with the inhibition of RNA synthesis. DUVERNAY *et al.*[30] have reported that the removal of the 10-carbomethoxy from marcellomycin and rudolfomycin caused reduction in *in vitro* and *in vivo* activity and the 10-carbomethoxy in the class II anthracycline was essential for inhibition of nucleolar RNA synthesis. As shown in Tables 1 and 3, 10-*epi*-ACM and γ-RMN-A with glycosidation at C-10 were far less potent, indicating that the group and the configuration at C-10 of anthracyclinone greatly affect the activity.

Table 6. Antitumor activity against L1210 leukemia in mice.[28,29]

Compounds	Optimal dose (mg/kg/day)	T/C (%)
Aclacinomycin A	3.75	203
Cinerubin A	1.5	163
2-Hydroxyaclacinomycin A	6.0	194
ε-Rhodomycinone-A	1.25	154
β-Rhodomycinone-A	5.0	214
Trisarubicinol	5.0	216
MA144 T1 (=aklavin)	20.0	159
MA144 S1	4.0	213
MA144 L1 (=*N*-monodemethyl ACM)	15.0	140
MA144 K1 (=*N*,*N*-didemethyl ACM)	30.0	105
Aclacinomycin Y	5.0	114
4‴-(*R*)-Aminoaclacinomycin A	2.5	129

Inoculum: L1210 cells, 2×10^5 cells/CDF_1 mouse, i.p. Treatment: 1~10 days, i.p.

Comparative studies on the cytotoxicity and inhibitory effect on nucleic acid synthesis of 4-demethoxy derivatives of DM and AM with their 4-demethoxy-11-deoxy derivatives[31] showed that the hydroxyl group at C-11 was effective in increasing the potency. In DM analogs having various side chains at C-9 such as methyl, ethyl, acetyl, acetonyl, hydroxyacetyl and 1-hydroxyethyl, there were no correlations between these groups and *in vitro* potency. With respect to the correlation between the *in vitro* and *in vivo* activity against L1210, there was almost no correlation (r=0.58). The correlation between IC_{50} values of cytotoxicity and nucleic acid synthesis was as follows: r=0.73 between cytotoxicity and DNA synthesis inhibition; r=0.66 between cytotoxicity and RNA synthesis inhibition.

In the course of counting the viable cells under the microscope, giant cells were observed in the cultures treated with AM, DM, CM and their analogs, PM, β-rhodomycin I, β-RMN-A and 7-OMEN belonging to group I anthracycline.

References

1) OKI, T.; Y. MATSUZAWA, A. YOSHIMOTO, K. NUMATA, I. KITAMURA, S. HORI, A. TAKAMATSU, H. UMEZAWA, M. ISHIZUKA, H. NAGANAWA, H. SUDA, M. HAMADA & T. TAKEUCHI: New antitumor antibiotics, aclacinomycins A and B. J. Antibiotics 28: 830~834, 1975

