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EFFECT OF DIVALENT CATIONS ON THE DAUNOMYCIN–DEOXYRIBONUCLEIC ACID COMPLEX

MYER M. FISHMAN and IRA SCHWARTZ

Department of Chemistry, The City College, City University of New York, New York, N.Y. 10031, U.S.A.

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Abstract—Studies have been carried out on the interaction of Cu^{2+} and Mg^{2+} with complexes formed between daunomycin and DNA. In the visible region, at pH 5·2, the addition of Cu^{2+} (10^{-4} M) to a denatured DNA–daunomycin complex causes a spectral shift from 505 nm to 540 and 582 nm. These peaks disappear at pH values above and below 5·2. Mg^{2+} (10^{-1} M), at pH 6·9, causes the appearance of peaks similar to those obtained with Cu^{2+}, but this occurs with either native or denatured DNA–daunomycin complexes. Daunomycin shows a fluorescence emission peak at 555 nm. In the presence of Cu^{2+}, there is a reduction in the fluorescence intensity of the DNA–daunomycin complex, at pH values above 6·0. Below this pH, Cu^{2+} has no effect. Mg^{2+}, on the other hand, has a marked quenching effect at pH 7·8. As the pH drops below 7·0, this effect gradually disappears. Below pH 3·0, where DNA has no effect on fluorescence, both Cu^{2+} and Mg^{2+} are without effect.

DAUNOMYCIN (Dm), a glycosidic antibiotic which inhibits DNA-dependent RNA synthesis, was first described by Di Marco *et al.*[1] The antibiotic forms a complex with DNA, as manifested by alterations in the physicochemical properties of the daunomycin,[2–4] and it is the binding of daunomycin to DNA which is presumed to be the cause of this inhibitory effect.

Several workers have studied the binding of metal ions to DNA.[5–8] Calendi *et al.*[4] investigated the binding of various cations to either daunomycin or to the daunomycin–DNA complex. In this report, we wish to compare and indicate differences in the binding of Cu^{2+} and Mg^{2+} to the DNA–antibiotic complex, especially with regard to their pH dependence.

METHODS

Calf-thymus DNA was purchased from the Worthington Biochemical Corp., Freehold, N.J. Stock solutions of DNA were prepared by dissolving 10 mg DNA in 10 ml of 5×10^{-3} M NaCl, with gentle rotation over a period of 4 days, at 4°. The solutions were then diluted to a final volume of 100 ml. Concentrations of DNA were determined spectrophotometrically, using E(p) = 6800 at 260 nm. DNA was denatured by heating in a boiling water bath at 100° for 10 min, followed by quick cooling in ice. Daunomycin was kindly supplied by Farmitalia, Milan, Italy. Stock solutions of daunomycin (10^{-3} M) were prepared by dissolving the daunomycin in 5×10^{-3} M NaCl and then storing at 4° in the dark. Tris–HCl buffer (0·1 M) and NaCl (5×10^{-3} M) were used to make the appropriate dilutions and to control the pH for each experimental run. Below pH 5·2, the Tris buffer was still present, even though the system was essentially unbuffered. Observed effects were thus pH and not Tris dependent. The pH was measured with a Corning model 5 pH meter.

All spectrophotometric measurements, in both the visible and u.v., were made with a Cary model 14 recording spectrophotometer. Fluorescence spectra were measured with a Hitachi MPF-2A fluorescence spectrophotometer. All fluorescence determinations were made against a standard solution of daunomycin ($2{\cdot}5 \times 10^{-5}$ M). This solution was also used to calibrate the instrument from day to day. Samples were excited at 466 nm and emission spectra were read between 500 and 640 nm.

Unless otherwise indicated, experiments were run at a molar ratio of 4:1 for DNA; Dm ($1{\cdot}0 \times 10^{-4}$ M; $2{\cdot}5 \times 10^{-5}$ M).

RESULTS

The visible and ultraviolet spectra of daunomycin and the Dm–DNA complex are shown in Fig. 1. In the visible, daunomycin has a maximum absorbance at 475 nm, but the addition of increasing amounts of DNA caused a decrease in the absorbance at 475 nm and a shift in the maximum toward the longer wavelength, namely 505 nm. In the u.v., daunomycin showed peaks at 233, 255 and 292 nm. The addition of DNA caused a decrease in the absorbance at 233 nm and the disappearance of the peak at 292 nm. This was taken to be evidence for complex formation. Only minor differences were observed when either native or denatured DNA was used, and the spectra remained essentially unchanged as the pH was varied between 2·4 and 7·8.

