of rheumatoid arthritis [9], and *N*-acetylcysteine has been found useful in the treatment of paracetamol poisoning [10]. Inhibitors of GAD are well known as convulsants [3–6] and this is a possible risk if compounds such as thiomalate or *N*-acetylcysteine are able to enter the brain following systemic administration.

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Effect of the methylation of aglycone hydroxyl groups on the biological and biochemical properties of daunorubicin*

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Daunorubicin and doxorubicin are well-known anthracycline antibiotics with chemotherapeutic efficacy in the treatment of several malignant disorders. Although different mechanisms by which the anthracyclines inhibit growth of tumor cells have been proposed [1, 2], DNA is thought to be the primary target of the biological action of these anticancer agents [3, 4]. Considerable interest has been devoted by many laboratories to studies of structure–activity relationships among anthracycline derivatives [4, 5]. A positive correlation between biological activity and DNA affinity has been found in several series of daunorubicin and doxorubicin derivatives [4]. Therefore, further investigations on the structural and conformational features of drug molecules relevant to the mechanism and specificity of their binding to DNA are of interest to identify essential and nonessential elements in their structures. We have undertaken a systematic study on the influence of methylation of hydroxyl groups in the chromophore. This report describes the relation between some biochemical and biological properties of daunorubicin derivatives obtained by methylation of hydroxyl groups at the 6, 9 and 11 positions (Fig. 1).

Daunorubicin derivatives were synthesized by Farmitalia Carlo Erba, Milan, Italy (G. Cassinelli *et al.*, manuscript in preparation).

Methods for the determination of the cytotoxic effect on HeLa cells have been previously described [7]. For the determination of intracellular drug levels, HeLa cells were incubated with drug (2 μM) at 37° for 15, 30, 60 and 120 min. Saturation of intracellular drug accumulation is reached for the tested compounds within 60 min. After incubation the cells were harvested and kept frozen at −20°. The cell

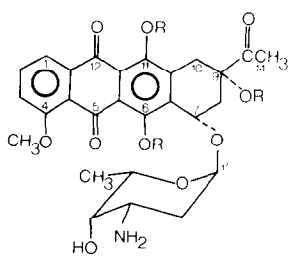

Fig. 1. Structure of daunorubicin (R = H) and derivatives (R = CH_3 or H, as indicated in the text).

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Table 1. Biologic and biochemical properties of daunorubicin derivatives

Antibiotic	Activity on HeLa cells ID_{50} (ng/ml)*	Intracellular drug content (μg/mg protein)†	Activity on P388 leukemia‡ Dose¶ (mg/kg)	T/C‖ (%)	Affinity parameters for calf thymus DNA ($2n \times K_{app}$)§
Daunorubicin	8	2.10 ± 0.07	4.4	148	9.9×10^5
9-*O*-Methyl-daunorubicin	900	1.23 ± 0.03	25.0	114	7.2×10^4
11-*O*-Methyl-daunorubicin	1600	0.11 ± 0.02	200.0**	122	1.0×10^4
6-*O*-Methyl-daunorubicin	2000	0.11 ± 0.05	50.0	127	1.4×10^4
6,9-Di-*O*-methyl-daunorubicin	>4000	—	12.5	100	0.8×10^4
6,11-Di-*O*-methyl-daunorubicin	>8000	—	4.0††	100	0.7×10^4

* Drug concentration required for 50% inhibition of colony-forming capacity. Cells were exposed to drugs for 24 hr.
† Intracellular drug content resulting from incubation of HeLa cells for 60 min with the compounds (2 μM) ± S.D.
‡ Single treatment i.p. on day 1.
¶ Optimal dose (nontoxic).
‖ Median survival time of treated mice over median survival time of control mice × 100.
§ K_{app} (the apparent binding constant) and n (the apparent number of binding sites per nucleotide) were determined from a Scatchard plot (r/m vs r), where r is the molar ratio of bound antibiotic per nucleotide and m is the molar concentration of free antibiotic. In this plot, K_{app} is the negative of the slope and n is the intercept of the linear region of the binding curve with the horizontal axis. $2n \times K_{app}$ values are the affinity parameters as defined by Muller and Crothers [22]. These values of K_{app} and n refer in all cases to an almost linear region of the binding isotherm at small values of r. The results obtained by the sedimentation technique are in good agreement with those obtained by different methods [23].
** Maximal dose tested.
†† This refers to preliminary experiments on L1210 leukemia. In this system, the optimal dose of daunorubicin was 4 mg/kg (T/C = 150%). Due to the lack of this compound, data on the activity on P388 leukemia are not available.

