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Effect of Various Substitutions in Positions 1, 2, 3, and 4 of 4-Demethoxydaunorubicin and 4-Demethoxyadriamycin

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Summary. *Previous studies on structure-activity relationships of anthracycline antitumor antibiotics have shown that removal of the methoxyl group at position 4 of the aglycone causes a marked increase in the potency of the compounds: 4-demethoxydaunorubicin and 4-demethoxyadriamycin had an antitumor effect similar to that of the parent compound at doses five to eight times lower, and they were active even when administered orally. This paper reports the effects of further substitutions at positions 1, 2, 3, and 4 of 4-demethoxy aglycone. The introduction of methyl groups at positions 2 and 3, or 1 and 4 resulted in decreased cytotoxicity and biological activity. The addition of a benzoyl ring at positions 2 and 3 decreased the activity further. 1,4-Dichloro-4-demethoxydaunorubicin and 2,3-dichloro-4-demethoxydaunorubicin were respectively as active and 2.5 times less active than was daunorubicin against HeLa cells in vitro while they were inactive against P388 and L1210 leukemias in vivo. 2,3-Dimethyl-4-demethoxyadriamycin showed an antitumor activity against mouse leukemias that was slightly higher than was that of adriamycin.*

Introduction

It has previously been shown that modifications of the tetracyclic aglycone of anthracycline antibiotics strongly modified the biological activity of these compounds. In particular, 4-demethoxydaunorubicin was effective against experimental mouse tumors at doses four to eight times lower than those effective for daunorubicin (Arcamone et al., 1976) and also exerted a significant antitumor activity at nontoxic doses when administered orally (Di Marco et al., 1977). 4-Demethoxyadriamycin was highly effective against experimental mouse tumors at doses about 10 times lower than those effective for adriamycin (Di Marco et al., 1978a).

It was, therefore, of interest to investigate whether further modifications in the D ring of the tetracyclic aglycone could lead to changes in the biological activity of this class of compounds.

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Materials and Methods

Compounds. The chemical structures of daunorubicin, adriamycin, and the derivatives investigated in the present study are presented in Table 1. The compounds were synthesized at the Farmitalia Research Laboratories, Milan, Italy (Arcamone et al., submitted for publication). All the drugs were dissolved in distilled water immediately before use.

In vitro Tests. The colony inhibition test was carried out as previously described (Arcamone et al., 1975) on HeLa cells (ATCC certified cell line No. 2). Dose-response curves for the various drugs were obtained for cells one day after seeding onto 60 mm Falcon plastic dishes (4×10^5 cells/plate) containing 4 ml of Eagle Basal Medium supplemented with 1% nonessential amino acids solution, 10% calf serum, and antibiotics (100 μg/ml each streptomycin and kanamycin and 100 U/ml penicillin) (Barranco and Novak, 1974; Twentyman and Bleehen, 1975). Drugs were added directly to the growth medium. At the end of the drug exposure period (24 h), the medium was removed, and the cells were trypsinized from the surface. After resuspension, counting, and diluting, the cells were plated on Falcon plastic dishes (200 cells/plate) and incubated for 6 days at 37° in 5% CO_2. At the end of this period the dishes were fixed and stained, and colonies that contained more than 50 cells were then counted.

In vivo Tests. The antitumor activity of the derivatives under study was tested on intraperitoneally (IP) transplanted L1210 and P388 leukemia and on intravenously (IV) transplanted Gross leukemia. Tests on L1210 and P388 leukemia were carried out in hybrid (C57BL/6 × DBA/2) F 1 (BDF 1) mice, and tests on Gross leukemia were performed on inbred C3H mice, as previously described (Arcamone et al., 1976; Di Marco et al., 1973, 1978b). The mice were supplied by the Charles River Breeding Laboratories, Calco, Italy. Each experimental group consisted of at least eight animals.