The addition of Cu^{2+}, at a concentration of 10^{-4} M, to the Dm–DNA complex gave rise to the visible spectra shown in Fig. 2. When added to the Dm–native DNA complex, there was a broadening of the peak. Together with the Dm–denatured

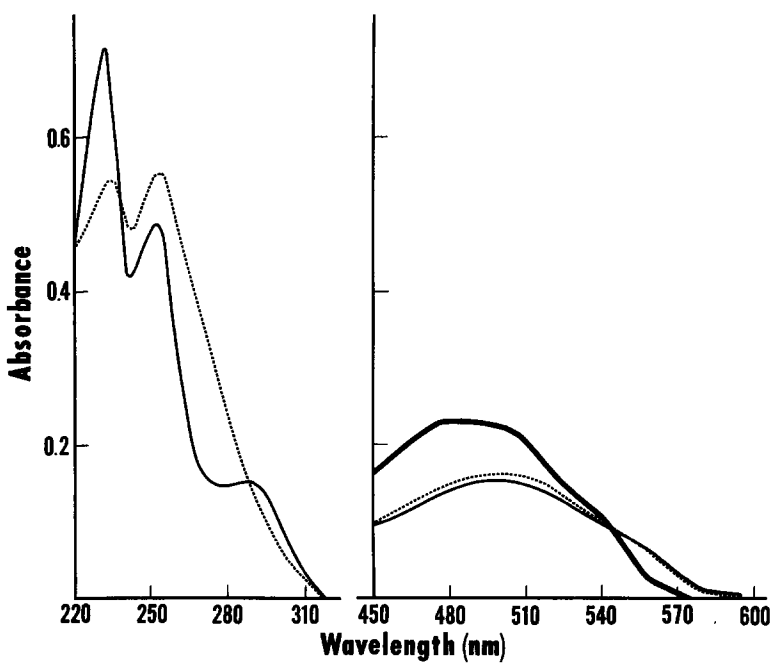

FIG. 1. Addition of DNA ($1{\cdot}0 \times 10^{-4}$ M) to daunomycin ($2{\cdot}5 \times 10^{-5}$ M). Key: ——, daunomycin; ·· ··, daunomycin plus native DNA; and ——, daunomycin plus denatured DNA

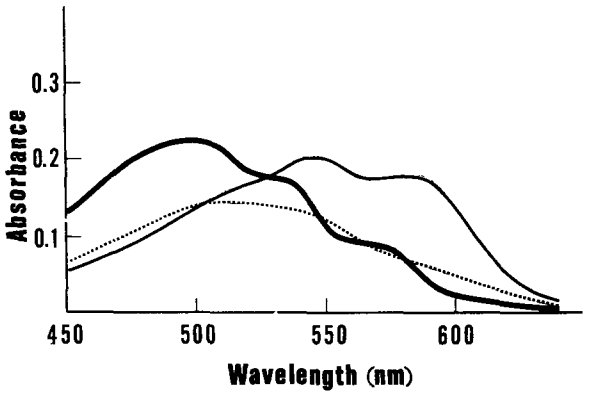

FIG. 2. Addition of Cu^{2+} to daunomycin or daunomycin–DNA complex. Key: ——, daunomycin ($5{\cdot}0 \times 10^{-5}$ M) plus Cu^{2+} ($3{\cdot}0 \times 10^{-4}$ M); · ·· ·, daunomycin plus native DNA ($2{\cdot}0 \times 10^{-4}$ M) plus Cu^{2+}; and ——, daunomycin plus denatured DNA plus Cu^{2+}.

