Biochemical Pharmacology, Vol. 30, No. 14, pp. 2021–2024, 1981.
Printed in Great Britain.

0006-2952/81/142021-04 $02.00/0

SHORT COMMUNICATIONS

The effect of physiological levels of divalent metal ions on the interaction of daunomycin with DNA: evidence of a ternary daunomycin–Cu^{2+}–DNA complex

(*Received* 21 *August* 1980; *accepted* 20 *February* 1981)

Daunomycin and its derivatives (especially adriamycin) are now widely used in the treatment of cancers although the use of daunomycin is now largely restricted to the treatment of acute leukaemia [1]. The chemical, physico-chemical, biochemical and structure-activity relationships of these drugs have recently been extensively reviewed [2–4]. Although the major effect of these drugs is thought to result from their ability to intercalate into DNA [4], thereby inhibiting both DNA and RNA synthesis [5], other modes of action have also been proposed and have been reviewed by Donehower *et al.* [6].

Unfortunately, the clinical use of these drugs is limited by their cardiotoxicity, and this topic has also been the subject of recent reviews [3, 7]. The intense efforts to date to ameliorate this cardiotoxicity involve three separate rationales [7]: a search for less cardiotoxic derivatives, coupling of the drugs to carrier molecules and a search for antagonists of the cardiotoxicity. The reason that these concerted efforts have proved to be only partially successful probably reflects our incomplete knowledge of the mode of action of these drugs (and the relative contribution of the different modes), the mechanisms involved in cardiotoxicity, and the factors affecting both of these processes.

Understanding of the effect of cellular components on the drug–DNA interaction has been improved by Johnston *et al.* [8] and others [9, 10] who studied the effect of histones on the interaction. Fishman and Schwartz [11] have also investigated the effects of high levels of Cu^{2+} and Mg^{2+} ions on the drug–DNA interaction. We have extended their preliminary study and report here on the effect of physiological levels of relevant divalent metal ions on the daunomycin–DNA interaction.

Materials and Methods

Calf thymus DNA (Worthington, U.S.A.) was dissolved in 0.15 M KNO_3 with gentle agitation over 4 days at 4° and filtered (3 μm, Millipore, U.S.A.) to remove dust and undissolved DNA. Several drops of AR chloroform were added to prevent bacterial growth. DNA concentrations were determined spectrophotometrically using an extinction coefficient of 6600 M^{-1} cm^{-1} at 259 nm.

Daunomycin hydrochloride and [^{3}H]-daunomycin were supplied by Farmitalia, Milan and were used without further purification. Drug solutions were prepared fresh each day and the concentration determined using an extinction coefficient of 11,500 M^{-1} cm^{-1} at 480 nm. All divalent metal ions were AR grade.

A Perkin–Elmer MPF-4 spectrofluorimeter was used for all fluorescence quenching studies. The excitation wavelength was 485 nm (15 nm band width) and the emission wavelength 570 nm (5 nm band width). For binding studies, twenty 50 μl increments of approximately 10^{-4} M DNA (nucleotide) were added to 2.5 ml of 1.0 μM daunomycin. The fluorescence intensity of the completely bound drug was established by adding saturating amounts of DNA (2–5, 50 μl additions of 1 mM DNA).

An Orion Cu^{2+} specific ion electrode was used to determine free Cu^{2+} ion concentrations. For Cu^{2+}-drug binding studies, 100 μl increments of 10^{-4} M daunomycin were added to 10.0 ml of 10^{-5} M $Cu(NO_3)_2$ and the potential recorded to ±0.1 mV with an Orion Digital Ionalyzer, Model 801A (Orion Research Inc., Cambridge, MA).

Polarographic studies were performed on a Metrohm E506 Polarecord (Switzerland) in the differential pulse mode with a mercury drop time of 2.0 sec, a standard calomel reference electrode and a platinum auxillary electrode. Solutions were degassed for 15 min with argon.

