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The interaction of Fe(III), adriamycin and daunomycin with nucleotides and DNA and their effects on cell growth of fibroblasts (NIH-3T3)

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The interactions of the iron complexes of the anthracycline antitumour drugs daunomycin (DN) and adriamycin (ADM) with the mononucleotide AMP, herring sperm DNA, plasmic pBR322 and immortalized 3T3 fibroblasts were studied. By means of Mössbauer spectroscopy it was demonstrated that DNA is a powerful ferric iron chelator as compared with AMP, which is not able to compete with DN or acetohydroxamic acid for ferric iron. The difference between AMP and DNA is postulated to be based on the chelate effect. The Mössbauer spectra of the ternary Fe–anthracycline–DNA systems differ from Fe–anthracycline binary complexes, indicating rearrangement reactions. Dialysis experiments clearly disclose the formation of a ternary Fe–ADM–pBR322 complex, the topology of which differs substantially from intercalating ADM. The effect of Fe–ADM complexes (3:1) on the growth of immortalized mouse embryonal fibroblasts (NIH-3T3) was studied in comparison with ADM alone. No significant difference on the inhibition of cell growth was noticed, suggesting comparable cytotoxicity for the compounds. In contrast to literature data, no evidence was found for DNA cleavage by ferric ADM at molar ratios as high as 1/100 (ADM/base pair), even if the ternary systems were prepared in the light and in the presence of reducing or oxidizing agents. Based on our observations it seems that the cytotoxicity of both ADM and Fe–ADM oligomer is not based primarily on intercalation or direct interaction with DNA.

Keywords: iron, anthracyclines, adriamycin, daunomycin, acetohydroxamic acid, ternary complexes, Mössbauer spectroscopy

Introduction

The anthracycline antitumour antibiotics adriamycin (ADM) and daunomycin (DN) have attained a central position in the chemotherapeutic control of cancer (Wiernik 1980, Gianni *et al.* 1983, Neidle & Waring 1983, Fujiwara & Hoshino 1986, Gariner-Suillerot 1989). Recently, these drugs have also been found to inhibit the infectivity and replication of human immunodeficiency virus *in vitro*. The usefulness of these drugs in chemotherapy is, however, tempered by toxic side effects which include conventional toxicities and a dose-limiting cumulative cardiotoxicity (Young *et al.* 1981). Various models on the mode of cytotoxicity of anthracyclines have been proposed, including change of membrane properties, in particular of heart mitochondria (Goormaghtigh *et al.* 1987, Nicolay *et al.* 1987, Garnier-Suillerot & Gattegno 1988, Bradmante *et al.* 1989), binding to DNA by intercalation (Chaires *et al.* 1989, Wang *et al.* 1987, Skorobogaty *et al.* 1988, Ragg *et al.* 1988, Gresh *et al.* 1989), formation of a ternary anthracycline–DNA–topoisomerase II cleavage complex (Fritzsche & Wähnert 1987), inhibition of the RNA polymerase-catalysed synthesis of RNA (Kriebardis *et al.* 1987), formation of reactive anthracycline degradation products (Olson *et al.* 1988) and the formation of reactive radicals by metabolic processes (Doroshow 1983, Ashnagar *et al.* 1984, Abdella & Fisher 1985). It has been experimentally difficult to decipher which of these various biochemical events are responsible for the cyto- and cardiotoxicity of ADM and DN.

As a consequence of their capability to intercalate into cellular DNA, anthracyclines are able to inhibit DNA

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replication and RNA transcription both *in vivo* and *in vitro* (Chaires 1986). Molecular biological approaches demonstrated a DNA sequence specificity of both drugs: DN has been shown to prefer two adjacent GC base pairs, flanked on the 5′ end by an AT pair (Phillips & Crothers 1986, Chaires *et al.* 1987), while ADM induces a block of transcription immediately upstream of CpA sequences in systems for *in vitro* transcription (Skorobogaty *et al.* 1988, Triest & Phillips 1989).

ADM and DN bind avidly to a range of transition metals (Fiallo & Garnier-Suillerto 1986b, Maletesta *et al.* 1988), in particular ferric iron (Fiallo & Garnier-Suillerot 1985, Gelvan & Samuni 1988, Gelvan *et al.* 1990). There is now increasing evidence that Fe^{3+} is involved in the cytoxic effects of anthracyclines *in vitro* (Demant & Norskov-Laurtsen 1986, Samuni *et al.* 1986, Gianni *et al.* 1988, Hasinoff & Davey 1988, Hasinoff 1989, Minotti 1989) and *in vivo* (Beraldo *et al.* 1985, Sinha *et al.* 1987, Bellamy *et al.* 1988, Mimnaugh *et al.* 1989). Especially important is the observation that ADM can bind adventitious ferric iron ions *in vivo* (Fantine & Garnier-Suillerot 1986, May & Williams 1980). In the last few years much effort has been devoted to clarify the nature of the Fe–anthracycline systems. Initially, formation of a triferric ADM complex was proposed (Gosalev *et al.* 1978, Muindi *et al.* 1984) for which unchanged antitumour activity and decreased toxicity was reported (Muindi *et al.* 1984). More recent experiments, however, indicate that oligomeric Fe^{3+}-anthracycline associations are formed at millimolar concentrations. In contrast to spectrophotometric studies, it has been proposed recently that the Fe–ADM systems dissociate at micromolar concentrations and at low pH (Gelvan & Samuni 1988). Accordingly, it was suggested that, at physiological pH and at the ADM concentrations used in pharmacology, very little, if any, iron should be bound to ADM (Gelvan & Samuni 1988), although the possibility that ternary complexes with other molecules (i.e. DNA) may be formed could not be ruled out.

