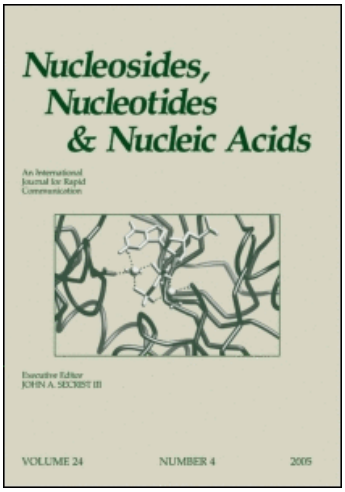

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DNA RECOGNITION BY QUINOLINE ANTIBIOTICS: USE OF BASE-MODIFIED DNA MOLECULES TO INVESTIGATE DETERMINANTS OF SEQUENCE-SPECIFIC BINDING OF LUZOPEPTIN.

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ABSTRACT

The luzopeptin antibiotics contain a cyclic decadepsipeptide to which are attached two quinoline chromophores that bisintercalate into DNA. Although they bind DNA less tightly than the structurally related quinoxaline antibiotics echinomycin and triostin A, the molecular basis of their interaction remains unclear. We have used the PCR in conjunction with novel nucleotides to create specifically modified DNA for footprinting experiments. In order to study the influence that removal, addition or relocation of the guanine 2-amino group, which normally identifies G.C base pairs from the minor groove, has on the interaction of luzopeptin antibiotics with DNA. The presence of a purine 2-amino group is not strictly required for binding of luzopeptin to DNA, but the exact location of this group can alter the position of preferred drug binding sites. It is, however, not the sole determinant of nucleotide sequence recognition in luzopeptin-DNA interaction. Nor can the selectivity of luzopeptin be attributed to the quinoline chromophores, suggesting that an analogue mode of DNA recognition may be operative. This is in contrast to the digital readout that seems to predominate with the quinoxaline antibiotics.

Abbreviations used: DNase I, deoxyribonuclease I; I, inosine; DAP, 2,6-diaminopurine.

Key words: Luzopeptin, sequence recognition, DNase I footprinting, inosine, diaminopurine.

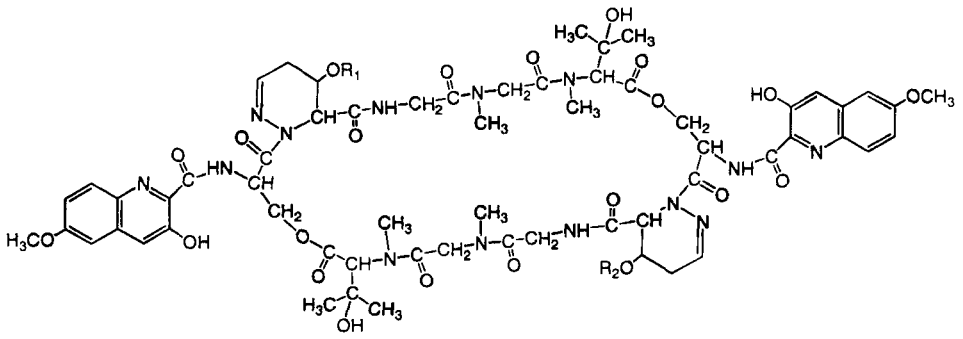
INTRODUCTION

The antibiotics echinomycin and triostin A represent a structurally unique group of antitumour agents that inhibit DNA synthesis. Both drugs contain two quinoxaline chromophores attached to a cyclic octadepsipeptide with a sulphur-containing cross-bridge. It is now well

established that they form bisintercalation complexes with DNA and bind selectively to CpG-containing sequences [1]. The luzopeptin group of antibiotics isolated from fermentation broths of *Actinomadura luzonensis* belong to the same class of DNA binding drugs but their structure differs in several respects (Figure 1). Luzopeptin C, and its mono and bis-acetylated analogues luzopeptin B and A, bear substituted quinoline rings (as opposed to quinoxalines) and the cyclic peptide is composed of ten amino acids. The lack of a sulphur cross-bridge and the presence of two cyclic imino acids derived from tetrahydropyridazine is typical of the luzopeptin-type antibiotic family which also includes quinaldopeptin [2] and the highly potent antitumour drug sandramycin [3,4]. Overall, the binding of the quinoline compounds to DNA resembles that of the quinoxaline antibiotics. The luzopeptins (also known as the BBM-928 series [5]) bisintercalate into DNA, unwinding the double helix by 40-50° as does echinomycin, and locate their symmetrical cyclic peptide ring within the minor groove of the helix [6,7]. The apparent association constant of luzopeptin A for supercoiled PM2 DNA (K_a = 1.9 x 10^7 M^{-1}) is about one order of magnitude higher than that measured with echinomycin [6]. The interaction with nucleic acids is so strong that it was speculated that luzopeptin might form covalent complexes with DNA [8]. In fact, however, there is no covalent binding [9] but intermolecular cross-linking of DNA through bifunctional intercalation may occur [10].

