Correlation between anthracycline structure and human DNA ligase inhibition

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A total of 19 anthracycline derivatives were tested for their ability to interfere *in vitro* with the action of the human replicative DNA ligase. Only those with the sugar devoid of unmodified amino groups or with large configurational modifications were found to be inactive. Maximal inhibition of DNA-joining activity was found to require a 4'-deoxy-3'-amino sugar. Self-adenylation of DNA ligase was largely insensitive to these drugs. An important general finding is that slight modifications of the anthracycline structure have pronounced effects on DNA-ligase-inhibitory activity and might be related to the specificity of anthracycline anti-tumour activity.

INTRODUCTION

Anthracyclines are an important class of antibiotics derived from *Streptomyces*. They are pigmented glycosides containing an amino sugar and a tetracyclic aglycone, an anthracyclonone [1]. Typical drugs of this class are doxorubicin (adriamycin) and daunomycin (daunorubicin), whose molecular formulae are shown in Fig. 1, structures I and VII respectively. These drugs are widely used in the clinical treatment of human cancers but, because of their toxicity, hundreds of anthracycline derivatives have been isolated or synthesized with the aim of improving their biological properties. Although interactions with membranes [2], Ca^{2+} transport [3] and sequence specificity [4] must be taken into account, the biological properties of these agents are usually attributed to their intercalative and reversible binding to DNA [5,6]. The activity of several DNA-directed enzymes is affected by intercalating anthracyclines [7]. There is also evidence that stabilization of the DNA topoisomerase II–DNA complex may play a substantial role in the cytotoxicity exerted by most, but not all, anthracyclines [8]. As it has been pointed out, closely related molecules, such as doxorubicin and daunomycin, are used to cure different kinds of tumours and show similar DNA-binding geometry [9] and very similar properties towards the stabilization of the DNA topoisomerase II–DNA complex [8]. It is therefore still unclear how anthracyclines attain their wide range of biological activities, and the existence of multiple targets must be considered. Recently we have found that DNA ligases are resistant to several intercalating anthracyclines, but extremely sensitive to those bearing a free amino group on the 3'-position of the sugar [10–12], and we have suggested that this enzyme should be considered as a potential intracellular target of anthracyclines [13]. In the present study we have analysed a group of 19 anthracycline derivatives which differ by the substitution pattern on the aglycone as well as on the sugar moiety. The study has identified some structural properties of anthracyclines which are connected with the inhibition of the DNA-ligase joining activity. It also shows that these properties derive from the interaction of these agents with the DNA substrate.

EXPERIMENTAL

Materials

Anthracycline derivatives, whose structure is shown in Fig. 1,

were kindly supplied by Dr. A. Suarato, Farmitalia–Carlo Erba (Milan, Italy). Stock solutions of anthracyclines were prepared in dimethyl sulphoxide or methanol and then diluted with reaction buffer up to the desired concentration. The final concentration of the organic solvent never exceeded 1%. The concentration of the stock solution was determined on dilution with methanol on the basis of the absorption coefficients reported in Table 1.

Enzyme purification

Purified human replicative DNA ligase was obtained from HeLa cells as described by Spadari *et al.* [14].

Polynucleotide-joining activity

Polynucleotide-joining activity of human DNA ligase I was assayed by the method of poly[d(A-T)] circularization [15]. A unit of human DNA ligase is defined as the amount of enzyme activity that converts 100 nmol of poly[d(A-T)] to an exonuclease III-resistant form after 30 min at 30 °C. Radioactivity in the control assay was less than 750 c.p.m., and the ligation obtained with the amount of DNA ligase used in the experiments corresponded to about 85 pmol of exonuclease-resistant material. At concentrations used in the present work, none of the tested compounds caused breakdown of the ligated polymer upon boiling for 3 min.