Compounds were dissolved in distilled water and administered IP, IV, or orally by stomach intubation, in a volume of 10 ml/kg

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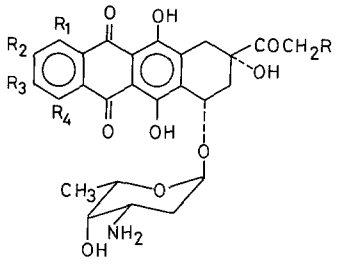
Table 1. Chemical structures of daunorubicin and adriamycin derivatives



Compound	R	R_1	R_2	R_3	R_4	Mol. wt.
Daunorubicin	H	H	H	H	OCH_3	563
4-Demethoxydaunorubicin	H	H	H	H	H	533
2,3-Dimethyl-4-demethoxydaunorubicin	H	H	CH_3	CH_3	H	561
(2,3-b)-Benzo-4-demethoxydaunorubicin	H	H			H	584
2,3-Dichloro-4-demethoxydaunorubicin	H	H	Cl	Cl	H	604
1,4-Dimethyl-4-demethoxydaunorubicin	H	CH_3	H	H	CH_3	561
1,4-Dichloro-4-demethoxydaunorubicin	H	Cl	H	H	Cl	602
Adriamycin	OH	H	H	H	OCH_3	579
4-Demethoxyadriamycin	OH	H	H	H	H	550
2,3-Dimethyl-4-demethoxyadriamycin	OH	H	CH_3	CH_3	H	577

body weight, according to different schedules of treatment. Toxicity was evaluated from the macroscopic autopsy findings, mainly from the reduction of spleen size.

Results

The activity of the compounds on the ability of HeLa cells to form colonies in shown in Table 2. As previously reported (Supino et al., 1977), the removal of the methoxyl group in position 4 of the tetracyclic aglycone of daunorubicin and adriamycin caused a marked increase of the cytotoxic activity against HeLa cells in vitro.

All the new substitutions reported in this paper caused a reduction in the cytotoxic activity. This reduction was radical in the case of benzo substitution and dichloro substitution at positions 2 and 3 of 4-demethoxydaunorubicin. The introduction of a methyl group in positions 2 and 3 of 4-demethoxydaunorubicin and 4-demethoxyadriamycin led to a decrease in activity, but the compounds obtained were still more active than daunorubicin and adriamycin. Substitution in positions 1 and 4 of 4-demethoxydaunorubicin brought the level of activity back to that of daunorubicin.

The results of the animal tests are reported in Tables 3–6. In mice bearing L1210 leukemia (Table 3) and mice bearing P388 leukemia (Table 4) and treated IP on day 1, as previously reported (Arcamone et al., 1976),

Table 2. Colony-forming ability of cultured HeLa cells after treatment with substituted 4-demethoxy derivatives of daunorubicin and adriamycin

Compound[a]	Concentration (ng/ml) required for 50% inhibition
Daunorubicin	10.00
4-Demethoxydaunorubicin	0.15
2,3-Dimethyl-4-demethoxydaunorubicin	5.80
(2,3-b)-Benzo-4-demethoxydaunorubicin	27.00
2,3-Dichloro-4-demethoxydaunorubicin	25.00
1,4-Dimethyl-4-demethoxydaunorubicin	10.05
1,4-Dichloro-4-demethoxydaunorubicin	7.15
Adriamycin	15.00
4-Demethoxyadriamycin	0.10
2,3-Dimethyl-4-demethoxyadriamycin	7.00

[a] HeLa cells were exposed to the drugs for 24 h

the optimum dose of 4-demethoxydaunorubicin, proved to be two to six times lower than the optimum dose of daunorubicin. 2,3-Dimethyl-4-demethoxydaunorubicin was about twice as potent as daunorubicin. All the other daunorubicin derivatives tested were less potent than daunorubicin, in agreement with the results observed in the in vitro test, with the exception of 1,4-dichloro-4-demethoxydaunorubicin. This compound was as toxic

Table 3. Activity of substituted 4-demethoxy derivatives of daunorubicin and adriamycin on L1210 leukemia in mice

