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Mutagenicity of Eight Anthracycline Derivatives in Five Strains of *Salmonella typhimurium*

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Abstract—*The mutagenicity of eight anthracycline derivatives, doxorubicin (adriamycin, ADM), daunorubicin (DNR), rubidazone (zorubicin), detorubicin (DTR), 4'-epi-adriamycin (4' epi-ADM), AD 32, N-leucyl daunorubicin (N-leu DNR) and aclacinomycin A, has been tested on* Salmonella typhimurium *TA 100, TA 98, TA 1535, TA 1537 and TA 1538. All but aclacinomycin A were mutagenic. Adriamycin and daunorubicin were mutagenic in all five strains, but only at very high doses (40–100 μg/plate) in TA 1535 and 1537. TA 98 was the most sensitive strain. In general S_9 mix (liver homogenate) increased the mutagenicity of the low doses of both compounds. Zorubicin, 4'-epi-adriamycin and detorubicin were mutagenic for TA 98 and TA 100 and slightly mutagenic in TA 1538. They were also activated by S_9 mix. N-leu DNR and AD 32 were slightly but significantly mutagenic for TA 98 and TA 1538 and were also activated by S_9 mix. Aclacinomycin A lacked mutagenic activity in the five strains even at cytotoxic doses, both in the presence and absence of S_9 mix. The results with AD 32, N-leu DNR and aclacinomycin A strengthen the hypothesis according to which amino moiety of the anthracycline glycosides is essential for mutagenesis.*

INTRODUCTION

THE ANTHRACYCLINE antibiotics, such as adriamycin [1], daunorubicin [2] and rubidazone [3, 4], have proven their therapeutic activity against a variety of human neoplasias. In addition to their antitumor activity, these compounds exhibit toxic side-effects. Cardiomyopathy or bone marrow toxicity are the most severe and are often dose-limiting. Alopecia is also frequently observed in treated patients.

Anthracycline antibiotics are DNA-intercalating agents and daunorubicin and adriamycin were shown to be carcinogenic in experimental animals [5, 6]. As expected, they were also highly mutagenic in a *Salmonella typhimurium* test [7]. Adriamycin is increasingly used in adjuvant therapy of potentially curable human neoplasias (breast cancer, malignant lymphomas, Hodgkin's lymphoma, etc.) and so it would be desirable to have anthracycline antibiotics with the same antitumor efficacy, lower toxicity and, if possible, no carcinogenicity.

Umezawa *et al.* [8] have reported the absence of any mutagenic effect of aclacinomycin A on *S. typhimurium* TA 98, with or without activation by S_9 mix. Their study suggested that the amino sugar moiety of anthracycline glycosides was essential for mutagenesis.

This study reports the comparative mutagenicities of 8 anthracyclines (Table 1), adriamycin, daunorubicin, rubidazone (zorubicin), detorubicin, 4'-epi-adriamycin, AD 32, *N*-leucyl daunorubicin (*N*-leu DNR) and aclacinomycin A, in five *S. typhimurium* strains, TA 98, TA 100, TA 1535, TA 1537 and TA 1538.

Three of these compounds contain a mono- or bisubstituted amino sugar (aclacinomycin A, *N*-leu DNR, AD 32) and we thus intended to test the hypothesis of Umezawa.

MATERIALS AND METHODS

Chemicals

Adriamycin and 4'-epi-adriamycin were kindly supplied by Farmitalia, Milan, Italy. Daunorubicin, detorubicin, rubidazone and *N*-leucyl daunorubicin were kindly supplied by Rhône-

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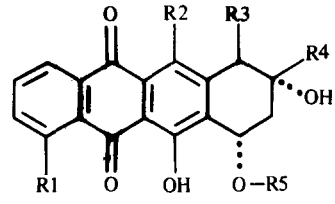
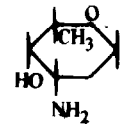
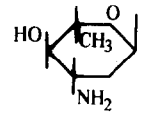
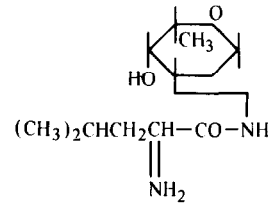
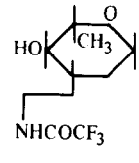
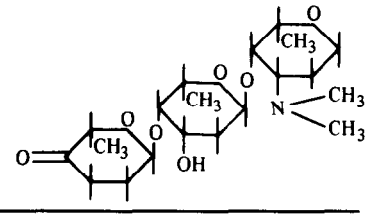
Table 1. Structures of the eight anthracycline derivatives