Unlike the quinoxaline compounds, luzopeptin does not exhibit any strict sequence selectivity. According to a previous footprinting study [8], the drug binds best to regions containing alternating A and T residues but association with non-AT sites is not excluded. Neither GC-rich nor purely AT sequences provide good binding sites for luzopeptin A, B or C. Different types of sequences with alternating purine-pyrimidine bases are tolerated [8]. In effect, the molecular basis for the recognition of particular sequences by quinoline antibiotics is poorly understood. However, NMR studies of the complexes between luzopeptin A and the short oligonucleotide duplexes $d(CATG)_2$ [11] or $d(GCATGC)_2$ [12] have revealed interesting characteristics. Both studies indicate that luzopeptin bisintercalates at the d(CA)-d(TG) steps. The two planar chromophores sandwich two Watson-Crick A•T base pairs. The recognition process is principally driven by the minor groove binding cyclic decadepsipeptide moiety rather than by the flanking quinoline chromophores which provide incremental stabilization [3]. A set of van der Waals contacts, particularly those between the CH_3 groups of the L-*N*-Me-Val residues and the minor groove surface, stabilizes the complex [11]. Hydrogen bonds are also involved. The glycine amides and the *trans* ester carbamoyl groups on both sides of the symmetrical cyclic peptide form hydrogen bonds with the 2-keto group of thymine and the 2-amino group of guanine residues respectively (open and closed circles in Figure 2). It is interesting to note that with the quinoxaline antibiotics, hydrogen bonds are only formed with the two G•C (echinomycin and triostin A) or A•T (TANDEM) base pairs clamped between the chromophores [1,13]. With the quinoline compounds, not only the central dinucleotide step (usually two A•T pairs) but also the adjacent G•C pairs are engaged in hydrogen bonds with the drug. These additional contacts could account for the higher affinity of the quinoline drugs for DNA compared to their quinoxaline counterparts.

To comprehend further the molecular rules that govern the recognition of different sequences by quinoline antibiotics, we have investigated the binding of luzopeptin A, B and C to DNA molecules in which the purine 2-amino group has been either removed from guanine, added



Luzopeptin A: $R_1 = R_2 = COCH_3$

Luzopeptin B: $R_1 = H$ $R_2 = COCH_3$

Luzopeptin C: $R_1 = R_2 = H$

FIGURE 1. Structure of the luzopeptin antibiotics.

luzopeptin-d(CATG)$_2$ luzopeptin-d(GTAC)$_2$

FIGURE 2. Schematic views of the complexes of luzopeptin with d(CATG)$_2$ and d(GTAC)$_2$ [11,12]. In both complexes, the two quinoline rings (thick vertical black bars) sandwich two adjacent AT base pairs while the cyclic peptide moiety (thin lines) lies within the minor groove. Drug-DNA hydrogen bonds between the glycine amides and the 2-keto group of thymines are represented by open circles. Hydrogen bonds between the *trans* ester carbamoyl groups on both sides of the symmetrical cyclic peptide and the 2-amino groups of guanine residues are indicated by closed circles.

to adenine, or both (Figure 3). A homologous series of 160 base pair fragments of DNA containing inosine and/or 2,6-diaminopurine residues (abbreviated DAP or D in a sequence for clarity) in place of guanosine and/or adenine residues respectively were synthesized by the polymerase chain reaction and subjected to DNase I cleavage in the absence and presence of the drugs. This strategy has previously been used with marked success to investigate the sequence-specific binding of proteins [14,15] and drugs [15-17] including the quinoxaline antibiotics [18,19]. It furnishes the most effective method to address directly the influence of the exocyclic 2-amino group of guanine which is also a key stuctural element of the DNA double helix [13,19,20]. We find that the presence of a purine 2-amino group is not strictly required for tight binding of luzopeptin to DNA; however, the addition, deletion or repositioning of the purine 2-amino substituent markedly changes the location of preferred drug binding sites. Thus this study provides a new structural basis for the recognition of DNA sequences by quinoline antibiotics.

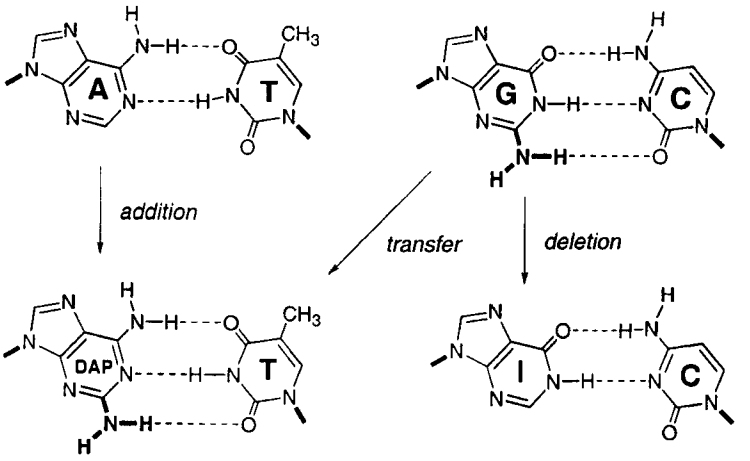


FIGURE 3. Structures of hydrogen-bonded purine-pyrimidine base pairs. Broken lines represent hydrogen bonds. I represents inosine; DAP represents 2,6-diaminopurine (2-aminoadenine).

EXPERIMENTAL

Antibiotic. Luzopeptins A, B and C were gifts from Dr M. Konishi (Bristol-Banyu Research Institute, Tokyo, Japan). Stock solutions of these antibiotics were prepared by direct weighing and, because of their low aqueous solubility, they were dissolved in dimethylsulphoxide (DMSO). These stock solutions were stored at 4°C and diluted to working concentrations immediately prior to use.