DNA ligase adenylation

DNA ligase adenylation was monitored by the technique previously described [16] in which DNA ligase is incubated at 37 °C for 30 min in the presence of 1.5 μM -adenosine 5'-[[^{35}S]-thio]triphosphate ($\text{ATP}^{[35\text{S}]}$; 40 Ci/mmol) in a reaction mixture also containing 50 mM-Tris/HCl, pH 8.0, 5 mM- MgCl_2 , 100 μg of BSA/ml, 1 mM-EDTA and 5 mM-dithioerythritol. Under these conditions, $\text{AMP}^{[35\text{S}]}$ binds covalently to DNA ligase and becomes acid-precipitable. Kinetic studies of human DNA ligase adenylation indicate that a plateau is reached within 15 min of the start of incubation (result not shown). Therefore reduced levels of adenylation after 30 min most probably indicate reduced affinity of the enzyme for the $\text{ATP}^{[35\text{S}]}$ substrate or increased instability of the adenylated enzyme.

Abbreviations used: $\text{ATP}^{[35\text{S}]}$, adenosine 5'-[[^{35}S]-thio]triphosphate; ID_{50} , dose causing 50% of maximal inhibition; numbered anthracycline derivatives are defined in Table 1 and their structures given in Fig. 1.

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Fig. 1. Structures of anthracycline derivatives studied in this work

Carbon atoms of anthracyclinone and sugar residues are numbered in the first structure. The names of the compounds are given in Table 1.

Table 1. Absorption coefficients (ϵ), and effects against human replicative DNA ligase, of test anthracyclines

No.	Anthracycline	ϵ ($M^{-1} \cdot cm^{-1}$)	Wavelength (nm)	ID ₅₀	
				Joining activity (drug/bp)	Self-adenylation (μM)
I	Doxorubicin (adriamycin)	13080	480	0.055 $\pm$ 0.006	690 $\pm$ 50
II	Epirubicin	13080	480	0.041 $\pm$ 0.005	450 $\pm$ 85
III	Esorubicin	13070	482	0.025 $\pm$ 0.003	570 $\pm$ 70
IV	4'-Deoxy-4'-iododoxorubicin	13090	480	0.050 $\pm$ 0.004	540 $\pm$ 65
V	3'-Deamino-3'-hydroxy-4'-epirubicin	12403	496	1.475 $\pm$ 0.350	$\geq$ 1000
VI	3'-Deamino-3'-hydroxy-4'-deoxy-4'-aminodoxorubicin	13340	495	0.085 $\pm$ 0.011	221 $\pm$ 45
VII	Daunorubicin (daunomycin)	12622	495	0.120 $\pm$ 0.023	$\geq$ 1000
VIII	4'-Deoxydaunorubicin	12971	495	0.023 $\pm$ 0.003	327 $\pm$ 51
IX	3'-Deamino-3'-epihydroxy-4'-deoxy-4'-aminodaunorubicin	12566	495	0.235 $\pm$ 0.058	$\geq$ 1000
X	4'-Deoxy-4'-aminodaunorubicin	12895	495	0.115 $\pm$ 0.020	620 $\pm$ 120
XI	4-Demethoxydoxorubicin	10945	482	0.117 $\pm$ 0.015	760 $\pm$ 95
XII	4'-Deoxy-4-demethoxydoxorubicin	10947	482	0.022 $\pm$ 0.003	$\geq$ 1000
XIII	3'-Deamino-3'-hydroxy-4'-epi-4-demethoxydoxorubicin	9460	482	0.370 $\pm$ 0.110	$\geq$ 1000
XIV	Idarubicin	10790	482	0.049 $\pm$ 0.012	n.d.
XV	(7 <i>R</i> ,9 <i>R</i>)-Idarubicin	10790	482	1.170 $\pm$ 0.350	690 $\pm$ 85
XVI	3'-Deamino-4'-deoxy-4'-epiamino-4-demethoxydaunorubicin	10308	482	0.170 $\pm$ 0.045	65 $\pm$ 17
XVII	3'-Deamino-3'-hydroxy-4'-epi-4-demethoxydaunorubicin	10946	482	0.780 $\pm$ 0.210	$\geq$ 1000
XVIII	3'-Deamino-3'-(3-cyanomorpholin-4-yl)doxorubicin	13080	496	0.725 $\pm$ 0.200	n.d.
XIX	4-Demethyl-6-deoxydaunorubicin (6-deoxycarminomycin)	11320	432	0.128 $\pm$ 0.039	770 $\pm$ 100