Equilibrium dialysis studies were carried out using double-knotted dialysis tubing (size 20/32, Visking Co., Chicago, IL), pre-treated with boiling 5 per cent bicarbonate (30 min) to remove plasticisers. 10.0 ml of 6 μM DNA was placed inside the dialysis bag and 10.0 ml of 2.10^{-5} M [^{3}H]–daunomycin and varying Cu^{2+} ion concentration outside the dialysis bag. After 5 days of equilibration at 20 ± 1°, 1.0 ml samples from both inside and outside the dialysis bag were added to 10.0 ml of TRIPOP scintillation fluid (75 per cent xylene, 25 per cent triton, 0.3 per cent PPO, 0.02 per cent POPOP) and total cpm over 10 min were recorded with a Model 3003 Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., IL).

Low mol. wt DNA was prepared by a 5 min ultrasonic treatment of 20 ml of 10^{-4} M DNA in 0.15 M KNO_3, using a Rapidis 600 sonicator. The average mol. wt of the DNA before and after this treatment has been established by velocity sedimentation in this laboratory to be 6.10^6 and 10^5 daltons respectively [12].

Thermal denaturation of DNA was monitored at 259 nm in a Gilford 240 spectrophotometer equipped with a Gilford 2527 thermoprogrammer (Gilford Instrument Labs., Oberlin, OH) at a heating rate of 0.5°/min. All solutions were degassed with helium immediately prior to heating.

Results

Daunomycin–Cu^{2+} complex. The thermodynamics of the drug–Cu^{2+} interaction was studied by titrating Cu^{2+} ions with daunomycin and monitoring free Cu^{2+} ion concentration with a Cu^{2+} specific ion electrode. The titration was carried out in 0.15 M KNO_3 to maintain a constant activity coefficient for Cu^{2+} ions. From a linear Scatchard plot of these results, the association constant for the daunomycin–Cu^{2+} interaction was calculated to be 6.3 10^5 M^{-1} at 20°. The number of binding sites for daunomycin on Cu^{2+} was 0.50, indicating a $Cu(daunomycin)_2^{2+}$ complex. This is probably a chelate of Cu^{2+} bridged between the carbonyl and phenolic groups of two drug molecules.

Effect of Cu^{2+}. The effect of Cu^{2+} on the drug–DNA interaction could not be studied by fluorimetric titration since quenching of the daunomycin fluorescence by Cu^{2+} ions was detectable even at metal ion levels as low as 0.2 μM, consistent with that noted by Fishman and Schwartz [11]. Furthermore, both the DNA–drug and Cu^{2+}–drug contributions to either spectral or fluorimetric measurements would vary with total drug concentration. We therefore resorted to equilibrium dialysis of DNA–[^{3}H] daunomycin in the presence of varying Cu^{2+} levels, even though this method has considerable errors associated with it [13]. At equilibrium, the amount of drug associated with DNA increased with Cu^{2+} ion concentration up to 5 μM Cu^{2+} ions but was not enhanced further by the presence of 50 μM Cu^{2+} ions (Fig. 1). The dialysis was carried out in 0.15 M KNO_3, and this restricted the maximum Donnan redistribution [14] to less than 1 per cent of the total drug and Cu^{2+} ions present.

The above result was somewhat unexpected but was

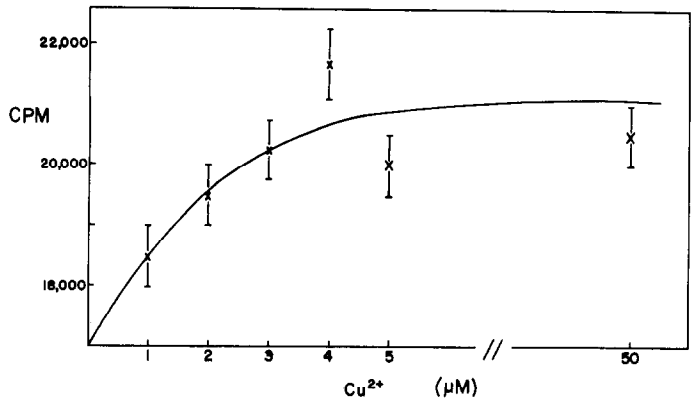

Fig. 1. Enhanced binding of [^{3}H]-daunomycin to DNA in the presence of micromolar levels of Cu^{2+} ions after equilibrium dialysis in 0.15 M KNO_3. Total radioactivity of the DNA–drug components was determined after 5 days of equilibration at 20°.

