Daunomycin modifies the sequence-selective recognition of DNA by actinomycin

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ABSTRACT

The antitumour antibiotic actinomycin D normally binds to DNA by intercalation at sequences containing the CpG step, but in the presence of daunomycin it has been reported to interact with poly(dA–dT). This observation has neither been confirmed nor explained. Here we have used a photoreactive 7-azido derivative of actinomycin to study the effect of daunomycin on its binding to three DNA fragments. Daunomycin did indeed alter the binding of actinomycin to the DNA, such that the antibiotic was displaced from its primary GpC sites onto secondary sites in the DNA, though not to AT regions especially. These findings suggest a possible scientific explanation for the increased toxicity seen during combination chemotherapy with these two drugs.

INTRODUCTION

The clinically useful antitumour drugs actinomycin and daunomycin form intercalation complexes with DNA and exert their antibiotic activities at the nucleic acid level (1). Both drugs recognize specific sequences in DNA: actinomycin binds selectively to GpC-containing sequences (2–6) whereas daunomycin shows a preference for 5'-(A/T)GC and 5'-(A/T)CG triplets (7–9). Although actinomycin does not normally intercalate into AT-rich sequences, Krugh and Young (10) reported that daunomycin or its analogue adriamycin facilitate strong binding of actinomycin to the synthetic polymer poly(dA–dT)·poly(dA–dT). We report below our investigation aimed at determining whether the synergistic effect observed by Krugh and Young with a synthetic polymer takes place in natural DNA containing mixed AT/GC sequences.

Our strategy was to examine the effect of daunomycin on the photoreaction of 7-azidoactinomycin (11,12) with DNA. The photolabelling experiments together with DNAase I footprinting assays reveal that daunomycin markedly affects the recognition of defined DNA sequences by actinomycin. The presence of intercalated daunomycin molecules appears to displace azidoactinomycin from its primary GpC sites and permits the

photosensitive compound to react at non-GpC-containing sequences, but not necessarily with AT-rich clusters. We interpret these observations in terms of drug-induced structural changes in DNA.

MATERIALS AND METHODS

Chemicals and biochemicals

Ammonium persulphate, Tris base [tris(hydroxy)methylamino-methane], acrylamide, bis-acrylamide, ultrapure urea, boric acid, tetramethylethylenediamine and dimethyl sulphate were from BDH. Formic acid, piperidine and formamide were from Aldrich Chemicals Inc. Calf thymus DNA (highly polymerized sodium salt) was purchased from Sigma Chemical Co. and deproteinized with two phenol/chloroform extractions then extensively dialyzed against the appropriate buffer. Bovine pancreatic deoxyribonuclease I (DNAase, EC 3.1.21.1, Sigma Chemical Co.) was stored as a 7200 U/ml solution in 20 mM NaCl, 2 mM MgCl₂, 2 mM MnCl₂, pH 7.3, at –20°C, and was freshly diluted to the desired concentration immediately prior to use. Restriction endonucleases *Eco*RI and *Pvu*II (Boehringer Mannheim, Germany) were used according to the supplier's recommended protocol in the activity buffer provided. Alkaline phosphatase, AMV reverse transcriptase and T4 polynucleotide kinase were from Pharmacia. [α -³²P]dATP and [γ -³²P]ATP (6000 Ci/mmol) were purchased from New England Nuclear. Unlabelled forms of ATP and dATP (ultrapure grade) were purchased from Pharmacia.

Drugs

The initial stock solution was made by dissolving 2 mg of actinomycin D (Sigma Chemical Co.) in 5 ml of water at 4°C. Daunomycin (Sigma Chemical Co.) was first dissolved in water to give a 1 mM stock solution. Subsequent dilutions of the drugs were made with 10 mM Tris, 10 mM NaCl buffer, adjusted to pH 7.0. Drug concentrations were determined photometrically applying molar extinction coefficients of 25,300 M⁻¹ cm⁻¹ at 425 nm and 11,500 M⁻¹ cm⁻¹ at 477 nm for actinomycin and daunomycin respectively. 7-Azidoactinomycin was synthesized

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according to a published procedure (11). Because of its photosensitivity, the drug was handled exclusively under photographic safelight conditions. The photoreactive compound was dissolved in water (1 mM stock solution), divided into small aliquots, quickly lyophilized and then stored in sealed 0.5 ml Eppendorf tubes at -70°C (in the dark). A fresh solution was made each time prior to the reaction with DNA by redissolving an aliquot in buffer to yield the desired concentration.