Compound	Dose[a]	MST[b]	Toxic deaths
Daunorubicin	2	141	
	2.9	144	
	4	150	8/76
	6.6	138	31/56
4-Demethoxydaunorubicin	0.5	118	
	0.75	137	
	1.0	150	
2,3-Dimethyl-4-demethoxydaunorubicin	0.6	125	
	1.25	131	
	2.5	168	1/10
(2,3-b)-Benzo-4-demethoxydaunorubicin	5	116	
	10	135	
	20	140	1/10
2,3-Dichloro-4-demethoxydaunorubicin	4.4	100	
	6.6	100	
	10	100	
	15	105	
	22.5	111	
	30	100	
	33.7	111	
	90	33	10/10
1,4-Dimethyl-4-demethoxydaunorubicin	2.9	132	
	4.4	125	
	6.6	147	
1,4-Dichloro-4-demethoxydaunorubicin	2.9	112	
	4.4	125	
	6.6	125	
	10	111	
	20	116	
	40	122	1/10
Adriamycin	2.9	141	
	4.4	142	1/18
	6.6	159	1/25
	10	163	2/23
4-Demethoxyadriamycin	0.25	155	
	0.50	166	
	0.75	189	1/7
	1.00	133	11/11
2,3-Dimethyl-4-demethoxyadriamycin	1.3	122	
	1.9	148	
	2.9	145	
	4.4	173	
	6.6	200	3/5

[a] Treatment i.p. on day 1 (mg/kg body weight)

[b] Median survival time expressed as percentage of untreated controls; average data from several experiments

as daunorubicin against HeLa cells in vitro (Table 2), but at the tolerated doses it did not show any appreciable antitumor activity against the L1210 leukemia in mice, and the activity it exerted against P388 leukemia was markedly lower than that of daunorubicin. 4-Demethoxyadriamycin, as previously reported, was about ten times more potent than adriamycin against L1210 leukemia.

The introduction of methyl groups in positions 2 and 3 of 4-demethoxyadriamycin led to a reduction in the potency of the compound: the resulting new derivative (2,3-dimethyl-4-demethoxyadriamycin) at the optimal

Table 4. Activity of substituted 4-demethoxy derivatives of daunorubicin and adriamycin against P388 leukemia in mice

Compound	Dose[a]	MST[b]	LTS[c]	Toxic deaths
Daunorubicin	2	200	2/10	
	4	205		
	4.4	170		
	6.6	140		2/6
	8	110		
4-Demethoxydaunorubicin	0.7	200	1/10	
	1	130		10/10
2,3-Dimethyl-4-demethoxydaunorubicin	1	185		
	2	230		
	4	350	1/10	4/9
1,4-Dimethyl-4-demethoxydaunorubicin	6.6	150		
	10	165		
	15	200		1/6
	22.5	140		6/6
1,4-Dichloro-4-demethoxydaunorubicin	10	135		
	15	140		
	22.5	140		

[a] Treatment i.p. on day 1 (mg/kg body weight)
[b] Median survival time expressed as percentage of untreated controls
[c] Long-term survivors (> 60 days)

Table 5. Activity of 2,3-dimethyl-4-demethoxyadriamycin compared with that of adriamycin on transplanted Gross leukemia in mice

Compound	Dose[a]	MST[b]	Toxic deaths
Adriamycin	4.5	183, 175	
	5.4	191, 191	
	6.5	208, 208	
2,3-Dimethyl-4-demethoxyadriamycin	2.1	225	
	2.6	216	
	3.1	241, 233	3/16
	3.7	258, 233	2/16
	4.5	208	8/8
	5.4	144	8/8

[a] Treatment i.v. on days 1–3 (mg/kg/day)
[b] Median survival time expressed as percentage of untreated controls (data from two experiments)

dose (4.4 mg/kg) exerted an antitumor activity against L1210 leukemia that was slightly higher than that observed after adriamycin treatment.

This compound was therefore tested against the transplanted Gross leukemia in C_3H mice treated IV on days 1, 2, and 3. The results reported in Table 5 show that 2,3-dimethyl-4-demethoxyadriamycin was also more potent and more active than adriamycin in this experimental system.