Compound	R1	R2	R3	R4	R5
Adriamycin (doxorubicin)	$-OCH_3$	-OH	$-H_2$	$-CO-CH_2OH$	
Daunorubicin	$-OCH_3$	-OH	$-H_2$	$-COCH_3$	id.
Rubidazone (zorubicin)	$-OCH_3$	-OH	$-H_2$	$-C(CH_3)=N-NH-C_6H_5$	id.
Detorubicin	$-OCH_3$	-OH	$-H_2$	$-C(=O)-CH_2-O-C(=O)-CH(OC_2H_5)_2$	id.
4'-Epi-adriamycin	$-OCH_3$	-OH	$-H_2$	$-C(=O)-CH_2-OH$	
N-Leucyl daunorubicin (RP 20132)	$-OCH_3$	-OH	$-H_2$	$-CO-CH_3$	
AD 32	$-OCH_3$	-OH	$-H_2$	$-CO-CH_2-O-CO-CH(OC_2H_5)_2$	
Aclacinomycin CO_2CH_3	OH	-H	A	$-CH_2-CH_3$	

Poulenc, Paris, France. AD 32 was a gift from Dr. M. Israel, Sydney Farber Institute, Boston, MA and aclacinomycin A was donated by Pr. K. Umezawa, Institute of Medical Science, University of Tokyo, Japan.

Media

The liquid medium used for growth of all the bacterial strains tested was nutrient broth (NB) containing 8 g of Difco Bacto Nutrient Broth and 5 g of NaCl per liter. The minimal media used for mutagenicity assays was 1.5% Bacto Difco Agar in Vogel-Bonner E Medium supplemented with 2% glucose.

Bacterial strains

Strains TA 1535, TA 1537, TA 1538, TA 98 and TA 100 of *Salmonella typhimurium* were kindly

provided by Dr. Bruce N. Ames, University of California, Berkeley, CA. The characteristics of each strain, the number of spontaneous revertants and the sensitivity to reference mutagenic agents were checked before each experiment.

Toxicity tests

The bacterial strain was diluted 10^5 and 10^6 times in Difco NB. Then 0.1 ml of the diluted bacterial strain and 0.1 ml of the test compound at the required concentration in water were mixed with 2 ml of soft agar (6 g/l), containing 0.25 mM biotin histidine (this concentration did not select the His^- revertant).

For each experiment control plates with solvent alone were used. Bacterial colonies obtained with each concentration of the test compound were counted and the percentage inhibition [9] for each concentration was calculated with the following formula:

$$1 = \frac{\text{No. of colonies with compound}}{\text{No. of colonies with solvent}} \times 100.$$

The percentage inhibition obtained at the various tested doses were plotted on logarithmic paper and the dose giving 50% inhibition (ID_{50}) was determined graphically.

Preparation of liver homogenate fraction (S_9) and S_9 mix (Ames et al. [9])

The liver fraction was obtained from rats having been subjected to enzyme induction with 500 mg/kg Aroclor 1254 5 days before being killed. Livers were homogenized with 0.15 M KCl (3 ml/g of liver) and the suspension was centrifuged for 10 min at 9000 ***g***. This S_9 fraction contained approximately 40 mg/ml protein and was frozen in liquid nitrogen until required.

The S_9 mix was obtained by adding 5 mM glucose-6-phosphate and 4 mM NADP in buffered medium (pH 7.4) containing 8 mM $MgCl_2$ and 33 mM KCl. The S_9 mix was freshly prepared for each experiment and stored at 0°C until used in the experiment. Its potency was controlled in standard tests using acetylaminofluorene or ethidium bromide.

Mutation tests (Ames et al. [9])

Microorganisms were grown in 20 ml of NB for 16 hr at 37°C. Aliquots of 0.1 ml of a culture of the test bacterial strain and 0.5 ml of S_9 mix were added when required to a tube containing 0.1 ml of the substance to test. This was mixed with 2 ml of melted top agar and poured into a plate containing minimal agar. After incubation at 37°C for 2 days, colonies were counted. For each experiment reference mutagens were used under the same conditions as the anthracycline derivatives tested: β-propionolactone (50 μg/plate) for strain TA 1535, hycanthone methane sulfonate (50 μg/plate) for TA 1537, acetylaminofluorene (50 μg/plate) with S_9 mix for TA 1538, niridazol (0,1 μg/plate) for TA 98 and TA 100, and ethidium bromide (60 μg/plate) with S_9 mix for TA 98.