2) OKI, T.; N. SHIBAMOTO, Y. MATSUZAWA, T. OGASAWARA, A. YOSHIMOTO, I. KITAMURA, T. INUI, H. NAGANAWA, T. TAKEUCHI & H. UMEZAWA: Production of nineteen anthracycline compounds by *Streptomyces galilaeus* MA144-M1. J. Antibiotics 30: 683~687, 1977
3) OKI, T.; I. KITAMURA, A. YOSHIMOTO, Y. MATSUZAWA, N. SHIBAMOTO, T. OGASAWARA, T. INUI, A. TAKAMATSU, T. TAKEUCHI, T. MASUDA, M. HAMADA, H. SUDA, M. ISHIZUKA, T. SAWA & H. UMEZAWA: Antitumor anthracycline antibiotics, aclacinomycin A and analogues. I. Taxonomy, production, isolation and physicochemical properties. J. Antibiotics 32: 791~800, 1979
4) MATSUZAWA, Y.; A. YOSHIMOTO, N. SIBAMOTO, H. TOBE, T. OKI, H. NAGANAWA, T. TAKEUCHI & H. UMEZAWA: New anthracycline metabolites from mutant strains of *Streptomyces galilaeus* MA144-M1. II. Structure of 2-hydroxyaklavinone and new aklavinone glycosides. J. Antibiotics 34: 959~964, 1981
5) OKI, T.; A. YOSHIMOTO, Y. MATSUZAWA, T. TAKEUCHI & H. UMEZAWA: New anthracycline antibiotic, 2-hydroxyaclacinomycin A. J. Antibiotics 34: 916~918, 1981
6) MATSUZAWA, Y. & T. OKI: Reduction and hydrolysis of 2-hydroxyaclacinomycin A. J. Antibiotics 34: 1495~1497, 1981
7) SOGA, K.; H. FURUSHO, S. MORI & T. OKI: New antitumor antibiotics: 13-Methylaclacinomycin A and its derivatives. J. Antibiotics 34: 770~773, 1981
8) TANAKA, H.; T. YOSHIOKA, Y. SHIMAUCHI, Y. MATSUZAWA, T. OKI & T. INUI: Chemical modification of anthracycline antibiotics. I. Demethoxycarbonylation, 10-epimerization and 4-*O*-methylation of aclacinomycin A. J. Antibiotics 33: 1323~1330, 1980
9) TANAKA, H.; T. YOSHIOKA, Y. SHIMAUCHI, Y. MATSUSHITA, Y. MATSUZAWA, T. OKI & T. INUI: Preparation of 4- and 6-*O*-methylaclacinomycin derivatives and their antitumor activities. J. Antibiotics 34: 905~908, 1981
10) TANAKA, H.; T. YOSHIOKA, Y. SHIMAUCHI, T. OKI & T. INUI: Chemical modification of anthracycline antibiotics. III. 4‴-Amino, 4‴-alkylamino and 4‴-amide derivatives of aclacinomycin A. J. Antibiotics 34: 850~855, 1981
11) OKI, T.; A. YOSHIMOTO, Y. MATSUZAWA, T. TAKEUCHI & H. UMEZAWA: Biosynthesis of anthracycline antibiotics by *Streptomyces galilaeus*. I. Glycosidation of various anthracyclinones by an aclacinomycin-negative mutant and biosynthesis of aclacinomycins from aklavinone. J. Antibiotics 33: 1331~1340, 1980
12) KITAMURA, I.; N. SHIBAMOTO, T. OKI, T. INUI, H. NAGANAWA, M. ISHIZUKA, T. TAKEUCHI & H. UMEZAWA: New anthracycline antibiotics, rhodirubins. J. Antibiotics 30: 616~618, 1977
13) MATSUZAWA, Y.; A. YOSHIMOTO, T. OKI, T. INUI, T. TAKEUCHI & H. UMEZAWA: New anthracyclic antibiotics roseorubicins A and B. J. Antibiotics 32: 420~424, 1979
14) KOMIYAMA, T.; Y. MATSUZAWA, T. OKI, T. INUI, Y. TAKAHASHI, H. NAGANAWA, T. TAKEUCHI & H. UMEZAWA: Baumycins, new antitumor antibiotics related to daunomycin. J. Antibiotics 30: 619~621, 1977
15) TAKAHASHI, Y.; H. NAGANAWA, T. TAKEUCHI, H. UMEZAWA, T. KOMIYAMA, T. OKI & T. INUI: The structure of baumycins A1, A2, B1, B2, C1 and C2. J. Antibiotics 30: 622~624, 1977
16) MATSUZAWA, Y.; A. YOSHIMOTO, K. KOUNO & T. OKI: Baumycin analogs isolated from *Actinomadura* sp. J. Antibiotics 34: 774~776, 1981
17) OKI, T.; Y. MATSUZAWA, K. KIYOSHIMA, A. YOSHIMOTO, H. NAGANAWA, T. TAKEUCHI & H. UMEZAWA: New anthracyclines, feudomycins, produced by the mutant from *Streptomyces coeruleorubidus* ME130-A4. J. Antibiotics 34: 783~790, 1981
18) YOSHIMOTO, A.; Y. MATSUZAWA, T. OKI, H. NAGANAWA, T. TAKEUCHI & H. UMEZAWA: Microbial conversion of ε-pyrromycinone and ε-isorhodomycinone to 1-hydroxy-13-dihydrodaunomycin and their bioactivities. J. Antibiotics 33: 1150~1157, 1980
19) YOSHIMOTO, A.; Y. MATSUZAWA, Y. MATSUSHITA, T. OKI, T. TAKEUCHI & H. UMEZAWA: Trisarubicinol, new antitumor anthracycline antibiotic. J. Antibiotics 34: 1492~1494, 1981
20) TANAKA, H.; T. YOSHIOKA, Y. SHIMAUCHI, Y. MATSUSHITA, Y. MATSUZAWA, T. OKI & T. ISHIKURA: Chemical modification of anthracycline antibiotics. IV. Synthesis of new anthracyclines with trisaccharide. J. Antibiotics, Submitted
21) OKI, T.: New anthracycline antibiotics. Jap. J. Antibiotics 30 Suppl.: S70~S84, 1977
22) CROOKE, S. T.; V. H. DUVERNAY, L. GALVAN & A. W. PRESTAYKO: Structure-activity relationships of anthracyclines relative to effects on macromolecular syntheses. Molecular Pharmacol. 14: 290~298, 1978
23) EGORIN, M. J.; R. E. CLAWSON, J. L. COHEN, L. A. ROSS & N. R. BACHUR: Cytofluorescence localization of anthracycline antibiotics. Cancer Res. 40: 4669~4676, 1980
24) DASKAL, Y.; C. WOODARD, S. T. CROOKE & H. BUSCH: Comparative ultrastructural studies of nucleoli of tumor cells treated with adriamycin and the newer anthracyclines, carminomycin and marcellomycin. Cancer Res. 38: 467~473, 1978