Chemicals and biochemicals. Ammonium persulphate, tris base, acrylamide, bis-acrylamide, ultrapure urea, boric acid, tetramethylethylenediamine and dimethyl sulphate were purchased from BDH. Formic acid, piperidine and formamide were from Aldrich. Bromophenol blue and xylene cyanol were from Serva. The nucleoside triphosphate labelled with [^{32}P] (γ-ATP) was obtained from NEN Dupont. Restriction endonucleases *EcoR*I and *Ava*I (Boehringer), *Taq* polymerase (Promega), DNase I (Sigma) and T4 polynucleotide kinase (Pharmacia) were each used according to the suppliers' recommended protocol in the activity buffers provided. The primers 5'-AATTCCGGTTACCTTTAATC and 5'-TCGGGAACCCCCACCACGGG, having a 5'-OH or 5'-NH_2 terminal group, were obtained from the Laboratory of Molecular Biology, Medical Research Council, Cambridge. Checks were carried out to ensure that the primers blocked with a 5'-NH_2 group were free from contaminants and could not be labelled by the kinase. All other chemicals were analytical grade reagents, and all solutions were prepared using doubly deionised Millipore filtered water.

Preparation, purification and labelling of DNA fragments containing natural and modified nucleotides. Plasmid pKMp27 [21] was isolated from *E. coli* by a standard sodium dodecyl sulphate-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 cleavage by the restriction enzymes. The 160 base pair *tyrT*(A93) fragment for use as a template was isolated from the plasmid by digestion with restriction enzymes *Eco*RI and *Ava*I. It is worth mentioning that this template DNA bore a 5'-phosphate due to the action of *Eco*RI and thus only the newly synthesized DNA (with normal or modified nucleotides) can be labelled by the kinase.

Polymerase Chain Reaction (PCR) and DNA labelling. The protocol used to incorporate inosine and/or 2,6-diaminopurine residues into DNA is comparable to those previously used to incorporate 7-deazapurine or inosine residues with only a few minor modifications [22-24]. PCR reaction mixtures contained 10 ng of *tyrT*(A93) template, 1 μM each of the appropriate pair of primers (one with a 5'-OH and one with a 5'-NH_2 terminal group) required to allow 5'-phosphorylation of the desired strand, 250 μM of each appropriate dNTP (dTTP, dCTP plus dATP or dDTP and dGTP or dITP according to the desired DNA), and 5 units of *Taq* polymerase in a volume of 50 μl containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.1% Triton X-100, and 1.5 mM $MgCl_2$. To prevent unwanted primer-template annealing before the cycles began, the reactions were heated to 60°C prior to adding the *Taq* polymerase. Finally, paraffin oil was added to each reaction to prevent evaporation. After an initial denaturing step of 3 min at 94°C, twenty amplification cycles were performed, each cycle consisting of the following segments: (a) for normal and DAP-DNA: 94°C for 1 min, 37°C for 2 min, and 72°C for 10 min. (b) for I-DNA and I+DAP-DNA: 84°C for 1 min, 30°C for 2 min, and 62°C for 10 min. After the last cycle, the extension segment was continued for an additional 10 min at 62° or 72°C, followed by a 5 min segment at 55°C and a 5 min segment at 37°C. The purpose of these final segments was to maximize annealing of full-length product and to minimise annealing of unused primer to full-length product. The reaction mixtures were then extracted with chloroform to remove the paraffin oil, and parallel reactions were pooled. Several extractions with n-butanol were performed to reduce the volume prior to loading the samples on to a 6% non-denaturing polyacrylamide gel. After electrophoresis for about 1 hour, a thin section of the gel was stained with ethidium bromide so as to locate the band of DNA under UV light. The same band of DNA free from ethidium was excised, crushed and soaked in elution buffer (500 mM ammonium acetate, 10 mM magnesium acetate) overnight at 37°C. This suspension was filtered through a Millipore 0.22 μm filter and the DNA was precipitated with ethanol. Following washing with 70% ethanol and vacuum drying of the precipitate, the purified DNA was resuspended in the kinase buffer. The purified PCR products were 5'-end labelled with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase according to a standard procedure for labelling blunt-ended DNA fragments. After completion of the reaction the nucleic acid was again purified by 6% polyacrylamide gel electrophoresis and extracted from the gel as described above. Finally, the labelled DNA was resuspended in 10 mM Tris-HCl buffer pH 7.0 containing 10 mM NaCl.

DNase I footprinting. Routine experiments were performed essentially as described recently [25]. The digestion of the samples (6 μl) of the labelled DNA fragment dissolved in 10 mM

Tris buffer (pH 7.0) containing 10 mM NaCl was initiated by adding 2 µl of a DNase I solution whose concentration had been adjusted to yield a final enzyme concentration of about 0.01 unit/ml in the reaction mixture. 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.

Electrophoresis and autoradiography. DNA cleavage products were resolved by polyacrylamide gel electrophoresis under denaturing conditions (0.3 mm thick, 8% acrylamide containing 8 M urea) capable of resolving DNA fragments differing in length by one nucleotide. Electrophoresis was continued until the bromophenol blue marker had run out of the gel (about 2.5 hours at 60 Watts, 1600 V in TBE buffer, BRL sequencer model S2). Gels were soaked in 10% acetic acid for 15 min, transferred to Whatman 3MM paper, dried under vacuum at 80°C, and subjected to autoradiography at -70°C with an intensifying screen. Exposure times of the X-ray films (Fuji R-X) were adjusted according to the number of counts per lane loaded on each individual gel (usually 24 hours).