confirmed by differential pulse polarographic measurements of free drug in the presence of DNA and DNA–Cu^{2+} (Fig. 2). When Cu^{2+} ions were added to daunomycin, the major quinone peak at −0.63 V was reduced as the drug–Cu^{2+} complex increased, and this complex exhibited a polarographic peak at −0.16 V. Free Cu^{2+} ions exhibited a characteristic wave at +0.013 V under identical conditions, with a baseline at −0.16 V identical to that of the supporting electrolyte, 0.15 M KNO_3. The broad wave at approximately −0.05 V is due to residual oxygen which could not be displaced, even by extended and vigorous degassing with argon.

When DNA was added to daunomycin, the polarographic wave at −0.63 V decreased in accord with the known ability of the drug to bind to DNA [15]. When Cu^{2+} ions were also added, the wave at −0.63 V was depressed further (Fig. 2), indicating a lowered concentration of free drug (i.e. additional daunomycin has now bound to either DNA or to Cu^{2+} ions). Since the −0.16 V wave, characteristic of the drug–Cu^{2+} complex, was not observed, the additional daunomycin therefore appears to be bound to the DNA present.

Thermal stability of DNA–daunomycin–Cu^{2+} solutions. The thermal stability of DNA was enhanced by daunomycin (17.2°) and decreased by Cu^{2+} ions (1.7°), consistent with previous observations [4, 16]. However, when both ligands were present together, the effect was not additive (Cu^{2+} ions induced a 4.2° destabilisation compared to the DNA–daunomycin complex at a drug:DNA ratio of 0.1), suggesting some form of interaction between DNA and the two ligands. This interaction was even more clearly demonstrated by a 7.7° destabilization induced by Cu^{2+} ions at a drug:DNA ratio of 0.11 where the additional 10 per cent of daunomycin (compared to the 0.1 ratio solution) would be expected to contribute to an *increase* of DNA thermal stability if the drug was binding independently (of Cu^{2+} ions) to DNA. This relative destabilization induced by increasing drug concentration is not due to a decrease of the amount of the intercalated form of the drug, since it can be calculated that less than 0.1 per cent of the drug exists as the Cu^{2+}-(daunomycin)$_2$ complex at the concentrations employed. The relative destabilization of DNA by increasing daunomycin concentrations (in the presence of Cu^{2+} ions) is therefore due to a ternary interaction involving DNA and both ligands.

Effect of daunomycin on DNA–Cu^{2+} interaction. Increasing amounts of daunomycin were added to a solution of DNA and Cu^{2+} ions. The free Cu^{2+} ion concentration was monitored with a Cu^{2+} specific ion electrode. At the Cu^{2+}:DNA nucleotide ratio of 6 employed, all high affinity Cu^{2+} binding sites on the DNA are saturated [17]. The minimum free Cu^{2+} ion concentration is therefore approximately 19.6 μM, consistent with the value of 19.8 μM detected experimentally.

The free Cu^{2+} ion concentration decreased with increasing daunomycin concentration (Fig. 3). A decrease of free Cu^{2+} ions must occur since a Cu^{2+}-daunomycin complex will be formed. However, the maximum possible decrease of the free Cu^{2+} ion concentration due to the formation of Cu–(daunomycin)$_2^{2+}$ complexes is negligible (Fig. 3) compared to the observed decrease of the free Cu^{2+} ion concentration when DNA was also initially present. Cu^{2+} ions