The possibility of time-dependent changes occurring in the Fe^{3+}–$(ADM)_3$ complex has also been investigated. By analysing the properties of the chelate at various times after preparation, it was found to degradate spontaneously to chelates with lower toxicity and altered iron-binding properties (Gelvan *et al.* 1990). In particular, it seems that Fe(III) accelerated ADM degradation to less toxic products; i.e. as ADM degrades, the iron is gradually transferred to one of the ADM degradation products and becomes less available to substitution by strong chelators (EDTA) and to reduction by biological reducing agents (ascorbate, cysteine or glutathione) (Gelvan *et al.* 1990). In contrast to the aged complex (24 h), administration of a freshly prepared (a few minutes) chelate is essentially equivalent to administration of free ADM (Gelvan *et al.* 1990). One of the goals of the present study was to validate these data.

Whereas a wealth of information is available on anthracycline–DNA binding (Kriebardis *et al.* 1987, Wang *et al.* 1987, Ragg *et al.* 1988, Skorobogaty *et al.* 1988, Gresh *et al.* 1989), little is known about the interaction of ferric anthracyclines with nucleotides and DNA (Eliot *et al.* 1984). At a DNA nucleotide/Fe–DN ratio of 1 and smaller, of a ternary DNA–Fe DN seems to be formed. Under more physiological conditions (i.e. nucleotide/drug ratio $\geqslant 50$), spectrophotometric results suggest that the free drug is intercalated between the base pairs after slow release of Fe^{3+} from Fe–$(DN)_3$ (Beraldo *et al.* 1985, Gelvan & Samuni 1988). The finding that the Fe–ADM complex is able to produce highly reactive oxygen-derived free radicals (Gianni *et al.* 1985, Lenkinski & Sierske 1985, Demant & Nørskov-Lauritsen 1986, Zweier *et al.* 1988) led to the suggestion that this complex is the drug active form *in vivo* and that its main (even if not the only: see Hannun *et al.* 1989) target is DNA. It has been reported that the complex is indeed able to bind DNA and catalyse its oxidative cleavage (Fantine & Garnier-Suillerot 1986).

As outlined above, the reported data are contradictory and accordingly our knowledge about Fe–anthracycline–DNA systems is still fragmentary. In order to illuminate this field in more detail we present an *in vitro* investigation, employing Mössbauer spectroscopic, and electrophoretic and dialysis techniques, of the interaction of the Fe and DN (1/3) and Fe–ADM (1/3) complexes with the mononucleotide AMP, poly A/U, herring sperm DNA and pBR322. Moreover, the effect of Fe–ADM complexes and of ADM alone on fibroblast growth in culture was analysed.

Materials and methods

Chemicals

3-Morpholinopropane sulphonic acid (MOPS) and DN–HCl were purchased from Fluka Chemie (Buchs, Switzerland) DN, ADM, acetohydroxamic acid (AHA), AMP, poly A/U and herring sperm DNA were from Sigma (Deisenhofen, Germany). ^{57}Fe (95% isotopically pure) was from Rohstoffeinfuhr (Düsseldorf, Germany). Desferrioxamine B was from the Institute stock. [^{14}C]ADM (specific activity 57 mCi mmol^{-1}) and $^{55}FeCl_3$ (specific acitity 1.6 mCi mg^{-1}) were purchased from Amersham (Italia, Milano, Italy). Doubly distilled water was used in all experiments. All other reagents were of analytical grade and purchased from Merck (Darmstadt, Germany)

Sample preparation

A $^{57}Fe(III)$ chloride stock solution was prepared by dissolving ^{57}Fe in a small volume of HCl. Prior to our experiments the pH of an aliquot was adjusted to 2.0 with KOH. The fresh preparation was required because of the tendency of Fe^{3+} to precipitate at pH 2. The metal concentration was determined photometrically with desferrioxamine B. DN and ADM were dissolved in 0.01 N HCl. MOPS buffer was chosen because of its low affinity for metal ions. The DN and ADM stock solutions were freshly prepared for each experimental series, and kept in the dark at −20°C until experiments were to be executed. Since DN and ADM are photochemically labile (Carmichael *et al.* 1983, Li & Chignell 1983, Nakano *et al.* 1984, Samuni *et al.* 1986) exposure of the solutions and of the various Fe–anthracycline systems to light was minimized. When iron

was mixed with anthracyclines, the pH was titrated with MOPS buffer within 1 h to pH 7.4 and adjusted to a final buffer concentration of 100 mM (Matzanke *et al.* 1992b). The final DN concentrations varied, depending on the experimental batch, between 1.5 and 6 mM while the iron concentrations varied bwtween 0.5 and 2 mM. Complex formation, pH adjustment and equilibration were performed at 4°C to slow down iron hydrolysis and to avoid possible degradation of anthracyclines (Jansen *et al.* 1985, Minotti 1989). The stability of ADM, ferric ADM and ferric daunomycin was checked by HPLC. The systems exhibited no significant decomposition within the time frame of the experiments when they were kept at 4°C in the dark.

Preparation of ternary complexes for in vitro *studies*

Plasmid DNA (pBR322) was mixed with the preformed binary Fe ADM complex at different molar ratios of ADM to DNA base pairs, either in the light or in the dark, in the presence or absence of reducing (glutathione) or oxidizing (hydrogen peroxide) agents. Unlabelled binary complexes were used to control 'DNA cleavage' activity of the metal chelate and in Mössbauer experiments: labelled complexes containing either [^{14}C]ADM or $^{55}Fe^{3+}$ were used in experiments aimed to analyse the DNA-binding properties of the binary complex and of ADM, respectively.