Quantitation by storage phosphor imaging. A Molecular Dynamics 425E PhosphorImager was used to collect data from storage screens exposed to the dried gels overnight at room temperature [26]. Base line-corrected scans were analyzed by integrating all the densities between two selected boundaries using ImageQuant version 3.3 software. Each resolved band was assigned to a particular bond within the *tyr*T(A93) fragment by comparison of its position relative to sequencing standards generated by treatment of the DNA with formic acid followed by piperidine-induced cleavage at the purine residues (G+A track), taking into account the difference in mobility of the standards due to their being one nucleotide shorter and bearing an additional 3' phosphate group which causes them to migrate approximately 1-1.5 bands faster than their counterparts generated by DNase I cleavage.

RESULTS

Luzopeptin A strongly affects the mobility of *tyr*T DNA molecules containing natural bases (Figure 4). A concentration as low as 2 µM suffices to decrease markedly the electrophoretic mobility of normal DNA on a non-denaturing polyacrylamide gel. This reflects the high affinity of the drug for duplex DNA. As mentioned in the introduction, the binding of luzopeptin is tight but non-covalent (reversible). With the modified DNA species, the same gel shifts can be easily detected with luzopeptin A (Figure 4) and they were also seen with luzopeptins B and C (gels not shown). With both inosine DNA and DAP DNA, the mobility shift is detectable at the lowest luzopeptin A concentration tested (2 µM). We can therefore immediately conclude that the deletion of the guanine 2-amino group (G→I substitution) does not prevent tight binding of luzopeptin to

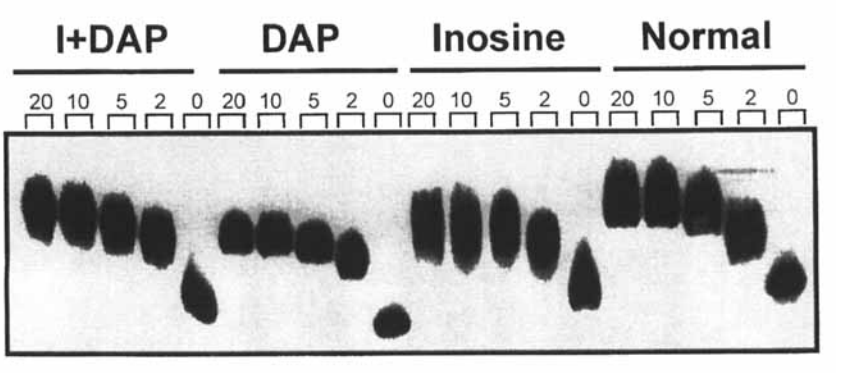


FIGURE 4. Effects of luzopeptin A on the mobility of the 160 base pair *tyrT* fragment containing normal or modified bases on a 6% non-denaturing polyacrylamide gel. The drug concentration (expressed as micromolar) is indicated at the top of each lane.

DNA. Similarly, the addition of an amino group to adenine residues (A→D substitution) apparently has no significant effect on the strength of interaction between the drug and DNA. It is interesting to note that echinomycin does not decrease the gel mobility of normal DNA, though it reduces considerably the electrophoretic mobility of DAP-containing DNA (unpublished observations) as a result of the increased affinity conferred by the A→D substitution [24].

Next we investigated the sequence preference of the luzopeptin antibiotics by means of DNase I footprinting methodology using normal and modified DNA. The 5'-[^{32}P]-labelled PCR fragments were incubated with various concentrations of the antibiotics for 30 min at room temperature to enable equilibration of the drug-DNA complexes and then the cutting reaction was initiated by adding DNase I. The resulting DNA cleavage products were resolved on sequencing gels. Examples of some of the numerous footprinting experiments performed with the normal and modified PCR products are illustrated in Figure 5. Luzopeptin A exerts weaker effects on the nuclease cleavage pattern of normal DNA than luzopeptins B and C. With the latter antibiotic, regions protected from DNase I cleavage can be clearly discerned around positions 20, 40 and 60 (Figure 5). A careful densitometric analysis of these gels, and all others, was performed to identify the sequences at the protected cleavage sites. One such differential cleavage plot - Figure 6, which was not derived from Figure 5, shows that luzopeptin B protects the sequences 5'-GTTACGGA (nucleotide positions 18-25), 5'-GCAACCAG (nucleotide positions 36-43) and 5'-ACGTAACA (nucleotide positions 57-64) from cutting by the nuclease. Between the footprints (presumptive drug binding sites) regions can be discerned where the cleavage by the enzyme is substantially enhanced. For example, luzopeptin B promotes DNase I cleavage by as much as one natural log at the sequences 5'-AAAATTA and 5'-TTTTTCTC centred around positions 30 and 50, respectively (Figure 6). These results with normal DNA are totally consistent with previous reports [8] and confirm that the luzopeptin antibiotics bind preferentially to sequences containing mixed A•T