DNA complex, at pH 5·2, the peak at 505 nm disappeared, with the appearance of two new peaks at 540 and 582 nm. However, as the pH was altered either above or below 5·2 (Fig. 3 A and B), both of these peaks disappeared. Finally at pH 3·6, the spectrum of daunomycin itself returned. This suggested a change in the type of ion binding to the complex. Interestingly, there were no significant changes in the u.v. spectra of either Cu^{2+}–Dm or the Cu^{2+}–Dm–DNA complex, over the wide range

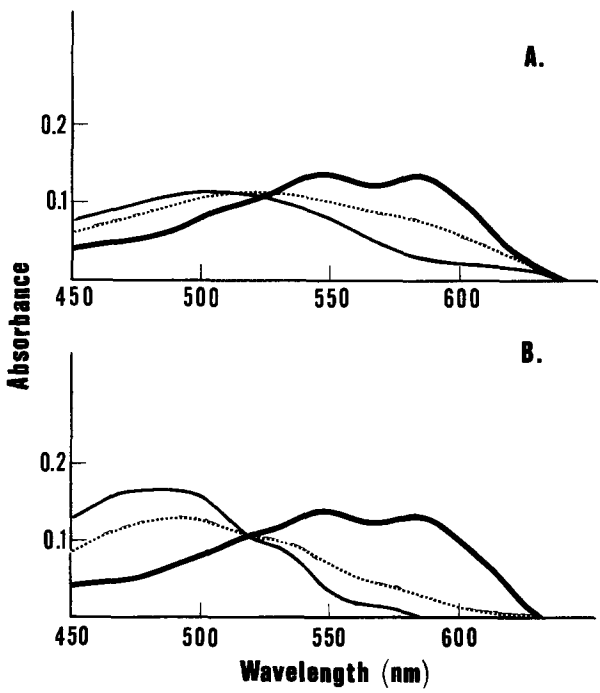

FIG. 3. Effect of pH on the daunomycin ($2{\cdot}5 \times 10^{-5}$ M)–denatured DNA ($1{\cdot}0 \times 10^{-4}$ M)–Cu^{2+} ($1{\cdot}0 \times 10^{-4}$ M) complex. (A) ——, pH 5·2; ·····, pH 6·4; and ——, pH 7·4. (B) ——, pH 5·2; ·····, pH 4·5; and ——, pH 3·6.

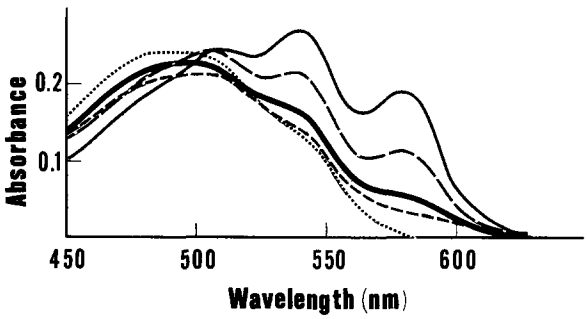

FIG. 4. Effect of pH on the daunomycin ($2{\cdot}5 \times 10^{-5}$ M)–DNA ($2{\cdot}0 \times 10^{-4}$ M)–Mg^{2+}($1{\cdot}0 \times 10^{-1}$ M) complex. Key: ·····, pH 5·8; -----, pH 6·1; ——, pH 6·9; – – – –, pH 7·4, and ——, pH 7·8

of pH values tested. The binding preference of Cu^{2+} for denatured DNA is similar to that observed in the actinomycin–DNA complex, as reported by Fishman and Rosenwasser.[9]

Studies were also carried out on the effect of Mg^{2+}. At concentrations of 10^{-4} M, similar to those for Cu^{2+}, there were no noticeable effects. However, as seen in Fig. 4, the addition of 10^{-1} M Mg^{2+} to the Dm–DNA complex was attended by spectral changes which were pH-dependent. As the pH was increased beyond 5·2, and particularly in the slightly alkaline region, there was a gradual appearance of the absorbance peaks at 540 and 582 nm, similar to that observed for the Cu^{2+}–Dm–denatured DNA complex. With Mg^{2+}, however, the change was observed with both native and denatured DNA. It should also be noted that at pH 7·8, the spectrum of the Mg^{2+}–Dm–DNA complex was the same as that of the Mg^{2+}–Dm complex.