25) UMEZAWA, H.; Y. TAKAHASHI, M. KINOSHITA, H. NAGANAWA, T. MASUDA, M. ISHIZUKA, K. TATSUTA & T. TAKEUCHI: Tetrahydropyranyl derivatives of daunomycin and adriamycin. J. Antibiotics 32: 1082~1084, 1979
26) UMEZAWA, K.; M. SAWAMURA, T. MATSUSHIMA & T. SUGIMURA: Mutagenicity of aclacinomycin A and daunomycin derivatives. Cancer Res. 38: 1782~1784, 1978
27) YOSHIMOTO, A.; T. OKI & T. INUI: Differential antimicrobial activities of anthracycline antibiotics on *rec*⁻ *Bacillus subtilis*. J. Antibiotics 31: 92~94, 1978
28) HORI, S.; M. SHIRAI, S. HIRANO, T. OKI, T. INUI, S. TSUKAGOSHI, M. ISHIZUKA, T. TAKEUCHI & H. UMEZAWA: Antitumor activity of new anthracycline antibiotics, aclacinomycin A and its analogs, and their toxicity. Gann 68: 685~690, 1977
29) MATSUSHITA, Y. *et al.*: Antitumor activity of various anthracycline glycosides. in preparation.
30) DUVERNAY, V. H.; J. M. ESSERY, T. W. DOYLE, W. T. BRADNER & S. T. CROOKE: The antitumor effects of anthracyclines. The importance of the carbomethoxy-group at position-10 of marcellomycin and rudolfomycin. Molecular Pharmacol. 15: 341~356, 1979
31) UMEZAWA, H.; Y. TAKAHASHI, M. KINOSHITA, H. NAGANAWA, K. TATSUTA & T. TAKEUCHI: Synthesis of 4-demethoxy-11-deoxy-analogs of daunomycin and adriamycin. J. Antibiotics 33: 1581~1585, 1980