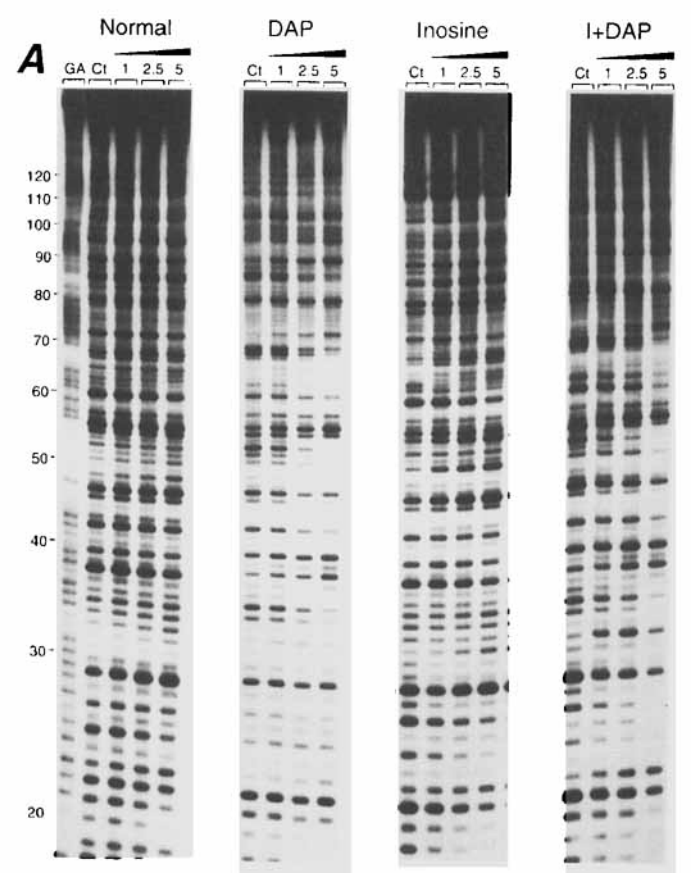
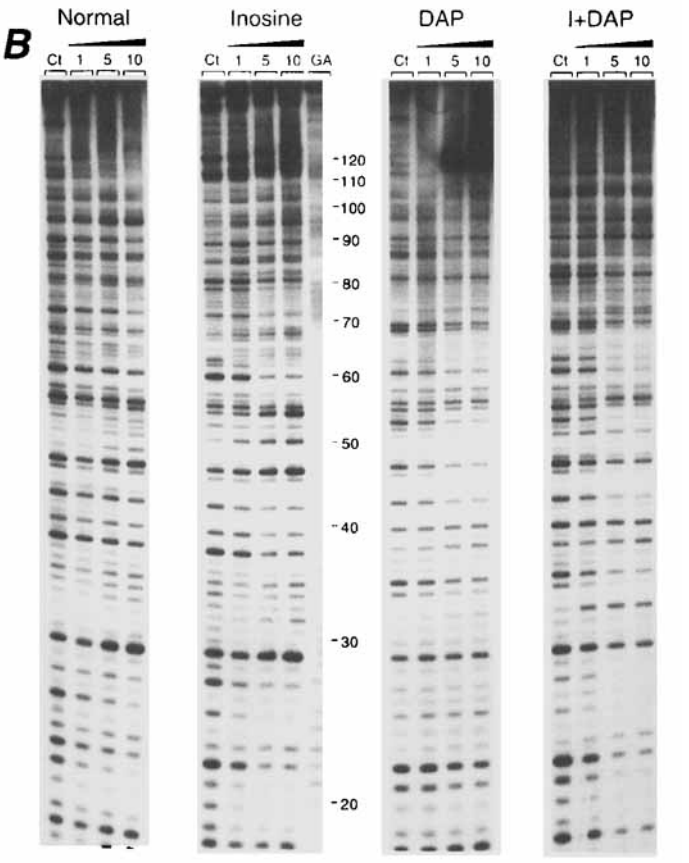


FIGURE 5. Selected autoradiographs showing DNase I footprinting of luzopeptins A, B and C on the *tyrT*(A93) DNA fragment containing natural nucleotides (Normal), inosine residues in place of guanosine (Inosine), 2,6-diaminopurine residues in place of adenine (DAP) or inosine and diaminopurine residues in place of guanosine and adenine respectively (I+DAP). In each case, the DNA was 5'-end labelled at the *Eco*RI site with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase. The products of DNase I digestion were resolved on an 8% polyacrylamide gel containing 8M urea. The concentration (μM) of the drug tested is shown at the top of each gel lane. Control tracks labelled "Ct" contained no drug. Tracks labelled G+A represent Maxam-Gilbert sequencing markers specific for purine residues. Numbers at the side of the gels refer to the numbering scheme used in Figure 6.



(continued)

and G•C base pairs. Binding to sequences composed of contiguous A•T pairs is clearly disfavoured.

At first sight the binding of the drug to the inosine DNA appears much the same as for normal DNA. Clear luzopeptin C footprints can be detected around positions 20, 40 and 60 with the inosine-substituted DNA (Figure 5), but there are some subtle differences (see below). Changes are much more obvious with the DAP-substituted DNA which evidently provides a good substrate for luzopeptin. For instance, marked footprints can be identified around nucleotide positions 36 and 52 in the DAP DNA whereas each of these regions lies close to a site of enhanced cleavage in normal or inosine-substituted DNA. A few regions where the cleavage by the enzyme has been slightly enhanced in the presence of the antibiotic can also be detected with the DAP DNA.