Mössbauer experiments

Equilibrated solutions were transferred to Mössbauer sample holders in the dark, frozen in liquid nitrogen and kept at this temperature until measurement. In order to achieve a good signal to noise ratio, sample thickness did not exceed 9 mm. Measurements were performed at 78 and 4.2 K. The Mössbauer cryostat was a helium bath type (MD306; Oxford Instruments, Oxford, UK) and a small field of 20 mT perpendicular to the γ-beam was applied to the tail of the bath cryostat using a permanent magnet. The Mössbauer spectra were recorded in the horizontal transmission geometry using a constant acceleration spectrometer operated in conjunction with a 512 channel analyser in the time-scale mode. The source was at room temperature and consisted of 1.85 GBq ^{57}Co diffused in rhodium foil (Amersham Buchler, Braunschweig, Germany). Isomer shifts are given relative to metallic iron (α-Fe) at room temperature. The calibration spectra exhibit a line width of typically 0.24 mm s^{-1}. Isomer shift, δ, quadrupole splitting, ΔE_Q, magnetic splitting, ΔE_M, and percentage of total absorption area were obtained by least-squares fits of Lorentzian lines to the experimental spectra. In the mixed ligand system Fe–AHA–DNA the $Fe(AHA)_3$ subspectrum was stripped from the measured spectra using the absorption pattern of the aqueous solution.

Electrophoretic analyses

Fe DNA, ADM–DNA and Fe–ADM–DNA complexes were analysed by gel electrophoresis on 1.0% agarose, in 20 mM MOPS, pH 7.4, either immediately after preparation of the mixtures or after a 3 h lag. After running, gels were stained in ethidium bromide (2 mg ml^{-1} in distilled water) for 20 min and photographed under UV illumination through a red filter. The negatives of the photographs were scanned, where necessary, at 560 nm using a Beckman DU-8 spectrophotometer to compare band intensities. The planimetry of the resulting densitometer scans was used to calculate the relative abundance of each DNA form. Radioactive bands were fluorographed by exposing dried gels to Kodak XS films at −80°C for appropriate intervals.

Equilibrium dialysis

$^{55}FeCl_3$–DNA, [^{14}C]ADM–DNA, $^{55}FeCl_3$–ADM–DNA and $FeCl_3$–[^{14}C]ADM–DNA complexes (final volume 1 ml each, in 20 mM MOPS, pH 7.4) were dialysed against 5 ml of 20 mM MOPS for 24 h. [^{14}C]ADM and $^{55}FeCl_3$ were also dialysed in parallel as controls. After dialysis, aliquots (100 ml), taken either from the dialysis bag or from the external buffer, were spotted onto filter paper discs, dried and counted. The number of counts per millilitre were used to calculate inside and outside concentrations of ADM and iron.

Cell cultures and drug effects on cell growth

Immortalized mouse embryonal fibroblases (NIH-3T3) were harvested and detached from a subconfluent culture by successively rinsing with PBS^0 (Ca^{2+}/Mg^{2+}-free phosphate buffer saline), 1 mM EDTA in PBS^0 and 1 mM EDTA in PBS^0 containing 5 mg ml^{-1} trypsin. Cells were counted and plated at a concentration of 2.0×10^5 cells per 24 cm^2 flask in Dulbecco's modified Eagle's medium (DMEM), supplemented with antibiotics and 10% fetal calf serum.

After 2–3 h (the minimum time necessary for the cells to adhere to the flask surface), sterile ADM or Fe–ADM complex was added to the medium at different concentrations (ranging from 0.017 to 17 mM ADM). After 1 h incubation, the medium was removed and cells were grown for an additional 72 h in drug-free fresh medium. Untreated cells were used as a control.

To measure the drug effect on cell growth, cells treated as described above were harvested by trypsinization, pelletted and dissolved in 0.1 M NaOH at 50°C for 30 min and the absorbance of the solution was then measured at 260 nm, essentially as described in (Giulotto *et al.* 1987).

Drug uptake in cell nuclei

To evaluate the amount of ADM or Fe–ADM complex accumulated in cell nuclei, contact-inhibited cells were incubated for 1 h with [^{14}C]drug and immediately harvested by trypsinization. Nuclei were purified as described (Di Liegro *et al.* 1985). Briefly, harvested cells were washed twice in cold PBS, resuspended in nuclei buffer (NB: 0.32 M sucrose; 50 mM sodium phosphate buffer, pH 6.5; 50 mM KCl; 0.15 mM spermine; 0.5 mM spermidine, 2.0 mM EDTA; 0.5 mM EGTA; 1.0 mM phenylmethylsulphonyl fluoride) and homogenized with several strokes at a tight-fitting pestle in a Dounce

homogenizer. Nuclei were collected by centrifuging at 1000 *g* for 10 min and further prufied by centrifugation through a cushion of 1.0 M sucrose in NB, at 42000 *g* for 30 min. The nuclear pellet was washed once in reticulocyte saline buffer (RSB: 10 mM NaCl; 10 mM Tris–HCl, pH 7.4; 1.5 mM $MgCl_2$). Drug uptake was evaluated by counting samples in a Beckman L-7500 scintillation counter.