At a pH of 2·4, the addition of Mg^{2+} to the Dm–DNA complex gave rise to spectra in both the visible and u.v. (Figs. 5 and 6) which closely resembled that of the free

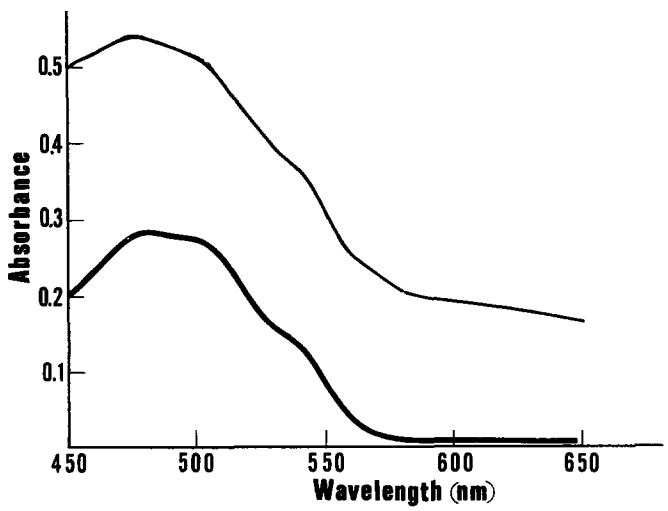

FIG. 5. Addition of Mg^{2+} to daunomycin–DNA complex at pH 2·4. Key: ——, daunomycin ($2{\cdot}5 \times 10^{-5}$ M); and ——, daunomycin plus DNA ($1{\cdot}0 \times 10^{-4}$ M) plus Mg^{2+} ($1{\cdot}0 \times 10^{-1}$ M).

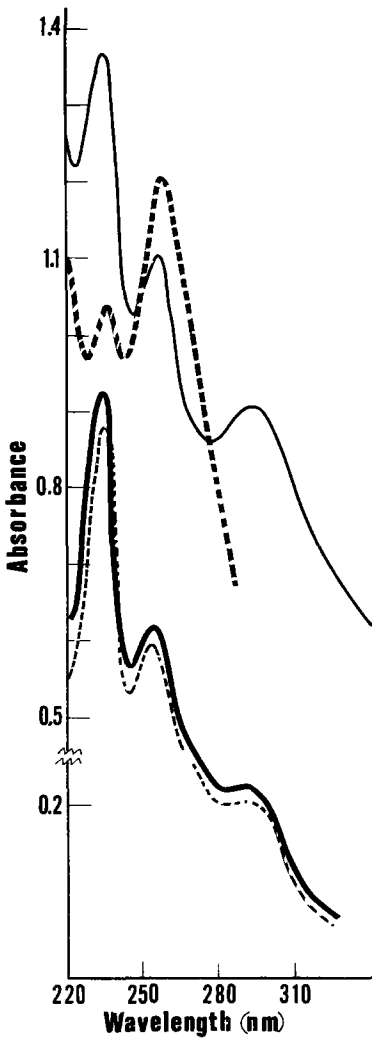

FIG. 6. Addition of Mg^{2+} ($1{\cdot}0 \times 10^{-1}$ M) to daunomycin or daunomycin–DNA complex at pH 2·4. Key: -----, daunomycin ($2{\cdot}5 \times 10^{-5}$ M); ——, daunomycin plus Mg^{2+}; ——, daunomycin plus DNA ($1{\cdot}0 \times 10^{-4}$ M); and – – – –, daunomycin plus DNA plus Mg^{2+}.

daunomycin, including the reappearance of the peak at 292 nm (at this pH, a high turbidity which required correction was observed in the samples). In view of the fact that at this pH the Dm–DNA complex remained intact, it would appear that it was the addition of Mg^{2+} which caused the dissociation of the complex.

Fluorescence data are given in Table 1 and Fig. 7. Daunomycin showed a characteristic spectrum over a wide range of pH with a peak at 555 nm and a shoulder of lesser emission at 578–580 nm. The addition of either native or denatured DNA markedly reduced the fluorescence intensity (FI) of the daunomycin. As noted by Calendi *et al.*,[4] an increase in the molar ratio of DNA:Dm was accompanied by a further decrease in the FI. Quenching, however, appeared to be at a maximum in the pH range 6·5 to 6·8.