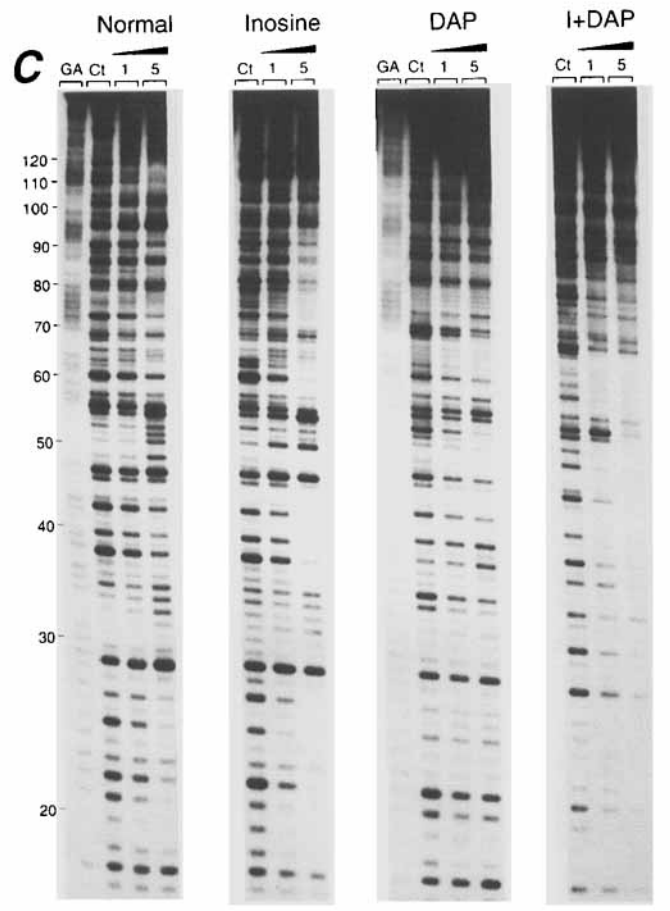


FIGURE 5. Continued.

The footprinting patterns observed with the doubly substituted I+DAP DNA are relatively complex. Densitometric analysis reveals that the combined G→I plus A→D substitutions create numerous new binding sites for the luzopeptins. As shown in Figure 6, luzopeptin B protects several 3-4 bp long sequences from cutting by DNase I. For example, the sequence 5'-TTDC at positions 33 and 68 provides a favoured binding site for the drug. The concentrations required to detect footprints with the I+DAP DNA are similar to those needed to observe selective binding on normal DNA. Therefore, the results indicate that the base substitutions do not markedly augment the affinity of the drug for DNA, in sharp contrast to what was observed with echinomycin and triostin A [19,24]. With the quinoxaline antibiotics, the A→D substitution potentiated the interaction of the drug with DNA considerably (up to 1000 fold). This is certainly not the case with the luzopeptins.

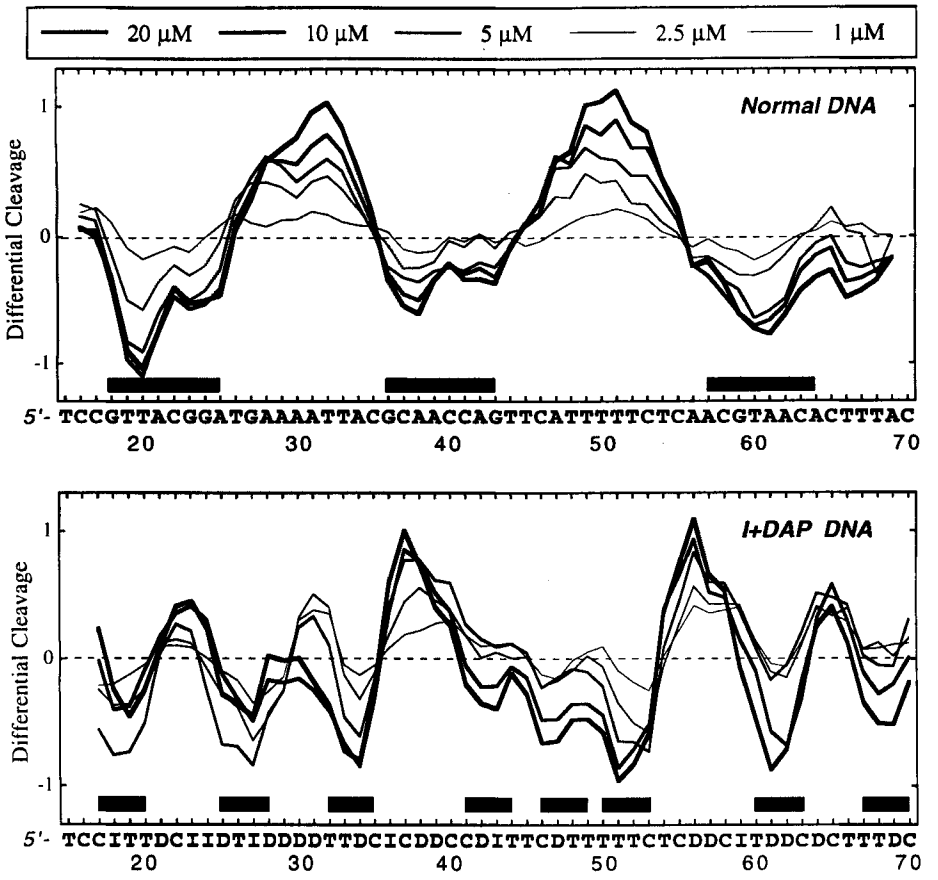


FIGURE 6. Representative differential cleavage plots comparing the susceptibility of the normal and the doubly-substituted I+DAP DNA to DNase I attack in the presence of luzopeptin B. Data were obtained from analysis of one of the large number of gels run, but not that illustrated in Figure 5. The sequence shown on the *x* axis corresponds to that of the Watson strand of the *tyrT*(A93) fragment containing natural bases. In the modified DNA, adenine and guanosine residues are replaced by diaminopurine and inosine residues. Positive and negative values correspond, respectively, to enhanced or diminished DNase I cutting at each internucleotide bond. The vertical scale is in units of $\ln(f_a) - \ln(f_c)$, where f_a is the fractional cleavage at any bond in the presence of the drug and f_c is the fractional cleavage of the same bond in the control. The results are displayed on a logarithmic scale for the sake of convenience. The filled rectangles above the sequences indicate the positions of the drug binding sites. The five superimposed plots were determined using increasing concentrations of luzopeptin B from 1 to 20 μM as specified at the top.

