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Biological and physio-chemical properties of some *N*-acyl-daunorubicin derivatives*

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Daunorubicin [1, 2], an antitumor antibiotic produced by the micro-organism *Streptomyces peucetius* [3], is used in treatment of acute leukemia and certain solid tumors in humans [4]. The biological activity of daunorubicin has been linked to its ability to interact with DNA [5, 6], particularly with A:T base pairs [7]. However, other reaction mechanisms have also been suggested [8, 9]. The possibility of DNA interaction and inhibition of DNA polymerase and reverse transcriptase activities was investigated by us for some *N*-acyl-daunorubicin derivatives [10].

The daunorubicin derivatives, *N*-octanoyl-, *N*-dodecanoyl-, *N*-hexadecanoyl- and *N*-isonicotinyl-daunorubins, were prepared essentially by the method of Aszalos *et al.* [11]. Their purity was determined by elemental analysis, quantitative u.v. spectroscopy, infrared (i.r.) and nuclear magnetic resonance (n.m.r.) spectroscopy and chromatography. These compounds have been found to be active against P388 lymphocytic leukemia in mice*. In order to explore their reaction mechanisms, the *N*-octanoyl and *N*-dodecanoyl derivatives were studied for their interaction with calf thymus DNA by circular dichroism (c.d.) spectroscopy, by thermal denaturation and by exhaustive dialysis. The difference spectra obtained with daunorubicin and *N*-octanoyl-daunorubicin are shown in Figs. 1 and 2. To obtain these spectra, measurements were made with DNA, with daunorubicin (or its derivatives) alone, and with DNA and daunorubicin combined. The spectrum obtained with DNA was subtracted from that of DNA and daunorubicin combined to obtain the difference c.d. spectrum according to Dalgleich *et al.* [12]. The c.d. spectrum of daunorubicin was used for comparison purposes. By this method, the contribution of each component is eliminated from the spectra, and the difference spectra express changes in the DNA molecule upon addition of the compounds.

For daunorubicin (Fig. 1). significant spectral changes can be seen between 230 and 200 nm with compound to DNA ratios (r) of 0.037 and 0.1. At such low concentrations, daunorubicin has very little c.d. contribution in this spectral area. At a higher concentration, where $r = 0.37$. the contribution is opposite to the c.d. changes obtained in the presence of DNA. Therefore, these spectral changes indicate changes in the DNA molecule due to daunorubicin interaction. Contrary to this, at high compound to DNA ratio, where $r = 0.37$, all tested *N*-acyl-daunorubicin derivatives show c.d. and difference c.d. spectra similar to those obtained with the derivative alone at that concentration (Fig. 2). Since it has been established that daunorubicin interacts with DNA [6] and since our results show that the difference c.d. spectra differences between daunorubicin and its *N*-acyl derivatives are significant between 230 and 200 nm in the presence of DNA, one may suggest that there is no interaction or only a weak interaction of the *N*-acyl derivatives with DNA.

For DNA denaturation experiments, calf thymus DNA was dissolved to a 10^{-4} M concentration in 5×10^{-3} M Na citrate buffer, pH 7.0, containing 10^{-2} M NaCl. To this solution, a 0.1% methanolic solution of daunorubicin or its derivative was added, to make $r = 0.037$ or 0.37. The temperature of thermal transition (T_m) was obtained by measuring the light absorption change at 260 nm with a 2525 Gilford Thermo-Programmer. It was found that, for daunorubicin at $r = 0.037$, the ΔT_m was $+2.3°$ and at $r = 0.37$ ΔT_m was $+10{,}7°$, suggesting helix stabilization or intercalation of daunorubicin with DNA. However, none of the *N*-acyl derivatives changed the T_m of DNA when added at this ratio, indicating lack of helix stabilization. Thus, the DNA denaturation experiments support the results of the c.d. experiments.

One other way to measure interaction of drugs with DNA quantitatively is by exhaustive dialysis. According to this method, the final concentration of daunorubicin or its derivative, which tightly bonds to DNA after exhaustive dialysis, can be measured. These studies were conducted according to White and White [13] using calf thymus DNA. To obtain molar ratios (r), extinctions were determined at 495 nm for daunorubicin (or its derivatives) in the concentration range of 0 to 10^{-4} M, in the presence of 10^{-4} M DNA. The results were plotted, and final r values were obtained from this chart after exhaustive dialysis followed by absorbancy measurements. It was found that the final r for daunorubicin is 0.18, in agreement with White and White. However, practically no *N*-acyl-daunorubicin derivatives could be found bound to DNA after exhaustive dialysis.