DNA purification and labelling

The plasmids pUC12, pBS and pCB2 were isolated from *E. coli* by a standard sodium dodecylsulphate–sodium hydroxide lysis procedure and purified by banding in CsCl–ethidium bromide gradients. Ethidium was removed by several isopropanol extractions followed by exhaustive dialysis against Tris–EDTA buffer. The purified plasmid was then precipitated and resuspended in appropriate buffer prior to digestion by the restriction enzymes. Three DNA fragments were used: a 178mer, a 265mer and a 170mer cut from plasmids pUC12, pBS and pCB2 respectively. To clone the 48 base pair AT-rich fragment, a synthetic 52mer oligonucleotide and its complement were mixed at a 1:1 ratio, heated to 70°C and slowly cooled to form the duplex. The sequences of the oligonucleotides were designed so that they could be cloned between the *Hind*III and *Bam*HI sites of the pUC12 vector. The vector plasmid pUC12 was double digested with *Bam*HI and *Hind*III prior to ligation with the insert. The resulting plasmid construct pCB2 was transformed into *Escherichia coli* TG1 cells. In each case the plasmid was double digested with *Eco*RI plus *Pvu*II for 3 h at 37°C then incubated with AMV reverse transcriptase in the presence of [α - ^{32}P]dATP to label specifically the 3'-end at the *Eco*RI site. The singly end-labelled DNA fragment was then purified by preparative non-denaturing polyacrylamide gel electrophoresis (6.5% acrylamide, 1.5 mm thick, 200 V, 2 h, in TBE buffer: 89 mM Tris base, 89 mM boric acid, 2.5 mM Na_2EDTA , pH 8.3). After rapid autoradiography to locate the DNA, the band was excised from the gel, minced with a blade and extracted overnight in 500 mM ammonium acetate, 10 mM magnesium acetate. The purified DNA was then precipitated twice with 70% ethanol prior to resuspension in 10 mM Tris, 10 mM NaCl buffer, pH 7.0.

DNAase I footprinting

DNAase I experiments were performed essentially according to the original protocol (13) using 10 mM Tris buffer, pH 7.0, containing 10 mM NaCl with a final enzyme concentration of about 0.01 U/ml. The extent of digestion was limited to less than 30% of the starting material so as to minimize the incidence of multiple cuts in any strand ('single-hit' kinetic conditions). Optimal enzyme dilutions were established in preliminary calibration experiments. After 3 min, the digestion was stopped by freeze drying, samples were lyophilized, washed once with 50 μl of water, lyophilized again and then resuspended in 4 μl of an 80% formamide solution containing tracking dyes. Samples were heated at 90°C for 4 min and chilled in ice for 4 min prior to electrophoresis as previously described (14).

Reaction of 7-azidoactinomycin with DNA

Complete details for the use of 7-azidoactinomycin and how to detect the sites of covalent attachment to DNA have recently been published (14). A freshly prepared 1 μM solution of 7-azidoactinomycin was mixed with the 3'-radiolabelled DNA in a final volume of 10 μl and incubated in the dark for 1 h.

After equilibration, tubes were placed in ice and covalent adduct formation was induced by illumination with a 100 W bulb at a distance of 2 cm from the top of the opened 0.5 ml Eppendorf tube. Illumination was performed for 5 min at 4°C in order to allow complete covalent DNA adduct formation and to convert the remaining unbound 7-azidoactinomycin into an unreactive hydroxylamine species. Control experiments were performed in the absence of drug as well as in the presence of light-inactivated drug (i.e. using a drug sample pre-irradiated for 5 min at 4°C) to ascertain that the cleavage products effectively result from drug binding and are not due to either spontaneous or light- or heat-induced degradation of the DNA. The 10 μl drug–DNA solutions were then freeze dried in dry ice prior to the piperidine treatment. The DNA samples were mixed with 25 μl of freshly diluted 0.5 M piperidine and heated at 90°C for 7 min. After freeze drying, samples were lyophilized repeatedly (up to 4 washes with 50 μl of deionized water) to remove all traces of piperidine salts. Dried samples were finally resuspended in 5 μl of 80% formamide containing 10 mM EDTA and 0.1% bromophenol blue–xylene cyanol as tracking dyes. Samples were heated to 90°C for 4 min and then chilled in an ice bath just before being loaded on a sequencing gel.

Quantitation by storage phosphorimaging

A Molecular Dynamics 425E PhosphorImager was used to collect data from the storage screens exposed to dried gels overnight at room temperature. Baseline-corrected scans were analysed by integrating all the densities between two selected boundaries using ImageQuant version 3.3 software.

RESULTS

The photoreactive analogue of actinomycin was allowed to react with a ^{32}P -labelled DNA fragment in the presence and absence of daunomycin. After equilibration (in the dark), the drug–DNA complexes were illuminated to trap azidoactinomycin at its binding sites. Photolysis of 7-azidoactinomycin results in the conversion of the azido moiety to a highly reactive nitrene which forms a covalent bond with DNA *in situ*. The sites of reaction are piperidine-labile and can be visualised on a sequencing gel; this technique has already been employed to adduce evidence for migration of antibiotic molecules between potential binding sites on DNA (14).