RESULTS

Effects of anthracycline derivatives on the DNA-joining activity of human replicative DNA ligase

Structural formulae of test drugs are presented in Fig. 1. Drug inhibition of the DNA-joining activity of human DNA ligase was tested in the poly[d(A-T)] circularization assay. The results are presented in Table 1. The most striking features were: (1) the inability of 3',4'-deamino or modified amino anthracyclines to inhibit the DNA-joining activity of human DNA ligase; (2) the generalized inhibitory activity associated with molecules bearing a free amino group on the sugar moiety; (3) the strongest inhibitory activity associated with molecules possessing a 3'-amino-4'-deoxy sugar.

Comparative effects of 3'-amino-4'-deoxy-anthracyclines

Six analogues with a 4'-deoxy sugar (III, IV, VIII, X, XII and XVI) were tested. The three possessing a 3'-amino-4'-deoxy-sugar carrying no other modifications (III, VIII and XII) exhibited the most potent inhibition of the joining activity of the human DNA ligase, both when compared with the 4'-hydroxy counterparts (Figs. 2a, 2b and 2c) and in absolute terms (Table 1). The modifications present on the aglycones, deriving from doxorubicin, daunorubicin and 4-demethoxydoxorubicin, have little influence on DNA-ligase-inhibitory potential (Table 1). A 4'-deoxy-4'-amino-sugar analogue carrying the amino group in the 4'-epi position (XVI) with no other modification on the sugar showed very low activity (Fig. 2c). The 3',4'-diamino-sugar anthracycline (X) showed an inhibition pattern similar to that of its 3'-amino-4'-hydroxy counterpart (VII, daunomycin), well below the values of inhibition of the 3'-deoxy-4'-aminodaunorubicin (VIII, Fig. 2b). Substitution of the hydroxy group with an iodine atom in 3' (IV) at low doses further reduced the inhibitory capability of the analogue (Fig. 2a).

Comparative effects of C-14 hydroxylation

The availability of other analogues allowed us to analyse the effects of other structural alterations. For example, the addition of a hydroxy group at C-14 of daunorubicin transforms it into doxorubicin, which is therefore an extremely similar molecule,

with almost identical properties with respect to stabilization of the DNA topoisomerase II-DNA complex and DNA-binding geometry [9] but, nevertheless, with different tumour specificity [8]. We found that doxorubicin (I) and daunorubicin (VII) differ by a factor of approx. 2.5 in their ID₅₀ for the joining activity of human DNA ligase (Table 1 and Fig. 3a), suggesting some positive influence of the hydroxy group in the C-14 position on the inhibitory activity. By contrast, 4-demethoxy molecules carrying these modifications at C-14 (XI, XIV) suggest a negative influence of the hydroxy group (Table 1 and Fig. 3b). In the case of both the very active 4'-deoxy (III, VIII) and the inactive 3'-deamino-3'-hydroxy-4'-epihydroxy molecules (XII, XVII), no effects of the C-14 modification were evident.

Comparative effects of 4-methoxyanthracyclines

A similar analysis is also possible for molecules differing only in the 4-demethoxy modification. Whereas doxorubicin (I) is more potent than its 4-demethoxy counterpart (XI) (Table 1 and Fig. 4a), daunorubicin (VII) is less potent than its 4-demethoxy counterpart (XIV) (Table 1 and Fig. 4d). Again, no significant differences were found between the two very active 4'-deoxy (III, XII) and between the two inactive 3'-deamino-3'-hydroxy-4'-epihydroxy (V, XIII) molecules.