A large number of gels were scanned with the phosphorimager and band intensities were quantified to construct differential cleavage plots like those shown in Figure 6. From the raw data it was not possible to establish any obvious recognition code for the luzopeptins. Initially we attempted to interpret the DNase I data by considering the nature of the dinucleotide step sandwiched by the drug, as was done previously for echinomycin and triostin [19,24]. But, as mentioned in the introduction, the binding of luzopeptins manifestly does not conform to any strict rule of sequence requirement, in contrast to the quinoxaline antibiotics. Accordingly, we elected to examine the notion that the quinoline compounds participate in hydrogen bonding interactions not only with the central dinucleotide step at a given site (usually two A•T pairs) but also with the flanking base pairs (usually G•C).

NMR studies with luzopeptin bound to the tetranucleotide sequences $(CATG)_2$ and $(GTAC)_2$ [11,12] showed that the quinoline rings sandwich the two central A•T base pairs and the cyclic decadepsipeptide moiety establishes key hydrogen bonds with the TG or GT steps. In particular, the glycine amides and the *trans* ester carbamoyl groups on both sides of the symmetrical cyclic peptide form hydrogen bonds with the 2-keto group of thymine and the 2-amino group of guanine respectively (open and closed circles in Figure 2). These considerations prompted us to enquire whether the presence or absence of purine 2-amino groups at particular positions within the binding site could explain the changes in the footprinting patterns observed upon incorporating DAP and/or inosine. From an examination of the data obtained from more than ten independent experiments we selected eight different tetranucleotide steps in the normal and modified *tyr*T fragments where footprinting was observed for Luzopeptin C with at least one of the four DNA species. Luzopeptin C was chosen because it provided the strongest footprints among the three drugs. For each site, we estimated the binding affinity of the ligand from the differential cleavage plots and determined whether or not the antibiotic could potentially establish the hydrogen bonding interactions mentioned above. In the summary schemes presented in Figure 7, a simple scheme of (+) or (–) symbols is utilized to indicate sites at which the drug binds or does not bind. Open and closed circles indicate the 2-keto group of thymine and the 2-amino group of guanine respectively, which are implicated in the aforementioned H-bonds. We have used an open square to represent the 2-keto group of cytosine which lies in a position identical to that found in thymine. When a C•G base pair occurs within the binding site it is possible that the glycine amide groups of luzopeptin could form a hydrogen bond to the cytosine C=O group. In addition, we have used a closed square symbol to indicate the 2-amino group of DAP residues which is structurally equivalent to the G amino group represented by a closed circle. It is instructive to consider the results obtained at each tetranucleotide sequence in turn.

At site 1 the drug sandwiches two A•T pairs, making potential hydrogen bonds with the T residues (○). There is also a possible H-bond between a *trans* ester carbamoyl group of the drug and the guanine 2-amino substituent (●). The deletion of the guanine 2-amino group (G→I substitution) or addition of an extra amino group on adenine residues (A→D substitution) has no significant effect on the capacity of the drug to interact at this type of site, as judged from the footprinting intensities. This corroborates the idea that the 2-amino group of guanine is not absolutely essential for the drug to recognize particular sequences in DNA.

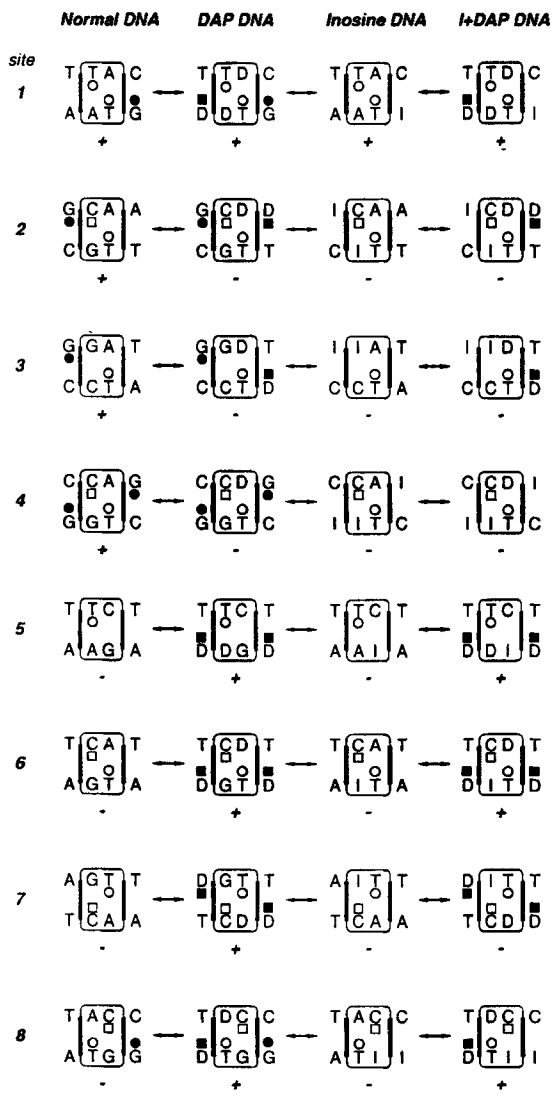


FIGURE 7. Summary of footprinting data for luzopeptin C, derived from a total of ten gels. Eight tetranucleotide sequences which can bind the drug were selected from differential cleavage plots (Figure 6 and the many others not shown) determined with normal and modified DNA. Sites are categorised semi-quantitatively simply as binding (+) or not (-). Symbols ○ and □ refer to the 2-keto groups of T and C whereas ● and ■ refer to the 2-amino group of G and D, respectively. For each sequence its disposition of hydrogen bonding groups in normal DNA and the three substituted DNA species is shown, together with a symbolic representation of the antibiotic positioned at the site as in Figure 2.