Results

Mössbauer spectroscopy

Figure 1(A) shows a Mössbauer spectrum of the Fe–DN (1/3) system at millimolar concentration prepared at pH 2. The system was allowed to equilibrate at pH 7.4 for 24 h. Two components are visible: a very broad magnetically split species [71.7(2)% of absorption area] and a quadrupole doublet [28.3(2)% of absorption area]. It remains spectroscopically stable over a time range of at least 50 h. The Mössbauer spectra of ferric ADM and DN at millimolar concentrations have been analysed elsewhere in detail (Matzanke *et al.* 1992a,b, Capolongo *et al.* 1996). The Mössbauer sextet observed in Figure 1(A) corresponds to superparamagnetic polynuclear aggregates which are magnetically highly anisotropic and exhibit a blocking temperature higher than 4.2 K. The quadrupole doublet is also related to a superparamagnetic system exhibiting, however, much less magnetic anisotropy and a blocking temperature lower than 4.2 K. These cooperative phenomena are a consequence of stacking effects of DN (Bathelmy-Clavey *et al.* 1974, Foster & Foreman 1974, Eksborg 1978, McLennan *et al.* 1985, Matzanke *et al.* 1992a,b, Capolongo *et al.* 1996). The ratio of the doublet and the sextet species of the Mössbauer spectra is a function of the metal/ligand ratio, with the ligand in excess only the doublet species is formed.

In Figure 1(B) the Mössbauer spectrum is depicted for the same system mixed, however, with a 468-fold molar excess of AMP. The spectrum is dominated by a broad and featureless magnetic component. Only a trace of a quadrupole doublet can be observed. The magnetic species is similar to the corresponding component in Figure 1(A). The spectra shown in Figure 1(C and D) display interaction of Fe–DN (1/3) with poly A/U (200-fold excess of nucleotides) and with herring sperm DNA (100-fold excess of nucleotides), respectively. The spectra are dominated by a broad magnetic sextet. Only traces of a quadrupole doublet are detectable. The spectral features of the magnetic components are similar to those found for the interaction of Fe–DN (1/3) with AMP and compare well with that in Figure 1(A).

Figure 2(A) displays the Mössbauer spectrum of Fe–ADM (1/3, pH 2). Again two components, a quadrupole doublet (30.6% of absorption area) and a broad magnetically split sextet (69.4% of absorption area), are discernable. Although the magnetic splitting of the sextet differs from that observed of Fe–DN (1/3), the general features of the spectra are the same. In Figure 2(B) the spectrum of the mixed system Fe–ADM (1/3) herring sperm DNA (100-fold excess) is shown after 24 h of equilibration with DNA. Whereas the magnetic species in Figure 2(A and B) are very similar, the quadrupole doublet has disappeared in the latter.

Due to the extreme line width of the ferric anthracycline spectra we decided, for comparison, to study the interaction between a monomeric complex of iron with DNA. We have chosen AHA. Ferric ions form stable 1/3 complexes with AHA in aqueous solution, exhibiting a stability constant of $\beta_3 = 28.3$ (Schwarzenbach & Schwarzenbach 1963). The corresponding Mössbauer spectrum is shown in Figure 3(A). The distinct, complex six-line pattern arises from a $S = 5/2$ system in a rhombic crystal field. No dramatic change of

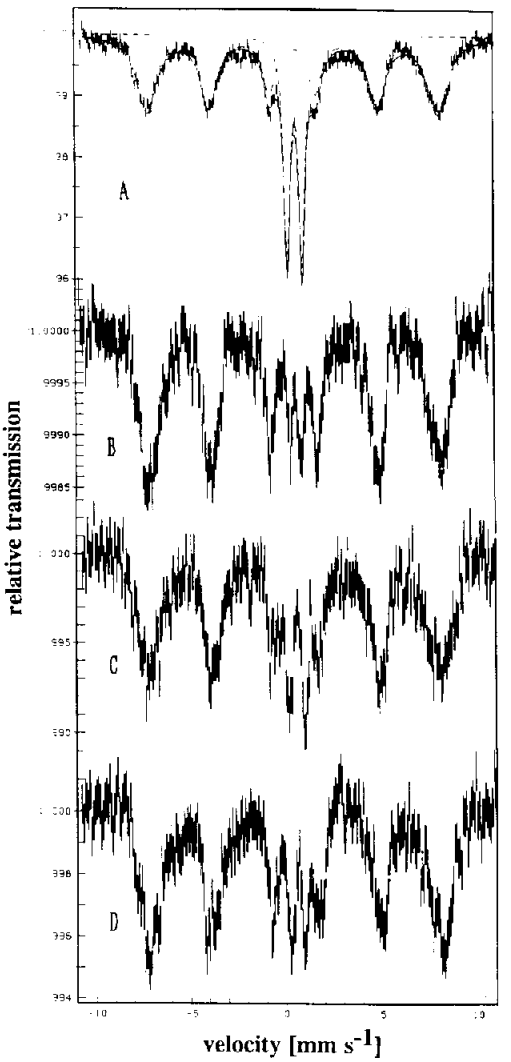

Figure 1. Mössbauer spectra of a Fe–DN (1/3) system prepared at pH 2 and equilibrated at pH 7.4 in 0.1 M MOPS buffer for 24 h: spectrum (**A**) without any addition (magnetic sextet species: $\delta = 0.48$ mm s^{-1}, $\Delta E_Q = -0.03$ mm s^{-1}, $\Delta E_M = 15.22$ mm s^{-1}, $\Gamma_{1/2} = 1.26$ mm; quadrupole doublet: $\delta - 0.59$ mm s^{-1}, $\Delta E_Q = 0.78$ mm s^{-1}, $\Gamma_{1/2} = 0.38$), (**B**) 200 mg AMP added, (**C**) +100 mg poly A/U and (**D**) +100 mg herring sperm DNA. The mixed systems (**B**)–(**D**) were allowed to equilibrate for an additional 24 h. The spectra were measured at 4.2 K in a field of 20 mT perpendicular to the γ-rays.