Comparative effects of 3'-amino-4'-hydroxyanthracyclines

As Fig. 5 shows, very little difference was found when a 3'-amino-4'-hydroxy molecule (I, doxorubicin) is compared with its closely related counterparts carrying either the hydroxy group in the epi position (II) or with the residues on the sugar inverted in their position (4'-amino-3'-hydroxy, VI). The 4-demethyl-6-deoxydaunorubicin (XIX) shows an activity comparable with that of daunorubicin (Table 1). The only tested analogue with a modified amino group in the 3'-position showed no significant inhibitory activity, and the (7*R*,9*R*)-4'-demethoxydaunorubicin [(7*R*,9*R*)-idarubicin] (XV) is the less active molecule of the group.

Effects of anthracycline derivatives on the self-adenylating activity of the human replicative DNA ligase

Of the 19 compounds, 17 were also tested for their ability to prevent the covalent binding of AMP[³⁵S] residue to the enzyme

Fig. 2. Comparison of the DNA-joining-inhibitory effects of 3'-amino-4'-deoxyanthracyclines (III in *a*, VIII in *b* and XII in *c*) with their corresponding 4'-hydroxy counterparts (I in *a*, VII in *b* and XI in *c*) and with the 4'-deoxy-4'-iodo derivative of III (*a*), with the 3',4'-diamino derivative of VIII (*b*) and with the 3'-deamino-4'-deoxy-4'-epiamino derivative of XII (*c*)

[^3H]Poly[d(A-T)] was allowed to react with 0.002 unit of enzyme for 30 min at 37 °C. Ligation values were plotted as the ratio between true initial velocity in the absence of drug (v_m) and the initial velocity in the presence of drug (v) against the ratio of drug concentration (μM) over DNA concentration (μM as bp) ('Drug/bp'). v_m corresponds to approximately 1.2×10^4 c.p.m. To allow an easier comparison of data presented in this Figure with those presented up to Fig. 5, we have used uniform scales in all these Figures. Lines passing beyond the last point shown represent lines to the next data point, which is not shown.

itself in the self-catalysed, DNA-independent, reaction with ATP[^{35}S] [16] (Fig. 6). Although most of the test anthracyclines were found to be inactive in this assay, the most active anthracycline derivative of this group is the 3'-deamino-4'-deoxy-4'-epiamino-4-demethoxydaunorubicin (XVI). Also, the second most potent anthracycline derivative is a 4'-amino sugar analogue carrying a hydroxy group in the 3' position of the sugar associated with the doxorubicin aglycone.

DISCUSSION

The results described here, of DNA-joining inhibition, were obtained with a substrate of poly[d(A-T)], whose nucleotide sequence has been reported to be recognized with medium strength by daunomycin [17] as substrate. Therefore, keeping in mind that some differences might, however, exist between poly[d(A-T)] and DNA recognition by the different anthracycline derivatives studied in the present work, we believe that our results can be readily extrapolated to DNA. And, indeed,

Fig. 3. Comparison of the DNA-joining-inhibitory effects of C-14-hydroxylated anthracyclines I, XI, III and XIII with their corresponding C-14-deoxy counterparts VII, XIV, VIII and XVII in (*a*), (*b*), (*c*) and (*d*) respectively

For more assay and Figure details, see the legend to Fig. 2.

Fig. 4. Comparison of the DNA-joining-inhibitory effects of 4-methoxyanthracyclines I, III, V and VII with their corresponding 4-demethoxy counterparts XI, XII, XIII and XIV in (*a*), (*b*), (*c*) and (*d*) respectively

For more assay and Figure details, see the legend to Fig. 2.

some derivatives have already given similar results in a reliable, but more difficult, DNA-circularization assay [10]. The present results well confirm previously described, more limited, observations on the inhibition of both bacteriophage T4 and human DNA ligase by amino-sugar anthracyclines [10,12]. The introduction in the test group of anthracyclines carrying deoxyamino-sugar gave rise to the discovery of a new class of human DNA ligase inhibitors (III, VIII and XII) more powerful than that previously described for bacteriophage-T4 DNA ligase (I, II, III and XIV). The slight influence of modifications on the aglycone residue of most active molecules stresses even more the role played by the 3'-amino group on the sugar during the inhibition of the human DNA ligase.