At sites 2-7 the drug sandwiches one A•T (or T•A) pair and one G•C (or C•G) pair. Site 2 normally provides quite a good binding site for luzopeptin, but in this case all base substitutions (be they G→I or A→D) completely abolish drug binding. This is perhaps surprising because with the A→D substitution the potential H-bond interaction would be maintained. Conceivably the insertion of an additional amino group could cause steric hindrance preventing the drug entering the minor groove. The situation is similar for sites 3 and 4. Here again, the binding of the drug to normal DNA is cancelled after the base substitutions. Evidently the interaction of luzopeptin with specific sites cannot be interpreted solely on the basis of the hydrogen bonding patterns deduced from the NMR experiments.

Sites 5 to 8 are more informative because, in these cases, the antibiotic binds neither to normal DNA nor to inosine DNA but binding is rendered possible by replacing adenines with DAP residues. At sites 5-7, the drug-DNA interaction is induced by the replacement of three A•T pairs with D•T pairs. It appears that the incorporation of a 2-amino group on the purine residues flanking the quinoline intercalation sites can permit the drug to establish new stabilising interactions responsible for the observed footprints at those sites. This is a sure indication that the purine 2-amino group must somehow contribute positively to the drug-DNA interaction, even if it does not represent an obligatory anchoring site. Luzopeptin also fails to bind to site 8 in normal DNA but interacts quite well with the corresponding DAP-containing sites. In this case, only two A•T pairs are replaced with D•T pairs. The many anomalies in the behaviour of sites equipped with ostensibly similar arrays of hydrogen bonding functionalities seem to preclude the drawing of any general conclusions, and underline the complexity of the molecular recognition process between luzopeptin and DNA.

DISCUSSION

Both the G→I and A→D substitutions exert weaker effects on the binding to DNA of luzopeptins A, B and C compared to what we have observed previously with the related quinoxalines triostin A and echinomycin [19,24]. Our earlier studies with the quinoxaline antibiotics demonstrated that the recognition of particular nucleotide sequences by these drugs was critically dependent upon the placement of the purine 2-amino group. The binding of echinomycin and triostin to CpG sites is primarily due to hydrogen bonding between the cyclic peptide of the antibiotic and the 2-amino groups of guanine residues. The fact that the luzopeptins contain quinoline rather than quinoxaline chromophores cannot explain the difference between the two groups of antibiotics in terms of DNA recognition. We recently reported that the purine 2-amino group serves equally well as a positive effector for sequence-recognition by the biosynthetic drug 2QN, which a bis-quinoline derivative of echinomycin [27].

The situation reported here with the luzopeptin antibiotics also differs from that described earlier with the synthetic des-tetramethyl analogue of triostin A code-named TANDEM. Unlike the naturally-occurring quinoxaline antibiotics which bind specifically to sequences centred around a CpG step, TANDEM selectively recognizes TpA sites [28-30]. TANDEM and the luzopeptins share

comparable sequence-selectivity but differ markedly in terms of binding strength. The affinity of TANDEM for DNA is considerably lower than that of luzopeptin. We have reported that the exocyclic guanine 2-amino group apparently plays no part in the recognition of TpA sites by TANDEM [19]. Indeed, using the same PCR-based strategy as employed here to synthesize modified DNA it was shown that the removal or addition of a purine 2-amino group has absolutely no influence on the interaction of TANDEM with TpA steps. We therefore concluded that the selective binding of TANDEM to TpA sites is not hydrogen bond driven and probably originates mainly from steric and/or stacking interactions [19] . The situation is more ambiguous with the luzopeptins. Here it is evident that the guanine 2-amino group plays some role in the recognition of DNA sequences by the antibiotics, but this substituent cannot be categorized either as a positive (as with echinomycin) or a negative (as with TANDEM) effector for luzopeptin binding.

NMR has revealed the existence of a hydrogen bonding interaction between the *trans* ester carbamoyl groups on both sides of the symmetrical cyclic peptide of luzopeptin and the 2-amino groups of guanine residues. Indirectly, our data suggest that these H-bond interactions only contribute to a minor extent to the formation of stable complexes. Clearly the direct interaction of the ligand with the exocyclic purine 2-amino group exposed in the minor groove is not sufficient to explain the remarkably tight binding of these drugs to sites such as CATG and GTAC. As with TANDEM, it seems most likely that sequence-selective binding of luzopeptin to DNA is more dependent on the sequence-dependent deformability of the DNA target site than on static complementarity between the ligand and the DNA bases. In other words, the recognition of DNA sequences by the luzopeptin antibiotics may correspond to a form of "analogue readout" whereby the DNA binding domain of the ligand recognizes some aspect of the global structure/conformation of the double helix. By contrast, the recognition of DNA sequences by the quinoxaline antibiotics such as echinomycin includes an important element of "digital readout" based on direct interaction of the ligand with each base pair independently of neighbouring base pairs.

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