The number of spontaneous revertant colonies were subtracted from those of the induced revertants.

For all experiments (toxicity and mutagenicity) 3 plates for each concentration of product were used. Each experiment was repeated at least 3 times (results are expressed as the mean of these data).

RESULTS

Toxicity tests

The toxicity of the 8 anthracyclines on the five *S. typhimurium* strains is shown in Table 2. The ID_{50} of AD 32 could not be determined because the compound was not toxic at 1000 μg/plate, a dose which corresponds to its maximal solubility in the test medium. The ID_{50} of *N*-leu DNR and of aclacinomycin A was also very high for all strains, especially for TA 100.

Mutation tests with Salmonella typhimurium

Table 3 is an example of spontaneous reversions induced by the reference mutagens in the five strains. As expected, the highest number of spontaneous revertants was observed in strain TA 100. All reference mutagens at the optimal doses induced a significant number of revertants. For each experiment the activity of S_9 mix was demonstrated by its capacity to activate the mutagenesis of acetylaminofluorene and ethidium bromide (the 2 compounds are not mutagenic without metabolic activation).

All compounds but aclacinomycin A were mutagenic for *S. typhimurium* TA 98: ADM, DNR, DTR were highly mutagenic and 4'-epi-ADM and zorubicin were also mutagenic but only at higher doses (Fig. 1). AD 32 and *N*-leu DNR were only slightly mutagenic at very high doses and aclacinomycin A had no mutagenicity, even at doses equal to the ID_{50} (Fig. 2). The S_9 mix activated the mutagenicity of the lowest active doses of ADM, DTR, 4'-epi-ADM, zorubicin, *N*-leu DNR and AD 32 but lowered the peak of revertants at higher concentrations. S_9 mix significantly decreased the mutagenicity of DNR on TA 98. Similar results were obtained with TA 1538 (Table 4), although the mutagenic effect was lower than with TA 98. AD 32 and *N*-leu DNR were mutagenic after activation but aclacinomycin A was not.

Table 2. Toxicity of 8 anthracycline derivatives in Salmonella typhimurium *TA 1535, TA 1537, TA 1538, TA 98 and TA 100*

	Doses giving 50% inhibition (μg/plate) (ID_{50}) with:				
	TA 1535	TA 1537	TA 1538	TA 98	TA 100
Doxorubicin (adriamycin)	50	100	50	30	20
Daunorubicin	80	115	25	80	60
Detorubicin	30	45	45	45	15
4'-Epi-adriamycin	20	25	12	100	16
Zorubicin (rubidazone)	25	100	10	20	35
N-Leucyl DNR	875	500	550	1200	1400
AD 32*	>1000	>1000	>1000	>1000	>1000
Aclacinomycin A	500	200	375	250	2500

*Toxicity tests were limited by the insolubility of AD 32 in medium at concentrations greater than 1000 μg/plate. At this concentration no toxicity was seen (except for TA 1538)-

Table 3. Mutagenic effect of AD 32 and N-leucyl daunorubicin on TA 98

Doses (μg/plate)	AD 32 S_9-	AD 32 S_9+	Doses (μg/plate)	*N*-Leucyl daunorubicin S_9-	*N*-Leucyl daunorubicin S_9+
40	3	15	40	0	0
200	36	17	200	11	92
400	62	37	350	26	110
800	80	89	600	38	84
1000	59	146	1000	53	31
2500	P	P	2500	T	T

P, Insoluble; T, toxic; S_9+, with and S_9-, without metabolic activation.

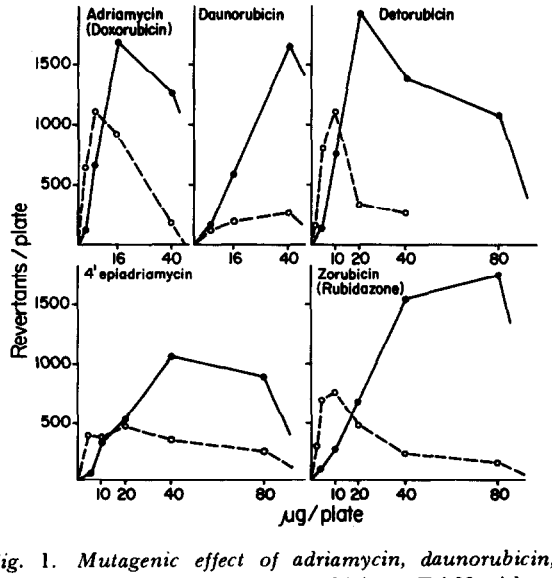


Fig. 1. Mutagenic effect of adriamycin, daunorubicin, detorubicin, 4'-epi-adriamycin, zorubicin on TA 98 without (●——●) and with (O----O) metabolic activation by rat S_9 mix.