Fig. 5. Comparison of the DNA-joining-inhibitory effects of a 3'-amino-4'-hydroxyanthracycline (I, doxorubicin) with its corresponding 4'-epihydroxy (II) and 3'-deamino-4'-deoxy-3'-hydroxy-4'-amino derivative (VI)

For more assay and Figure details, see the legend to Fig. 2.

These anthracycline derivatives are very poor inhibitors of the first step of the DNA-joining reaction: the DNA-independent adenylation of the human ligase. Doxorubicin (I) had already been reported to be inactive against the same reaction catalysed by the bacteriophage T4 DNA ligase [11], and the present results strengthen the conclusion that, in the case of anthracyclines also, inhibition of DNA ligase activity is obtained via interaction with the substrate, DNA, as already proposed for other DNA-binding drugs such as ethidium bromide and 4',6-diamidino-2-phenylindole [11]. Therefore, just as in the case of topoisomerase II-targeted [8] and bacteriophage-T4 DNA ligase-inhibiting anthracyclines [10], DNA intercalation is required, but not in itself sufficient, for the activity against human replicative DNA ligase.

Crystallographic studies indicate that, during DNA intercalation, the anthracycline aglycone lies between basepairs per-

pendicular to the axis of the double helix [9]. At the same time the sugar residue hangs into the minor groove with the 3'-amino group well exposed into the same groove. Our findings appear to confer to the position occupied by the positively charged 3'-amino group a strategic role for the inactivation of the DNA ligase. Therefore our observations go well beyond the reported biological inactivity of the aglycone chromophore without an amino sugar [1]. It is still unclear what kind of interactions occur between the 'hanging' amino group and the enzyme. However, the interaction must be very specific, since it does not seem to depend on simple spatial occupancy or charge. In fact anthracyclines with the amino group modified in a cyanomorpholine residue or substituted with a hydroxy group are inactive, whereas a 3',4'-diamino-sugar anthracycline is less active than anthracyclines with a single amino group at 3'. No information has so far been obtained for DNA ligase inhibition by 9'-deoxy molecules. The importance of these molecules relies on the observed reduction of cytotoxicity of anthracyclines carrying this modification [18].

However, small modifications in positions 4 and 14 of the aglycone do not significantly alter the strong inhibition mediated by the three very active 3'-amino-4'-deoxyanthracyclines. By contrast, the same modifications introduced in 3'-amino-4'-hydroxy molecules result in significant variation of the inhibitory response. Similar variations have not been detected in the case of the instability of the DNA topoisomerase II-DNA complexes [8]. Therefore the observed variation of the inhibitory response in the case of replicative DNA ligase activity acquires special significance. In fact it is known that closely related anthracyclines are commonly used to cure different tumours [1]. A working hypothesis can therefore be made based on the existence of different levels of multiple enzymic targets for anthracyclines in

Fig. 6. Concentration-dependence of the inhibitory effect of 17 test anthracyclines upon human DNA ligase self-adenylating activity

Incubation with ATP[³⁵S] was performed with 0.007 unit of enzyme dialysed at -20°C against 10 mM-sodium phosphate (pH 7.2)/150 mM-NaCl/50 % (v/v) ethylene glycol/50 μM -sodium pyrophosphate. Adenylation values were plotted as the ratio between the initial velocity in the absence of drug and the initial velocity in the presence of drug (v_m/v) against concentrations of drugs. v_m corresponds to approx. 10^4 c.p.m.

different tumour-cell lines. If the human replicative DNA ligase is one of these targets, the differential sensitivity of the enzyme to closely related anthracyclines could explain the different sensitivity of different tumour cell lines to anthracyclines. If this hypothesis is correct, some DNA topoisomerase II-undestabilizing anthracycline [8] should be found active against human DNA ligase.

We are grateful to Dr. A. Suarato and Dr. J. Spudich for critical reading the manuscript, and to Ms. M. T. Chiesa for her technical assistance. This work was supported by a grant of the Associazione Italiana per la Ricerca sul Cancro (AIRC).

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Received 28 February 1991/3 April 1991; accepted 12 April 1991