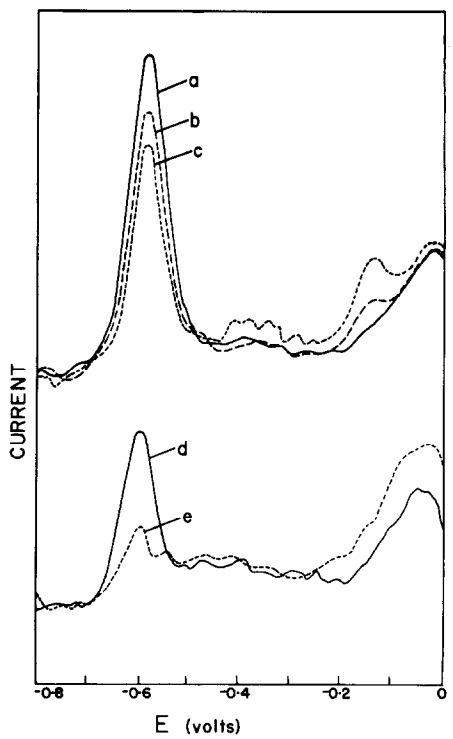

Fig. 2. Differential pulse polarograms of daunomycin (5 μM) in 0.15 M KNO_3 in the presence of: (a) no added Cu^{2+} or DNA; (b) Cu^{2+} (0.5 μM); (c) Cu^{2+} (1 μM); (d) DNA (5 μM); (e) DNA (5 μM) + Cu^{2+} (2 μM).

therefore appear to have enhanced affinity for the drug–DNA complex compared to the binding to DNA.

Ternary DNA-daunomycin-Cu^{2+} precipitates. During the course of our studies on the effect of daunomycin on DNA–Cu^{2+} complexes and the effect of Cu^{2+} ion on DNA–daunomycin complexes, we repeatedly observed a red to purple precipitate. The colour depended on the relative amount of Cu^{2+} ions present and was not due to pH changes. Precipitation was instantaneous at higher Cu^{2+} ion levels (25–100 μM; Da = 40 μM, DNA = 6 μM) and was observed down to Cu^{2+} ion levels as low as 2.5 μM (at the lower Cu^{2+} ion levels precipitation was time dependent—solutions were routinely left to stand for 1 hr and then centrifuged). At lower DNA concentrations of 3 and 1 μM, precipitation was observed down to Cu^{2+} levels of 5 and 100 μM respectively. These precipitation results were identical for DNA of molecular weight 6.10^6 or 5.10^5 daltons.

Effect of other divalent metal ions. The physiologically important divalent metal ions Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+} and Cd^{2+} had no effect on the Scatchard plot of the binding of daunomycin to DNA (n_{app} = 0.16 sites per nucleotide, K_{app} = 2.8 10^6 M^{-1}: see reference [18] for exact definitions of these terms) at metal ion levels corresponding to normal blood or serum levels [19–21]. At approximately 10-fold higher than normal levels, Mg^{2+} (10 mM) and Ca^{2+} (25 mM) reduced K_{app} to 1.10^6 M^{-1} and $0.6.10^6$ M^{-1} respectively, while Zn^{2+} (1 mM) reduced n_{app} to 0.13. (These elevated levels were to take account of the fact that specific cells or neoplastic cells could have metal ion levels considerably higher than normal.) Although 2 M Mg^{2+} is known to displace daunomycin from DNA [15, 22] the effect of Mg^{2+} ions at closer to physiological levels has not previously been appreciated.

Discussion

The mean total plasma Cu level has been determined [20, 21] as 114 μg/100 ml (*ca.* 2 10^{-5} M), of which only 7–10 μg/100 ml (1–1.5 μM) is associated with serum albumin (several high affinity sites exist but the majority are low affinity sites [23], with an association constant of 10^4 M^{-1}) and is therefore available to participate in equilibria with other ligands. Since the association constant for the drug–Cu^{2+} interaction is 6 10^5 M^{-1}, a significant amount of the drug may therefore exist as the Cu^{2+} complex when in blood.

At total Cu^{2+} ion concentrations as low as 1 μM,daunomycin binding to DNA appears to be enhanced (Figs. 1 and 2) and is consistent with the increase of DNA degradation by adriamycin when in the presence of Cu^{2+} ions [24]. In addition to the enhanced binding of daunomycin to DNA in the presence of low levels of Cu^{2+} ions, the metal ion itself also exhibits enhanced binding to DNA–daunomycin compared to binding in the absence of the drug.