In Figure 1, the results of photo-affinity cleavage reactions performed with azidoactinomycin on a 178mer restriction fragment are compared with conventional DNAase I footprinting patterns determined with the parent antibiotic in the presence and absence of daunomycin. In agreement with previous studies (2–6), the DNAase I footprints observed with actinomycin alone are centred on the GpC steps at positions 46, 71 and 78. The footprints seen with daunomycin are consistent with the known preferential binding sequences for this drug (7–9). However, when both actinomycin and daunomycin are bound to DNA, footprints develop at several new sites along the DNA helix. The footprints at the GpC steps become weaker but the cleavage by the nuclease at the AT-rich region spanning nucleotide positions 32–43 remains enhanced in the presence of actinomycin plus daunomycin. The footprinting data strongly suggest that the recognition of particular DNA sequences by the antibiotics has been altered; however, due to the reversible nature of binding by both drugs it is impossible to distinguish the effect of one antibiotic from the other. This ambiguity is an inherent problem

Figure 1. Comparison of the photoreaction with 7-azidoactinomycin and the footprinting plots showing the binding of actinomycin to DNA. The substrate was a 3'-end-labelled 178 base pair *EcoRI*–*PvuII* restriction fragment from plasmid pUC12. The histogram (upper panel) refers to the piperidine-induced cleavage reactions after irradiation of the DNA with 5 μ M azidoactinomycin in the presence or absence of 2 μ M daunomycin (14). Cleavage intensity values are in arbitrary units derived from the densities of the bands in the PhosphorImager scans. The differential cleavage plots (lower panel) compare the susceptibility of the 178mer fragment to DNAase I cutting in the presence of actinomycin (5 μ M), daunomycin (2 μ M) or both drugs together. Note the inverted ordinate scale which is expressed in units of $\ln(f_a) - \ln(f_c)$, where f_a is the fractional cleavage at any bond in the presence of the antibiotic and f_c is the fractional cleavage of the same bond in the control, given closely similar extents of overall digestion. Each line drawn represents a 3-bond running average of individual data points, calculated by averaging the value of $\ln(f_a) - \ln(f_c)$ at any bond with those of its two nearest neighbours. Only the region of the restriction fragment which was analysed by densitometry is shown.

in dual drug footprinting studies, and normally would preclude any assessment as to which ligand was affecting the binding of the other, and at what sites. However, by the expedient of using a photoactivatable derivative of one antibiotic we can trap it on its instantaneous binding sites at will by switching on the light, and thus focus exclusively on its distribution irrespective of the presence or absence of the 'competing' ligand. By this means the photolabelling experiments with azidoactinomycin furnish unequivocal evidence that the locations of the binding sites of actinomycin have changed (Figure 1). In the absence of daunomycin, the photoreaction occurs exclusively at the GpC and GpG steps which constitute tight and weak actinomycin binding sites, respectively (1–6, 11, 12, 15–18). In the presence of the anthracycline, azidoactinomycin is seen to react at numerous other positions in the DNA (notably at most G·C-containing sequences). However, it is evident that the AT-rich sequence between positions 28 and 43 remains inaccessible to the drug, although some definite reaction occurs at A and T nucleotides closer to the 5' end of the fragment.

To delineate further the influence of daunomycin on the sequence-selective binding of actinomycin, we extended our

investigation to two other restriction fragments containing different types of DNA sequences. The autoradiographs (Figure 2) and corresponding quantitative analyses (Figure 3) confirm that daunomycin exerts a marked effect on the sequence-selective binding of actinomycin to DNA. The 265mer DNA fragment contains numerous GpC and GpG steps to which actinomycin binds avidly in the absence of anthracycline. However, in the presence of daunomycin, the piperidine-sensitive sites of photoreaction are located principally at non-GpC steps. The strongest reactions occur at GpG steps, but the thymine residue within the triplet sequence 5'-CTA also appears as a site susceptible to alkylation by the azido compound. There is significant reaction at many other sites including A and T nucleotides, but no more than in the absence of daunomycin.