TABLE 1. FLUORESCENCE INTENSITY OF DAUNOMYCIN–DNA COMPLEXES*

				Effect of Cu^{2+} (1.0×10^{-4} M)			Effect of Mg^{2+} (1.0×10^{-1} M)		
pH	Dm	Dm–nDNA	Dm–dDNA	Dm	Dm–nDNA	Dm–dDNA	Dm	Dm–nDNA	Dm–dDNA
7·8	68·6	28·3	29·9				16·2	14·4	14·4
7·5				35·1	6·5	3·7			
7·1	72·0	21·2	6·2	33·0	5·2	1·1	46·6	31·0	24·7
6·8	69·5	17·8	6 7	25·3	4·0	0·8	51·2	34·8	31·0
6·5	68·0	13·2	11·7	25·5	4·0	0·7	54·0	40·2	34·0
6·1	70·4	21·8	16·5	36·8	8·3	1·9	58·9	44·2	39 0
5·8	71·3	22 0	16·5	66 0	21·0	8 0	70·1	47·5	47·5
5·5	77·4	26·3	15·2	70·3	22·5	12·0	70·1	48·0	46·5
4·6	72·1	22·2	17·1				72·1	49·6	
4·3				73·6	28 9	19·5			
3·5	73·4	29·9	29·5				69·9	48·4	54·0
2·3	74·3	53·7	63·7	74·3	65·8	72·0	70·0	63·1	68·0
1·5	74·3	69·1	68·0				68 4	67·3	66·0

* Excitation at 466 nm, emission at 555 nm. DNA, 1.0×10^{-4} M; daunomycin, 2.5×10^{-5} M

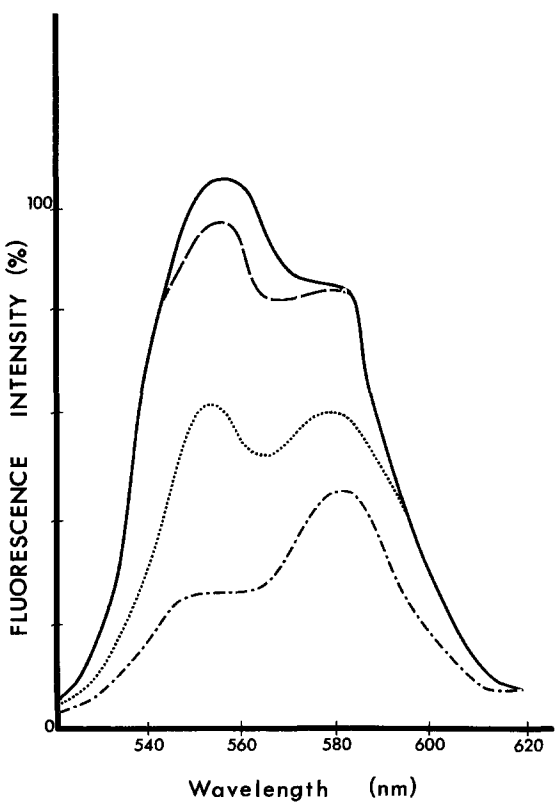

FIG. 7. Fluorescence of daunomycin (2.5×10^{-5} M). Key: ——, daunomycin, pH 7·9; – – – –, daunomycin plus 10^{-1} M Mg^{2+}, pH 6·8; ·····, daunomycin plus 10^{-1} M Mg^{2+}, pH 7·5; and –·–·–, daunomycin plus 10^{-1} M Mg^{2+}, pH 8·0.

Cu^{2+}, at a concentration of 10^{-4} M, influenced the fluorescence of Dm as well as the Dm–DNA complexes. The FI was reduced in all three instances, together with what appeared to be a maximum in quenching at pH 6·5 to 6·8. Below pH 6·0, Cu^{2+} had no effect on fluorescence.

Unlike Cu^{2+}, Mg^{2+} had no effect on fluorescence at concentrations less than 10^{-1} M. At this concentration, and at pH above 7·0, a quenching was observed, together with a change in the emission spectrum (Fig. 7). The peak at 555 nm was decreased and the one at 580 nm was sharply increased. As the pH was decreased below 7·0, the quenching effect changed rapidly so that below pH 6·0, the Mg^{2+} was without effect. The Mg^{2+} also influenced the FI of the complex in that the observed maximum in quenching at pH 6·5 to 6·8 of the Dm–DNA complex was eliminated. Here, the Mg^{2+} had the same effect on both native and denatured DNA. Below pH 3·0, where neither native nor denatured DNA had any influence on the fluorescence of daunomycin, the Mg^{2+}, too, had no effect.