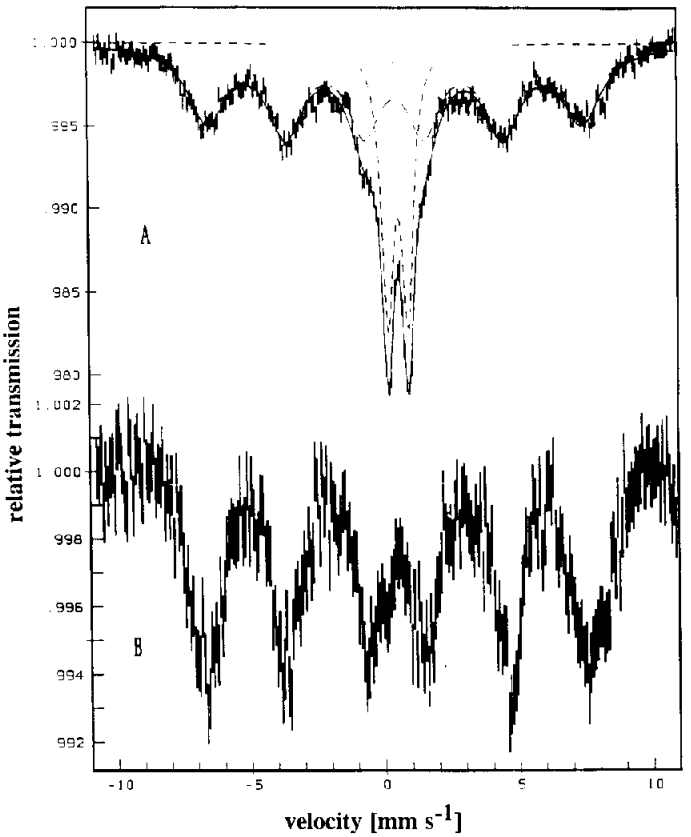

Figure 2. Mössbauer spectra of Fe/ADM(1/3) without any addition (**A**) (sextet: $\delta = 0.48(3)$ mm s^{-1}, $\Delta E_Q = -0.01$ mm s^{-1}, $\Delta E_M = 14.11$ mm s^{-1}, $\Gamma_{1/2} = 1.31(9)$ mm s^{-1}; doublet: $\delta = 0.58$ mm s^{-1}, $\Delta E_Q = 0.67$ mm s^{-1}, $\Gamma_{1/2} = 0.39$ mm s^{-1}) and with 100 mg herring sperm DNA (**B**). Preparation procedures and Mössbauer measurements are the same as described in Figure 1.

the ferric iron ligand sphere is observed when $Fe(AHA)_3$ is mixed with AMP (468-fold excess, Figure 3B), merely a slight line broadening is detectable, which probably arises from relaxation effects. Surprisingly, however, a drastic change is observed when $Fe(AHA)_3$ is mixed with herring sperm DNA (100-fold excess; Figure 3C). The spectrum is complex and contains at least two magnetic components. Twenty percent of the total absorption area arises from $Fe(AHA)_3$ as demonstrated by subtracting the spectrum of $Fe(AHA)_3$ (Figure 3A) from the Fe–AHA–DNA spectrum (Figure 3C); however, the residual spectrum (Figure 3D) is not well defined.

In vitro *studies*

Figure 4 shows an agarose gel on which plasmid DNA, DNA–ADM and DNA–Fe–ADM was run (at a ratio of 1 nmol ADM per 100 nmol DNA base pairs). Surprisingly, there is no evidence of drug-induced nicking of DNA (evaluated as the ratio between relaxed and supercoiled forms of pBR322 DNA), even in the presence of either a reducing or an oxidizing agent. The same holds true when the ternary systems are prepared in the light.

In order to clarify this point, we repeated the electrophoresis at higher ratios of ADM to DNA base pairs. The corresponding agarose gel stained with ethidium bromide is shown in Figure 5(A). Lane (b) of the gel displays the DNA–ADM system at a ratio of 1 nmol ADM per 10 nmol DNA base pairs. The supercoiled form of pBR322 had disappeared indicating that the DNA is relaxed (see for comparison Figure 5B, lane b). However, again no similar effect is found in the various DNA–Fe and DNA–ADM–Fe systems. This observation suggests that ADM, when alone, intercalates into DNA and induces relaxation; on the other hand, if complexed with iron, ADM seems unable to do the same. Moreover, a small amount of DNA remains at the origin in both the Fe–DNA and the Fe–ADM–DNA system.