From the above *in vitro* experiments, one can conclude tentatively that, unlike daunorubicin, the *N*-acyl-daunorubicin derivatives do not interact specifically with DNA. Therefore, it seems that a free–NH_2 group in the sugar moiety is required for its interaction with DNA. This preliminary conclusion is supported further by *in vitro* experiments on the interaction of nucleic acid polymerizing enzymes with the antibiotic and with its *N*-acyl derivatives (Table 1). Avian myeloblastosis viral (AMV) reverse transcriptase was purified according to a modified procedure of Abrell and Gallo [14] as described by Sethi and Sethi [15]. DNA polymerase α and DNA polymerase β activities were partially purified from NIH-Swiss mouse embryos [16]. Conditions for the enzyme assays and inhibition of enzyme activities have been described previously [7, 15, 16]. Two μg each of template-primer poly (rA), oligo (dT), poly (rC), oligo (dG) and 1.6 μg viral reverse transcriptase were used. Fifteen μg activated calf thymus DNA, and 11.5 μg and 7.5 μg DNA polymerase α and β, respectively, were employed. Control experiments contained 4% dimethylsulfoxide (DMSO). Data in Table 1 show that DNA polymerase α and DNA polymerase β activitities are inhibited by daunorubicin but not by its *N*-acyl derivatives. These enzyme activities also are inhibited by the antibiotic when poly d(A-T) was used [7]. By increasing the poly d(A-T) concentration in a daunomycin-inhibited-enzyme reaction, the enzyme activity was restored to more than 50 per cent [7]. Furthermore, poly (rA), oligo (dT) reaction with reverse transcriptase is inhibited partially by daunorubicin but with poly (rC), oligo (dG), there is a stimulation of enzyme activity (Table 1). The reason for this stimulation is unknown. Similar results have been reported by Chandra *et al.* [17].

To test the *in vitro* biological activity of these daunorubicin derivatives, the inhibition of proliferation of LLC monkey cells (ATCC-CCL 7.1) was investigated. For these studies, compounds were dissolved in dimethylsulfoxide, diluted 10-fold with the medium, filtered through a 0.22 μm millipore filter, and aliquots added to the cultured cells. Cultivation of the cells and estimation of the inhibition were performed as described by Evans *et al.* [18]. From these results (Table 2), one can conclude that the *N*-acyl derivatives are active against the monkey cells. Their activity is two to five times less than that of the parent compound. It should be mentioned that the *in vivo* titrations† reflect about the same

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† A. Aszalos, unpublished results. National Cancer Institute evaluations in the P388 lymphocrytic leukemia model.

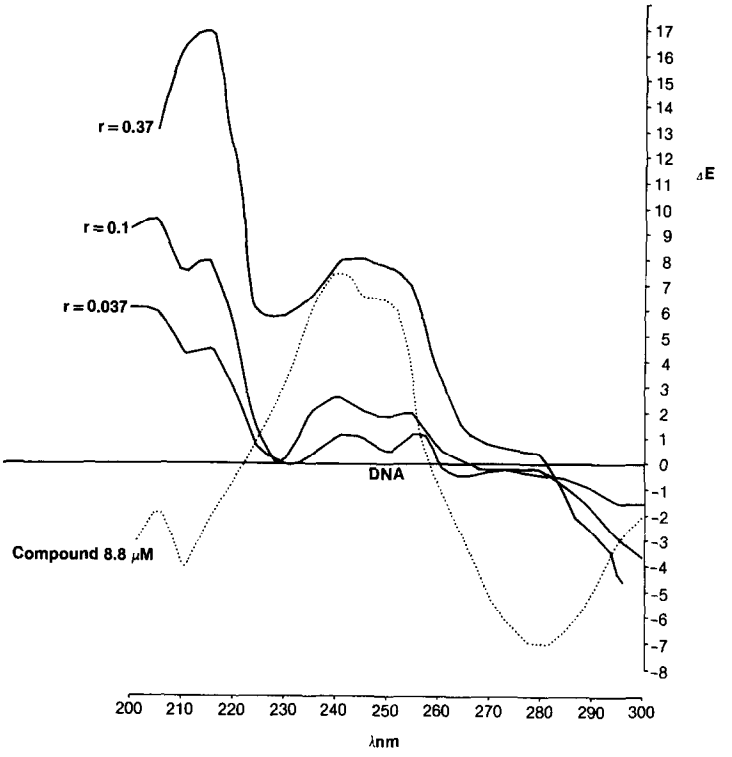

Fig. 1. Difference c.d. spectra of calf thymus DNA and different concentrations of daunorubicin–DNA complexes. DNA (Worthington Biochemicals) was dissolved in a 23.8 μM concentration in phosphate buffer, pH 6.0. To this solution, a 0.1 % concentration of daunorubicin was added in methanol solution to form molar ratio (r) of daunorubicin to DNA of 0.037, 0.1 and 0.37, corresponding to 3, 10 and 30 nucleotides/molecule of daunorubicin. The DNA was free of single stranded DNA as evidenced by the fact that no change in absorbance was observed between 25 and 70°, but there was a sharp change in absorbance above 70°. The DNA concentration in solution was determined by its absorbance at 260 nm. The residual protein in the DNA preparation was shown to be minimal by determining the ratio of absorbancies of 260 and 280 nm ($A_{260/280}$ = 2.2). Circular dichroism measurements were made with the appropriate concentrations of daunorubicin, DNA and daunorubicins plus DNA, but in separate cuvettes. The composit c.d. spectrum of daunorubicin and DNA was then subtracted from the c.d. spectrum obtained with DNA plus added daunorubicin. Results were expressed in terms of different c.d. spectra according to Dalgleish *et al.* [12].