These experiments with natural DNAs of random sequence demonstrate that daunomycin does influence the recognition of DNA sequences by actinomycin, but not to the extent of facilitating strong binding of actinomycin to AT-sequences as observed by Krugh and Young (10). However, neither the 178mer nor the 265mer fragment contain the extended sequences with alternating A·T base pairs which were used in the earlier

Figure 2. DNA photolabelling with 7-azidoactinomycin. The left and right panels show the photoreaction of 7-azidoactinomycin with a 265mer and 170mer DNA fragment from plasmid pBS and pCB2, respectively. In each case the *EcoRI*–*PvuII* restriction fragment was 3'-end-labelled at the *EcoRI* site and reacted with 7-azidoactinomycin (2 μ M) in the absence (lane marked 'Act') and presence of increasing concentrations of daunomycin (0.1–1 μ M as indicated at the top of each lane). The lane marked 'Dau' contained 2 μ M daunomycin without azidoactinomycin; the bands of weak intensity seen in this lane are identical to those seen in the control lane (marked 'Ct', containing no drugs) and reflect the sensitivity of the DNA itself to piperidine treatment. The numbers at the side of each autoradiograph refer to the nucleotide sequence of the corresponding DNA fragment as shown in Figure 3. The cloned 48 base pair AT-rich sequence in the 170mer fragment lies between the arrows.

work (10). To test directly whether daunomycin might induce reaction of azidoactinomycin with (A·T)_n tracts, we synthesized a 48 base pair oligonucleotide containing runs of alternating A·T base pairs spaced by CpG steps. This sequence, which totally lacks canonical binding sites for actinomycin (actinomycin binds to GpC but not to CpG steps), was then inserted into a plasmid. A 170 base pair restriction fragment containing the 48 base pair insert was cut out and used as a substrate for the azido compound. As shown in Figures 2 and 3, upon irradiation in the presence of daunomycin, azidoactinomycin strongly alkylates the guanine

residues at CpG and GpG sites, but little or no reaction is observed within the (A·T)_n tracts. It is noteworthy that in the presence of daunomycin, actinomycin reacts efficiently with the guanine residue within 5'-ACGT sequences, but much less well with the guanine within the 5'-TCGA sequence (position 32). This is in accord with the relative strength of binding to these tetranucleotide sequences deduced by Rill, Marsch and Graves (personal communication) from a detailed analysis of patterns of alkylation by 7-azidoactinomycin on over 1200 nucleotides in natural DNA sequences, which they have attributed primarily

Figure 3. DNA strand cleavage produced by azidoactinomycin on the 265mer fragment (upper panel) and the 170mer fragment containing the 48 base pair AT-rich insert sequence (lower panel), in the presence and absence of daunomycin. Details as described in the legend to Figure 1.

to the influence of the 5'-flanking T on the reactivity of the G. Others have also observed non-classical binding of actinomycin to the 5'-TCGA sequence (17).

DISCUSSION

The results reported here demonstrate that daunomycin exerts a marked influence on the sequence-selective binding of actinomycin to DNA. The anthracycline antibiotic diverts actinomycin from binding to its canonical GpC sequences so that it reacts with sequences which are not usually considered as potential binding sites, such as 5'-ACGT. Under the conditions used in this study, daunomycin apparently does not facilitate the binding of actinomycin to AT sequences. This difference from the findings of Krugh and Young (10) might be partially explained by the different concentrations of DNA and ligand used in footprinting experiments compared to circular dichroism; the concentrations used here were approximately an order of magnitude lower than those employed previously (10), though we were at pains to maintain comparable ratios of antibiotics to DNA base pairs (~1 actinomycin molecule/2-5 base-pairs in most experiments, which should have been sufficient to saturate all but weak sites). In fact, the intercalator:DNA ratios used here

are likely to be more interesting from the point of view of therapeutic applications of actinomycin and daunomycin.

Structural deformations of the double helix induced by the intercalation of daunomycin most likely account for the redistribution of the photosensitive antibiotic. Based on the recent evidence that actinomycin shuffles along the helix to locate its preferred sequence-dependent binding sites (14), we believe that it is the daunomycin-induced changes in minor groove width which are responsible for the altered configuration of actinomycin binding sites. Ongoing research will seek to explore this hypothesis.

The present findings may have a clinical correlate, since actinomycin and daunomycin have been used in combination chemotherapy. In the late 1970s, synergistic interaction between the two antibiotics leading to better antitumour activity was reported (19), but the combined use of daunomycin and actinomycin also resulted in increased toxicity for patients (20), thus limiting the clinical application of the drug combination. Our findings suggest a possible scientific explanation for the increased toxicity observed at the clinical level, namely that the simultaneous administration of daunomycin broadens the range of DNA sequences recognised by actinomycin, blunting its selectivity of attack on tumour cells. Although toxicological

problems are believed to arise mostly from drug-membrane interactions (leading to e.g. cardiotoxicity), rather than from direct binding to DNA, it would be a strange coincidence if the effects we have observed *in vitro* were to play no part in the increased biological effect of actinomycin and daunomycin administered together.

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