In order to gain information about the association between DNA, ADM and iron, radioactive labeled [^{14}C]ADM and ^{55}Fe were employed in the gel electrophoresis experiments previously described. Figure 5(B) displays a fluorogram of the gel shown in Figure 5(A). On lane (b) (ADM–DNA binary complex) radioactive ADM is clearly visible in a position corresponding to that of relaxed circular DNA, confirming the result shown in Figure 5(A, lane b). Lane (c) displays the interaction of ferric iron with plasmid DNA. The major portion of iron precipitates in the gel slot (lane c) and at last some of the metal is bound to DNA yielding the non-mobile form of DNA. Only a minor portion of iron is migrating in the gel. The system

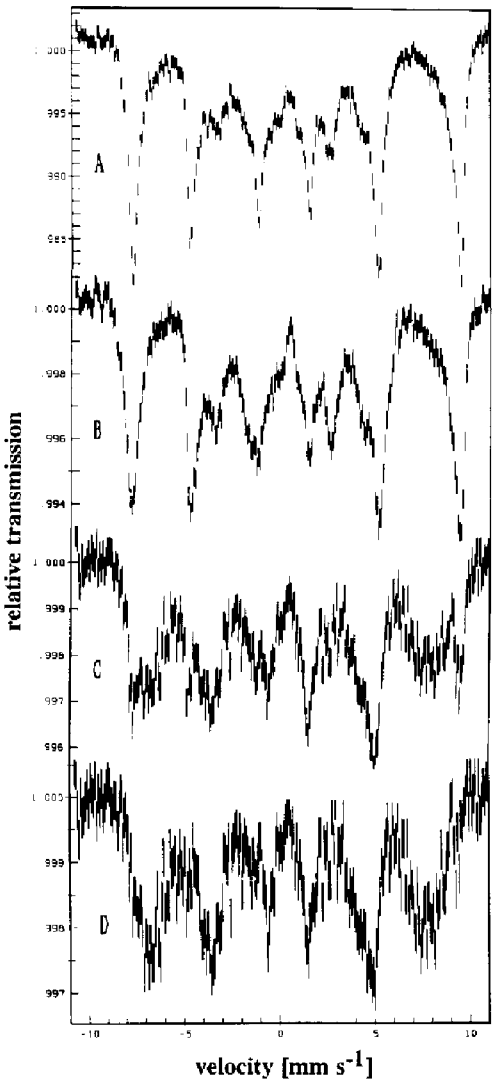

Figure 3. Mössbauer spectra of an frozen aqueous solution of $Fe(AHA)_3$ **(A)**, of $Fe(AHA)_3$ mixed with 200 mg AMP **(B)** and of $Fe(AHA)_3$ mixed with 150 mg herring sperm DNA **(C)**. Spectrum **(D)** represents spectrum **(C)** after subtraction of spectrum **(A)** (20% of the total absorption area). Sample preparation and recording of the spectra were performed under the same conditions as described in Figure 1.

Fe–[^{14}C]ADM–DNA is displayed on lane (d) (ADM/base pair ratio = 1/50) and on lane (e) (ADM/base pair ratio = 1/10). In the ternary system only a small amount of ADM is bound to DNA which remains, in contrast to the binary system, in its supercoiled form. A portion of ADM is retained at the origin. The migration pattern of the same ternary systems, labelled with ^{55}Fe, are shown in lanes (f) and (g). Again significant activity is retained in the pockets. We attribute the finding of ^{55}Fe and of [^{14}C]ADM activity at the origin to an electrophoretically non-mobile ternary Fe–ADM–DNA complex. In addition to a ADM/base pair ratio of 1/10, activity is smeared along the lane. This indicates

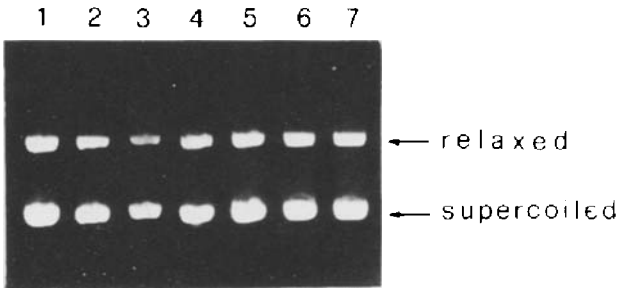

Figure 4. Electrophoretic analysis of plasmid DNA (pBR322) complexed or not with either ADM or Fe ADM chelate. Binary and ternary complexes were prepared as described in the text, at a molar ratio of 1 nmol ADM per 100 nmol DNA base pairs, and analysed on a 1% agarose gel in 20 mM MOPS: **(1)** untreated pBR322; **(2)** plasmid DNA–ADM; **(3)** DNA–ADM complex prepared in the presence of 10 mM hydrogen peroxide; **(4)** DNA–ADM complex prepared in the presence of 10 mM glutathione; **(5)** Fe–ADM ternary complex; **(6)** DNA–Fe–ADM ternary complex prepared in the presence of 10 mM hydrogen peroxide; **(7)** DNA–Fe–ADM ternary complex prepared in the presence of 10 mM glutathione. The mixtures used in this particular experiment were 3 h aged; identical results (not shown) were obtained with freshly prepared samples.