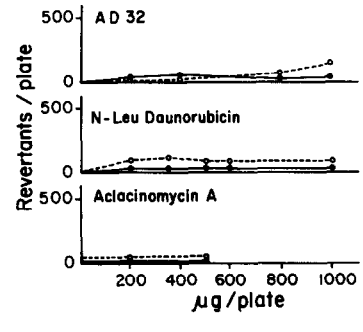


Fig. 2. Mutagenic effect of AD 32, N-leu daunorubicin and aclacynomicin on TA 98 without (●——●) and with (O----O) metabolic activation by rat S_9 mix.

ADM, DNR, DTR, zorubicin and 4'-epi-adriamycin were also mutagenic in *S. typhimurium* TA 100, whereas AD 32, *N*-leu DNR and aclacinomycin A were inactive; finally, only ADM and DNR were mutagenic in TA 1535 and TA 1537, but only at toxic concentrations.

Table 4. *Mutagenic effect of the eight anthracycline derivatives on TA* 1535, *TA* 1537, *TA* 1538 *and TA* 100

Compounds	Doses (μg/plate)	Induced revertants/plate TA 1535 S_9-	TA 1535 S_9+	TA 1537 S_9-	TA 1537 S_9+	TA 1538 S_9-	TA 1538 S_9+	TA 100 S_9-	TA 100 S_9+
	2.56	—	—	—	—	—	—	65	141
Adriamycin	6.4	0	0	0	15	12	77	65	98
	16	0	0	3	5	44	138	169	133
(doxorubicin)	40	0	150	12	—	46	133	247	7
	100	T	T	711	T	252	T	T	T
	200	T	T	T	T	T	T	T	T
	2.56	—	—	—	—	8	7	65	0
	6.4	0	0	0	0	21	13	109	0
	16	0	0	0	0	69	16	141	57
Daunorubicin	40	0	0	0	6	136	11	150	0
	100	0	0	0	87	T	T	T	T
	150	T	T	T	T	T	T	T	T
	0.16	0	0	0	0	0	0	27	0
	0.8	0	0	0	0	0	0	0	0
Zorubicin	4	0	0	0	0	2	9	16	102
	20	0	0	0	0	31	119	132	184
(rubidazone)	40	—	—	—	—	97	210	275	218
	80	—	—	—	—	47	1196	380	149
	100	T	T	T	T	T	T	T	T
	0.16	0	0	0	0	0	9	50	0
	0.8	0	0	0	0	0	4	65	15
	4	0	0	0	0	22	36	112	98
Detorubicin	20	0	0	0	0	63	6	231	315
	40	—	—	—	—	86	0	—	—
	100	T	T	T	T	T	T	T	T
	0.16	0	0	0	0	0	3	2	0
	0.8	0	0	0	0	0	4	19	15
	4	0	0	0	0	6	22	95	98
4'Epi-adriamycin	20	0	0	0	0	39	18	680	315
	40	0	0	0	0	34	0	578	638
	80	0	13	0	0	0	0	1054	494
	100	T	T	T	T	T	T	T	T
	0								
	6.4	0	0	0	0	0	0	0	0
	16	0	0	0	0	0	0	0	0
	25.6	0	0	0	0	0	0	0	0
	40	0	0	0	0	0	0	0	0
Aclacinomycin	64	0	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0	0
A	160	0	0	0	0	0	0	0	0
	250	0	0	0	0	0	0	0	0
	400	0	0	T	T	T	T	0	0
	1000	T	T	T	T	T	T	0	0
	2500	T	T	T	T	T	T	T	T
	8	0	0	0	0	5	9	0	53
	40	0	0	0	0	5	19	0	54
AD 32	200	0	0	0	0	11	136	0	0
	1000	0	0	0	0	30	159	0	0
	2500	P	P	P	P	P	P	P	P
	4	0	0	0	0	0	0	0	0
	20	0	0	0	0	0	0	0	0
	40	0	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0	0
	200	0	0	0	0	0	0	0	0
N-Leu DNR	350	0	0	0	0	0	0	0	0
	500	0	0	0	0	0	0	0	0
	600	0	0	0	0	0	0	0	0
	1000	T	T	T	T	T	T	T	T

T, Toxic effect; P, insoluble; —, non-tested dose; S_9+, with and S_9-, without metabolic activation.