Table 6 shows the results obtained after oral administration to mice bearing Gross leukemia of 4-demethoxy derivatives of daunorubicin and adriamycin substituted at positions 2 and 3, in comparison with their parent compounds. As previously reported (Di Marco et al., 1977), adriamycin was not active when administered orally. Daunorubicin, when administered orally at a dose tenfold higher than the optimal IV dose, showed an antitumor activity lower than that obtained after IV treatment. After removal of the methoxyl group in position 4, the resulting compounds had a significant antitumor activity even after oral administration. Following the introduction of methyl groups in positions 2 and 3, the activity after oral treatment was maintained but there was a decrease in the potency of the compounds,

Table 6. Effect of oral administration in mice bearing Gross leukemia

Compound	Dose[a]	MST[b]	Toxic deaths
Daunorubicin	25	133	
	50	150	
	100	166	4/8
4-Demethoxydaunorubicin	1.13	133	
	1.70	150	
	2.55	200	2/20
	3.82	200	11/20
2,3-Dimethyl-4-demethoxydaunorubicin	5.7	186	
	8.6	223	2/18
	12.9	116	8/8
2,3-Dichloro-4-demethoxydaunorubicin	43.5	100	
	65.1	100	
Adriamycin	200	100	
4-Demethoxyadriamycin	4	117	
	5.7	133	
	8.6	183	2/15
	12.9	183	
2,3-Dimethyl-4-demethoxyadriamycin	12.9	125	
	19.3	158	
	28.9	183	

[a] Oral treatment on days 1–3 (mg/kg/day)
[b] Median survival time expressed as percentage of untreated controls

which had to be administered at doses about three times higher than the corresponding 4-demethoxy derivatives. Following the introduction of chlorine groups in positions 2 and 3, a further drop in activity was observed: 2,3-dichloro-4-demethoxydaunorubicin was inactive when administered orally at the doses tested.

Discussion

The markedly higher biological activity of 4-demethoxydaunorubicin and 4-demethoxyadriamycin in comparison with the parent compounds and the high antitumor activity of these derivatives even after oral treatment are important findings that can give a more complete understanding of the structure-activity relationships of this class of antitumor drugs. The synthesis of derivatives bearing various substitutions at positions 1, 2, 3, and 4 of the D ring of the aglycone allows a comparison of the effects brought about by the different substituents.

The introduction of methyl groups in positions 2 and 3, or 1 and 4 of the demethoxy aglycone resulted in decreased cytotoxicity and biological activity, evaluated on the basis of the nontoxic dose that gave the more pronounced increase in the lifespan of tumor-bearing mice after both oral and IP treatment. The addition of a benzoyl ring at positions 2 and 3 decreased the activity further. Assuming that the binding to DNA is the most important mechanism of action of anthracyclines (Zunino et al., 1972), the observed changes in activity are presumably connected with changes in Van der Waals' radii of substituents in the considered positions (1.2 for hydrogen; 2.0 for a methyl group). This is not, however, the only factor that conditions the antitumor activity. In fact, 1,4-dichloro-4-demethoxydaunorubicin was almost as active as daunorubicin against HeLa cells in vitro, and was inactive or only marginally active against the L1210 or P388 leukemias in mice treated with a single IP administration. Similarly, 2,3-dichloro-4-demethoxydaunorubicin was only 2.5 times less active than daunorubicin in vitro and was devoid of any antitumor activity against L1210 leukemia[1] (Van der Waals' radius of the chlorine atom is 1.8). In order to explain these discrepancies between the in vitro and in vivo test results, we can suppose that chlorine-substituted compounds are rapidly inactivated and/or metabolized in the animal body. Once again, the antitumor activity of compounds that belong to the anthracycline group cannot be predicted on the basis of the in vitro findings (Di Marco et al., 1978b).

Of the derivatives discussed here, only 2,3-dimethyl-4-demethoxyadriamycin caused slightly more pronounced increase in the lifespan than that effected by

[1] In order to rule out technical errors, in vitro and in vivo experiments with these compounds were performed simultaneously

adriamycin in mice bearing ascitic L1210 leukemia and systemic Gross leukemia. This result may be of practical interest, but further studies involving a greater variety of tumors and toxicological investigations, particularly with regard to cardiotoxicity, are necessary to ascertain whether this compounds has a therapeutic advantage over adriamycin.

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