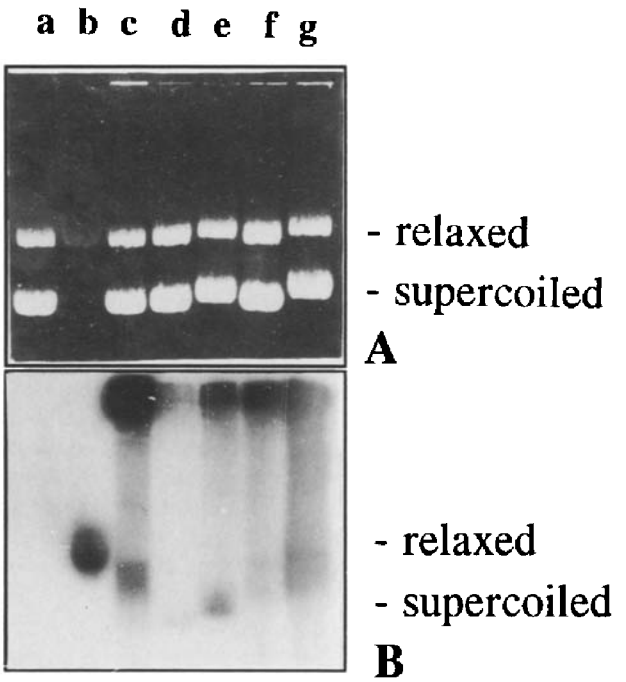

Figure 5. Interaction of ADM and Fe–ADM chelate with plasmic DNA. **(A)** Ethidium bromide staining; **(B)** fluorography. Binary and ternary complexes were prepared as described in the text and analysed on a 1% agarose gel, in 20 mM MOPS: (a) pBR322; (b) plasmid DNA–[^{14}C]ADM complex (1 nmol ADM per 10 DNA base pairs); (c) DNA–$^{55}FeCl_3$ complex; (d) DNA–[^{14}C]ADM–$FeCl_3$ complex (1 nmol ADM per 50 DNA base pairs); (e) DNA–[^{14}C]ADM–$FeCl_3$ complex (1 nmol ADM per 10 DNA base pairs); (f) DNA–ADM–$^{55}FeCl_3$ complex (1 nmol ADM per 50 DNA base pairs); (g) DNA–ADM–$^{55}FeCl_3$ complex (1 nmol ADM per base pairs).

Table 1. Equilibrium dialysis of ADM and $FeCl_3$ free or bound in complexes

Samples	[^{14}C]ADM (in)/[^{14}C]ADM (out)	$^{55}FeCl_3$ (in)/$^{55}FeCl_3$ (out)
Simple compounds		
[^{14}C]ADM	1.03 ± 0.43 (3)	
$^{55}FeCl_3$		1160 ± 517 (3)
Binary complexes		
[^{14}C]ADM–$FeCl_3$	4.9 ± 1.7 (3)	
ADM–$^{55}FeCl_3$		479 ± 154 (3)
[^{14}C]ADM–DNA	90 ± 16.9 (3)	
$^{55}FeCl_3$–DNA		102 ± 16 (3)
Ternary systems		
[^{14}C]ADM–$FeCl_3$–DNA	18.9 ± 5.6 (3)	
ADM–$^{55}FeCl_3$–DNA		308 ± 36 (3)

The measured counts per minutes were used to calculate inside and outside concentrations of drug and iron. Numbers in parentheses indicate the number of independent experiments.

dissociation of ferric iron from an electrophoreticly mobile form of the Fe–ADM–DNA aggregate. Moreover, some significant amount of ^{55}Fe is detectable at approximately the position of relaxed DNA. In summary, the ternary systems exhibit two different forms: a major non-mobile pool (small amounts of DNA, most of metal and drug) and a smaller mobile one (large amounts of DNA, small amounts of metal or drug). Mobile forms indicate DNA binding but there is no coincidence in the position of [^{14}C]ADM and ^{55}Fe. It seems that the ternary system decomposes during electrophoresis, at least in part.

Because we could not rule out the possibility that the electric field might influence the stability of the ternary complex, we further tested the properties of the complexes in dialysis experiments (MMCO cutoff of dialysis membranes: 12–14 kDa). These data are summarized in Table 1. As expected, free iron precipitates due to its low solubility ($K_{Sp} = 10^{-38.3}$) and is retained in the dialysis bag. ADM, on the other hand, diffuses freely out of the bag and readily equilibrates. After dialysis of the Fe–ADM system, iron is again kept back in the tube but also most of the ligand (83%) is also retained, indicating the formation of polymeric Fe–ADM complexes. A minor portion of the ligand remains mobile and is able to diffuse. In the ADM–DNA system almost all the drug is retained in the dialysis tube. This is expected and reflects intercalation of ADM into DNA. The binding of ADM to DNA in the ternary complex is less tight compared with the binary system.

Drug sensitivity of fibroblasts

3T3 cells were treated for 1 h with either ADM or Fe–ADM (1/3 system). After 72 h of additional growth, cell cultures were analysed. The corresponding data are reported in Table 2. Compared with a untreated control the cell numbers of cultures after drug treatment dropped considerably. The survival rate was dependent of the concentration of the administered drug. However, treatment with Fe–ADM compared with ADM alone did not result in any significant difference of morphology analysed by microscopy (data not shown) and survival rate.

Table 2. Sensitivity of 3T3 cells to treatment with either ADM or $FeCl_3$–ADM complex

ADM (μM)	Cell survival (%)	$FeCl_3$–ADM[a] (μM)	Cell survival (%)
	100	—	100
0.17	86 ± 4 (3)	0.17	95 ± 2 (3)
1.7	50 ± 5 (3)	1.7	48 ± 6 (3)
17	19 ± 3 (3)	17	21 ± 4 (3)

[a]The reported concentrations refer to ADM.
Numbers in parentheses indicate the number of independent experiments.

Drug uptake in fibroblast nuclei

Cells were treated as described in Materials and methods; in each experiment, two flasks were processed in parallel for each condition. After harvesting the cells, nuclei were purified as described above. Aliquots of the final nuclear pellets were used to measure DNA content and [^{14}C]ADM uptake. We have calculated an average value of 1 nmol of ADM per 1000 nmol of DNA base pairs, after treatment with either ADM or Fe–ADM complex.