Table 1. Comparison of inhibition of avian myeloblostosis virus (AMV)-reverse transcriptase and of NIH-Swiss mouse embryo DNA polymerase α and β with different template-primers by daunorubicin and by *N*-acyl-daunorubicins*

Enzyme	AMV-reverse transcriptase				DNA polymerase α		DNA polymerase β	
Template-primer	Poly(rA)·oligo(dT)†		Poly(rC)·oligo(dG)‡		Act.	DNA§, ‖	Act.	DNA‖, ¶
Compounds (μg/ml)	100	50	100	50	100	50	100	50
Daunorubicin-HCl	37	56	168	134	13	34	2	10
N-octanoyl-daunorubicin	74	94	82	89	121	119	106	101
N-dodecacyl-daunorubizin	96	86	91	99	119	117	99	107
N-isonicotinyl-daunorubicin	85	104	107	116	115	104	97	95

* Data is expressed as per cent control activity.
† Control activity with DMSO: 40 pmoles [^{3}H]dTMP incorporated (100 per cent).
‡ Control activity with DMSO: 15 pmoles [^{3}H]dGMP incorporated (100 per cent).
§ Control activity with DMSO: 243 pmoles [^{3}H] dTMP incorporated (100 per cent).
¶ Control activity with DMSO: 148 pmoles [^{3}H]dTMP incorporated (100 per cent).
‖ Activated DNA or gapped DNA was prepared from calf thymus DNA by incubating 6 mg DNA with 0.1 mg of pancreatic DNA-asI and 10 ml buffer containing 0.5 mM Tris–HCl, pH 7.0, 50 mM $MgCl_2$ and 5 mg bovine serum albumin, for 15 min at 37°. The reaction was terminated by chilling and the DNA-as was inactivated by heating at 70° for 5 min.

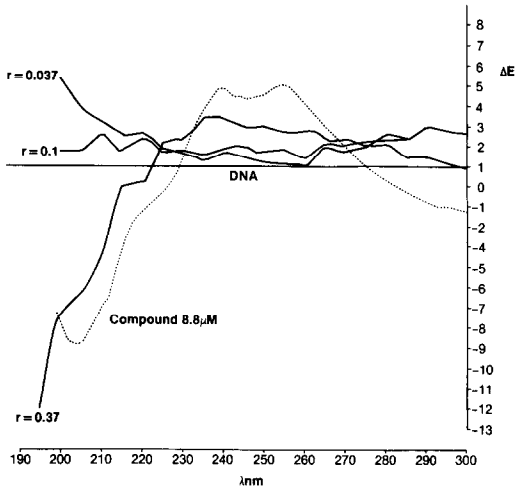

Fig. 2. Difference c.d. spectra of calf thymus DNA and different concentrations of *N*-octanoyl-daunorubicin in the presence of DNA. Experiments were carried out as described in the legend of Fig. 1 except that *N*-octanoyl-daunorubicin was used instead of daunorubicin.

quantitative relationships.

When *N*-acyl-daunorubicins are studied in the presence of growing mammalian cells for 3 days and are recovered by extraction, after Triton X-100 treatment [19], into ethyl-acetate, no hydrolysis to daunorubicin can be detected by thin-layer chromatography (silica gel 60 Merck solvent $CHCl_3$–MeOH–H_2O (120:20:1); daunorubicin (d) R_f: 0.1; *n*-octanoyl-d R_f: 0.76; *N*-dodecanoyl-d R_f: 0.8]. This result parallels that obtained with the *N*-trifluoro-acetyl derivative of adriamycin valerate, in which no hydrolysis of the trifluoro-acetyl residue was found in the *in vivo* studies [20].

One can conclude from the above experiments that, unlike the parent compound, the *N*-acyl daunorubicins do not interact specifically with DNA, or inhibit mammalian or viral DNA polymerase activity, but retain biological activity against mammalian cells *in vitro*. These results suggest that the biological activity of *N*-acyl-daunorubicin derivatives may be due to something other than a DNA interaction mechanism.

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Table 2. Inhibition of proliferation of LLC-MK_2 (ATCC-CCL 7.1) monkey cells by daunorubicin and by *N*-acyl-daunorubicins.

Test compound	Concn of test compound (μg/ml)	Cell yield (Control 2.3×10^6)	Dose at 50% inhibition (μg/ml)
Daunorubicin (d)	1.0	1.47×10^5	0.1
	0.1	1.38×10^6	
	0.01	2.16×10^6	
N-octanoyl-d	1.0	2.77 ± 10^5	0.4
	0.1	2.10×10^6	
	0.01	2.91×10^6	
N-dodecanoyl-d	10.1	6.8×10^5	0.5
	1.0	1.5×10^5	
	0.1	2.0×10^6	
N-hexadecanoyl-d	10.0	4.4×10^4	0.5
	1.0	3.1×10^5	
	0.1	2.0×10^6	
N-isonicotinyl-d	10.0	8.8×10^4	0.2
	1.0	1.7×10^5	
	0.1	1.6×10^6	

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