DISCUSSION

In nucleic acids, there are two classes of binding sites available for interaction, namely the heterocyclic bases and the negatively charged phosphate groups. It has been shown by several investigators[7,10,11] that Cu^{2+} does bind to the PO_4 groups, but binds preferentially to the nitrogen bases, thereby decreasing the melting temperature (T_m) of DNA and destabilizing the double helix. Mg^{2+}, on the other hand, binds exclusively to the PO_4 groups, thereby increasing the T_m by stabilizing the helix. Thus Cu^{2+} and Mg^{2+} appear to have opposite effects.

In our studies, these differences in behavior of the divalent cations were reflected in their effect on the spectral characteristics of the DNA–daunomycin complex. Cu^{2+}, at a concentration of 10^{-4} M, caused changes in the visible spectrum of daunomycin itself as well as that of the denatured DNA–daunomycin complex, even at a molar concentration ratio of 4:1 for the latter. No such effect was observable when Cu^{2+} was added to the native DNA–daunomycin complex. If we assume that daunomycin through the O and/or OH groups of the aglycone moiety, interacts with the PO_4 groups on the DNA backbone, then the sugar moiety together with its amine group (Fig. 8) might be masked by the double helical configuration of the native DNA. Thus, Cu^{2+}, which is known to form complexes with nitrogen-containing ligands, would have no specific effect on the native DNA–daunomycin complex because of an inability to combine with the inaccessible NH_2 group. On the other hand, it would be free to complex with the NH_2 group

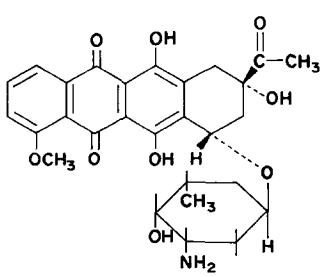

FIG. 8. Structure of daunomycin.

in either free daunomycin or that exposed in the sugar moiety when the double-stranded DNA denatures and unfolds. This would account for the appearance of the peaks at 540 and 582 nm (Fig. 3A and B), which were obtained at pH 5·2.

As the pH of the denatured DNA–daunomycin–cu complex was shifted from 5·2, the observed maxima at 540 and 582 nm disappeared, suggesting a dissociation of the NH_2–cu complex. As the pH was reduced to 3·6, the visible spectrum of the free daunomycin appeared but there remained a very much reduced fluorescence intensity. At this pH, daunomycin, in the presence of 10^{-4} M Cu^{2+}, exhibits no quenching. Studies in our own laboratories[8,9] as well as others[12] have demonstrated that Cu^{2+} binds to the amine group of guanine in denatured DNA. Cu^{2+} could thus hold the DNA as well as the daunomycin through a chelated complex formed between NH_2 and one or more OH groups, allowing for the apparent reduced FI. It would also still allow for what appeared to be the spectrum of the free daunomycin, i.e. that due to the aglycone chromophore.

Unlike Cu^{2+} (10^{-4} M), Mg^{2+} was completely without effect at concentrations less than 10^{-1} M. At this concentration, Mg^{2+} exerted what appeared to be a dual effect: (1) due to the high salt concentration, and (2) due to the specific divalent cation. In the main, there was a much closer correlation between spectral properties in the u.v. and visible region and the fluorescence characteristics, unlike that found with Cu^{2+}.

In the presence of 10^{-1} M Mg^{2+}, the DNA–daunomycin complex appeared to be most stable in the pH range 6·5 to 7·0. As the pH was reduced to 2·4 or raised to 8·0, Mg^{2+} tended to dissociate the complex. At pH 2·4, there was no further Mg^{2+} activity, and a spectral distribution for free daunomycin was found in both the u.v. and the visible. Above pH 6·5 and continuing on to pH 8·0, as the complex was dissociating, there was a strengthening of the daunomycin–Mg^{2+} complex, as evidenced by the appearance of the spectral maxima at 540 and 582 nm, very similar to the effect with Cu^{2+}. Parallel with these maxima, quenching of fluorescence was greatest at pH 7·8. Thus, it would appear that Mg^{2+}, much like Cu^{2+}, but at concentrations 10^3 greater, will form strong coordination complexes which involve the amino group of the sugar moiety and the O and/or OH groups of the aglycone portion of the daunomycin molecule.

Further studies are currently underway to elucidate the mechanism of binding.

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