Discussion

Spectroscopic changes are not observed in the Mössbauer spectra of the magnetically split sextet aggregates of ferric DN compared to the mixed systems with AMP (Figure 1B), poly A/U (Figure 1C) and herring sperm DNA (Figure 1D), although the extreme line widths could mask additional magnetic components. However, the doublet species almost disappears in all mixed systems presented here. In the presence of nucleotides or DNA the equilibrium between

doublet and sextet aggregates is obviously shifted almost completely to the side of the sextet species, indicating release of DN and ADM. From this it can be concluded that a rearrangement of the complex takes place in the ternary systems. However, it could not be discerned at this level whether the disappearance of the doublet indicates the formation of a new ternary complex or whether it reflects dissociation of the binary complex followed by intercalation of the free drug.

Since no Mössbauer spectroscopic data were available on ferric iron binding to DNA it could not be completely excluded at this point that the extremely broad line widths of the sextet mask an additional species which would indicate metal–DNA binding or the formation of ternary complexes. Therefore we investigated the interaction of the monomeric complex $Fe(AHA)_3$ with DNA. The stability constant of $Fe(AHA)_3$ ($\beta_3 = 28.3$; Schwarzenbach & Schwarzenbach 1963) is comparable to that of $Fe(DN)_3$ ($\beta_3 = 28.4$; Beraldo *et al.* 1985). AHA has no extended π system which, therefore, excludes intercalation and stacking effects (Bathelmy-Clavey *et al.* 1974, Foster & Foreman 1974, Eksborg 1978, McLennan *et al.* 1985) and consequently no interaction with AMP (Figure 3B) can be observed. Surprisingly, however, the interaction of $Fe(AHA)_3$ with DNA results in the formation of a second magnetic species (Figure 3C and D). A ligand function of OH^- and formation of $Fe(OH)_3$ can be excluded in this system, because $Fe(AHA)_3$ is very stable in aqueous solution at pH 7 (e.g. Figure 3A and B). Therefore DNA itself must be the competing ligand. Since mononucleotides are unable to abstract iron from the $Fe(AHA)_3$ complex (Figure 3B), the ability of DNA to compete for iron must be a consequence of chelate effects (Pitt & Martell 1980). The chelate effect arises because of (i) favourable stability constants associated with the formation of compact multidentate structures and (ii) a concentration factor (Pitt & Martell 1980). The spectral pattern of the Fe–DNA or Fe–AHA–DNA complex is extremely broad and ill defined. The structure of this system is not clear yet and should be analysed in detail in a future study. However, it is likely that the phosphate groups participate in iron binding.

Based on these experiments it is safe to state the DNA is a potent ferric iron chelator capable of extracting iron from $Fe(AHA)_3$ or of forming ternary Fe–AHA–DNA complexes. Because the complex formation constants of ferric anthracyclines are comparable to those of $Fe(AHA)_3$, DNA should similarly extract iron from Fe–anthracycline forming Fe–DNA binary complexes or Fe–anthracycline DNA ternary complexes. Unfortunately, the extremely broad line widths of magnetic split Mössbauer species prevent a detailed analysis.

The dialysis experiments clearly demonstrate that the uncomplexed drug binds DNA more tightly (in/out ratio: 90) than the Fe–ADM complex (in/out ratio: 18.9). Moreover, in the ternary complex ADM is much tighter bound than in the Fe–anthracycline system. From this it can be concluded that, in fact, a ternary Fe–ADM–pBR322 complex is formed. A small amount (5.6%) of the drug is not bonded to DNA in the ternary system and can diffuse as indicated by the in/out ratio of the equilibrium dialysis. If intercalatable DNA would be present in this system, the in/out ratio should be comparable to that of the drug–DNA system. This is not the case. We attribute, therefore, this finding to topological differences between pure DNA and the ternary complex hindering the free drug from intercalation.

On the other hand, the fluorograms of the gel electrophoresis experiments clearly disclose three forms of association of the ternary Fe–ADM–DNA systems: (i) a non-mobile ternary complex; (ii) a small portion of ADM–DNA binary complex in the supercoiled form (due to the small amount of free ADM the drug is obviously unable to grossly relax the DNA) and (iii) a small portion of an Fe–relaxed DNA complex. Most of the DNA in these systems is mobile on the agarose gels, carrying only small amounts of drug or ferric iron, whereas the bulk of the drug and iron are retained at the origin with small amounts of pBR322. Based on the electrophoresis results it would be expected that the concentration of supercoiled DNA should be sufficient to bind mobile ADM via intercalation. Since this is not the case, we therefore infer that the three diverse forms of interaction between ferric anthracyclines and DNA found in the gel electrophoresis are a result of the applied fields and hence do not reflect the equilibrium in solution.

In summary, these results suggest that the interaction of the Fe–anthraccyline complex with DNA differs substantially from the intercalating binary system anthracycline–DNA. In particular, anthracyclines in the ternary system are less tightly bound to DNA and probably do not intercalate.

In cell cultures of 3T3 fibroblasts we could not observe significant differences of the cytotoxic action between ADM and the freshly prepared Fe ADM oligomeric complex and only small quantities of the drug are found in the nucleus. Together these observations suggest that the cytotoxicity of both ADM and Fe–ADM oligomer is probably not based primarily on intercalation or direct interaction with DNA. In addition, these results confirm data reported for a different immortalized cell line. It remains to be disclosed in a future study whether ferric anthracycline oligomers are less cardiotoxic than the free drug. If this would turn out to be true, a potential application of ferric anthracycline oligomers in clincal therapy of cancers might be at hand.

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