DISCUSSION

The mutagenicity of DNA intercalating anthracycline antibiotics has been reported. McCann *et al.* [10] and Seino *et al.* [11] demonstrated the mutagenic effects of ADM and DNR on *S. typhimurium* TA 100 and TA 98. They also reported that the addition of S_9 mix reduced this activity. It was further confirmed [7] that both compounds produced malignant transformation *in vivo*. Our results confirm the results obtained *in vitro* and show that both compounds are also mutagenic, but to a lesser extent and only at high doses in TA 1537, which is sensitive to frameshift mutagenic actions. Therefore ADM and DNR have the greatest mutagenic and probably carcinogenic potentials.

DTR,4-epi-ADM and zorubicin represent a group of anthracycline antibiotics with intermediate mutagenic potency. They are active only in strains TA 98, TA 100 and TA 1538, which are sensitive to intercalating agents.

AD 32 and *N*-leu DNR have very little mutagenicity. The great tolerance to high doses of AD 32 and *N*-leu DNR in all bacterial strains tested raises the problem of the uptake of the drug by the bacteria.

Indeed, Kanter and Schwartz [12] have shown in a human lymphoblastoid cell line that the uptake and the cellular levels after a 2-hr incubation were much lower for AD 32 than for ADM and that both were several fold lower than those of DNR. Even if the absence of mutagenicity was due to differences of drug uptake, such a difference may be useful in the human situation. Indeed, it is possible that a given drug may be therapeutically useful with reduced toxicity as a result of lower concentrations of the compound in the normal target organ. Recent work of Jaenke *et al.* [13] suggested that the reduced cardiotoxicity of some anthracyclines may be directly related to the relative qualitative and quantitative accumulation of drug metabolites in the myocardium.

Finally, aclacinomycin A was not mutagenic in the five *S. typhimurium* strains tested with or without S_9 mix activation. We did not measure the drug uptake by the *S. typhimurium* strains, but the results of the toxicity test (Table 2) show that aclacinomycin indeed penetrated the bacteria. This result confirms and extends those of Umezawa *et al.* [8] in TA 98. These authors compared the mutagenicity of various derivatives of aclacinomycin A and DNR and suggested that substitution on the amino group of the anthracycline glycoside was responsible for the lack of mutagenicity of aclacinomycin A. It is believed that anthracycline antibiotics act by binding with DNA by intercalation of the anthracyclic moiety between nucleotide bases. It has been suggested that the amino group may form an electrostatic bond with the phosphate group of DNA [14]. If we consider (1) the report by Di Marco *et al.* [15] that *N*-acetylation of DNR decreases its inhibition of DNA synthesis, (2) the study of Umezawa *et al.* [8] and (3) the present study, which shows that among the eight anthracyclines tested only the three with a substituted amino group on the anthracycline glycoside have little or no mutagenic activity, then the role of this amino group in anthracycline mutagenicity appears very probable.

It seems that the 2 parts of the anthracyclic derivative molecules have different properties in their activity and/or quinone derivatives [16]. The anthracycline moiety provides free radicals [17] and is known to intercalate with DNA [14]. The amino sugar moiety stabilizes the DNA intercalation with its amino function [14]. The monosubstitution of the amino group decreases the mutagenic properties (*N*-leu DNR and AD 32) and bisubstitution abolishes it (aclacinomycin A) without modifying anticancer properties. The modification of the anthracycline part of the molecule (5 iminodaunorubicin: imino substitution) abolishes the mutagenic properties but also decreases the anticancer properties [17]. In addition, in the absence of metabolic activation the anthracycline derivatives exhibited a mutagenic activity. This implies that these derivatives are capable of intercalating, even without metabolic activation. After metabolism the derivatives retain their mutagenic properties and exhibit a greater mutagenicity for certain doses after activation. It may thus be concluded that certain metabolites, especially those reported by Moore and Czerniak [16] or those forming free radicals [17], present the possibility of reacting with DNA by forming covalent bonds [16].

It is interesting to note that these molecules with reduced mutagenicity also have a lower cardiotoxicity, as shown by Dantchev *et al.* [18]. This finding should stimulate the search for other anthracycline antibiotics substituted on this amino group.

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