The enhanced binding of *both* daunomycin and Cu^{2+} ions to DNA (compared to the binding to DNA in the absence of the second ligand) suggests a (Cu^{2+}–daunomycin) complex with DNA, this complex having a higher affinity for DNA than daunomycin itself. This conclusion is consistent with the non-additivity of the drug and Cu^{2+} ion contributions to DNA thermal stability, as this result also indicates the formation of a ternary DNA–drug–Cu^{2+} complex. The existence of such a ternary complex is most clearly manifested by the formation of DNA–daunomycin–Cu^{2+} precipitates.

Given the evidence for the existence of the ternary complex, it is tempting to suggest that the precipitate involves the intercalated form of the drug on one DNA molecule, bridged by a Cu^{2+} complex to daunomycin intercalated on another DNA molecule (i.e. a DNA–daunomycin–Cu^{2+}–daunomycin–DNA interstrand complex). With increasing concentrations of daunomycin and/or Cu^{2+} ions, the interstrand crosslinking would be enhanced, resulting in precipitation of the complex. DNA serum levels in various cancer patients [25] is (180 ± 40) ng/ml (cf. normal level of 13 ± 3 ng/ml), but can rise to 5000 ng/ml (i.e. DNA nucleotide blood levels encompass the 0.6–15 μM range in cancer patients). It is therefore disturbing to detect these precipitates at 'clinical' levels of DNA, Cu^{2+} and daunomycin. The daunomycin concentration was maintained at 40 μM for these precipitation studies, since this is within the range clinically employed [26] in order to minimise cardiotoxicity (the maximum suggested level of 550 mg/m^2 corresponds to approximately 50 μM blood level of the drug). Even at Cu^{2+} levels as low as 2.5 μM, precipitation

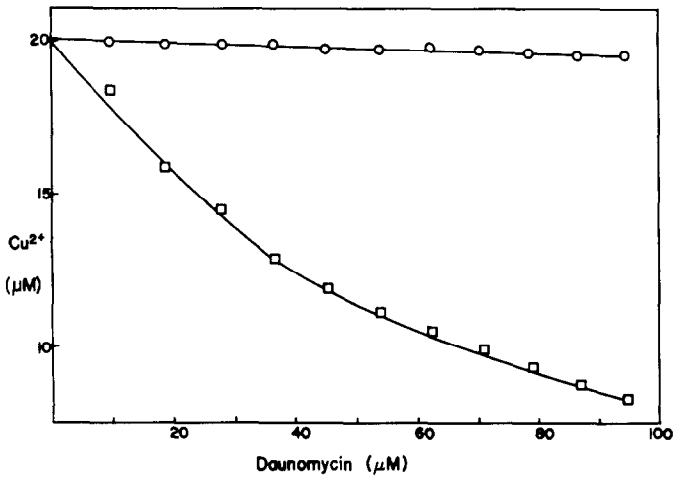

Fig. 3. Effect of daunomycin concentration on the binding of Cu^{2+} ions to DNA (□) in 0.15 M KNO_3, 25°. Initial Cu^{2+} and DNA concentrations were 20 μM and 6.2 μM respectively. All Cu^{2+} concentrations shown have been corrected to allow for dilution upon addition of daunomycin (100 μl additions, 0.96 μM). For comparison, the binding of Cu^{2+} ions to daunomycin is also shown (○).

was visibly evident with 6 μM of DNA nucleotide.

The clinical implications of these *in vitro* effects are, of course, completely unknown. However, it does appear that the possible role of ternary DNA–daunomycin–Cu^{2+} complexes should be considered with respect to the mechanism of action of daunomycin, the pharmacokinetics of the drug, membrane permeability, electrocardiographic changes and congestive heart failure, etc. induced by daunomycin.

SUMMARY

A ternary DNA–daunomycin–Cu^{2+} complex has been indicated by the enhanced daunomycin–DNA interaction in the presence of Cu^{2+} ions, the enhanced binding of Cu^{2+} to DNA in the presence of daunomycin, and non-additive thermal stabilization of DNA by Cu^{2+} and daunomycin. The ternary complex manifests itself most dramatically in the form of a precipitate at micro-molar levels of drug, Cu^{2+} ion and DNA nucleotide.

Acknowledgements—This work was supported by a grant from the Australian Research Grants Committee. We thank Farmitalia, Milan for the gift of daunomycin.

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