suspension was extracted with $AgNo_3$ and *n*-butyl alcohol as previously described [8]. Since *O*-methylation is accompanied by a change in the optical properties of the antibiotic, drug concentration in the organic phases was determined by fluorescence quantitation (Perkin–Elmer MPF 44A spectrofluorometer), at the optimal excitation and emission wavelength, using standards of each compound. *In vivo* antitumor studies were carried out on P388 and L1210 leukemia systems as previously reported [6]. Binding parameters for the drug–DNA interaction were determined according to the sedimentation method described by Blodgett and Yielding [9]. This method was adopted since attempts to determine accurate binding parameters by the fluorescence quenching method [10] were unsuccessful, as a consequence of change in optical properties following methylation.

Table 1 presents data on five compounds from this series. In all compounds studied, *O*-methylation resulted in a dramatically reduced biological activity. The following order of decreasing cytotoxic activity on HeLa cells was observed: 9-*O*-methyl-daunorubicin > 11-*O*-methyl-daunorubicin > 6-*O*-methyl-daunorubicin > 6,9-di-*O*-methyl-daunorubicin > 6,11-di-*O*-methyl-daunorubicin. All compounds, even at the highest nontoxic doses, lost significant antitumor effects on P388 leukemia in mice. Only 6-*O*-methyl-daunorubicin at the optimal dose (about 10 times greater than that of the parent compound) showed a very weak antitumor effect. To evaluate the possible influence of the changes of the physicochemical properties of these antibiotics in their cellular pharmacodynamics, the drug uptake by HeLa cells was determined. The results obtained at 60 min, reported in Table 1, show that methylation of any hydroxyl group generally caused a reduction of intracellular drug accumulation. This effect is less marked in the case of the methylation at the 9 position.

Comparison between uptake versus biological effects suggests that intracellular drug accumulation may not be the only factor accounting for the dramatic reduction of cytotoxicity *in vitro* and the loss of antitumor activity of the compounds of this series. For example, 9-*O*-methyl-daunorubicin, whose uptake by HeLa cells *in vitro* was about half that of daunorubicin, was about 100 times less active than daunorubicin *in vitro*, about five times less potent *in vivo*, and completely devoid of antitumor activity. Moreover, changes in cellular pharmacodynamics are expected to influence potency rather than efficacy [4, 11, 12]. Indeed, results reported in Table 1 show that methylation of the hydroxyl groups in the aglycone also markedly reduced the DNA binding affinity. The most dramatic effects on DNA binding ability were observed following methylation at the 6 and/or 11 position. This behavior is in agreement with preliminary observations [13].

The importance of the C-9 hydroxyl group has been emphasized [5, 13–16]. Since the planarity of the tetracyclic ring system should be retained following the methylation of any hydroxyl group of the aglycone, it was surprising to observe such a marked alteration in DNA binding properties. Since the hydroxyl groups at C-6 and C-11 are hydrogen bonded to the adjacent quinone carbonyl groups at C-5 and C-12 [17–19], methylation is expected to modify the charge distribution in the planar chromophore. Indeed, spectral studies of daunorubicin and derivatives used in this investigation indicated that there are considerable electronic differences in the chromophore following *O*-methylation at the 6 and/or 11 position. It is likely that electronic factors contribute to changes in the DNA binding properties of these compounds. This interpretation is in agreement with the observation that 5-iminodaunorubicin, which has appreciably altered electronic properties, apparently binds less tightly to DNA than daunorubicin does [20]. Therefore, although changes in the electronic character of the aglycone have been correlated with possible reduction of the cardiotoxic effects [5, 19–21], the electronic properties of the

chromophore could have a relevant role in DNA binding and biological activity. Obviously, the reduced DNA binding ability of derivatives methylated at the 6- and/or 11-*OH* groups could arise from coupled steric–electronic factors or pure steric effects. An estimation of the relative importance of these factors requires further investigation on the effects of various structural changes in the aglycone moiety.

In summary, methylation of any hydroxyl group of the aglycone moiety of daunorubicin destroyed *in vivo* antitumor activity almost completely, although most of the examined derivatives retained some cytotoxic and toxic activity at very high doses. The loss of antitumor activity was associated with a reduction in intracellular accumulation and a dramatic reduction in DNA binding. The results suggest that the alteration in the strength of binding to DNA plays a relatively major role in the antitumor activity at very high doses. The loss of antitumor activity our previous observations [4, 6].

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