New Binding Sites for Antitumor Antibiotics Created by Relocating the Purine 2-Amino Group in DNA

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Most antibiotics and drugs bind to DNA in the minor groove of the helix and recognize their preferred nucleotide sequences by a combination of van der Waals interactions and hydrogen bonds.[1] For GC-specific ligands, the critical element of recognition is usually acceptance of a hydrogen bond from the 2-amino group of guanine, the only donor group exposed in the minor groove.[2] Additional stabilization may come from formation of a hydrogen bond to the neighboring purine ring N(3) atom. Thus the quasirotationally symmetrical peptide antibiotics echinomycin[3] and actinomycin[4] recognize their cognate CpG and GpC target sites by three and four hydrogen bonds, respectively.[5,6] Here, we show that if a 2-amino group is added to the adenine bases by virtue of substitution with 2,6-diaminopurine (DAP), new binding sites are created to which the antibiotics bind in preference to their canonical guanine-containing sites.

The effect of replacing adenine residues in DNA with DAP is to add an extra NH···CO hydrogen bond to the normal A·T base pair in much the same steric relationship to the other constituents of the minor groove as the NH···CO hydrogen bond of a G·C pair. Thus a small molecule seeking the characteristic disposition of hydrogen-bonding elements which identify a G·C pair would find them in a DAP·T pair,[7] and in DNA entirely substituted with DAP residues, every base pair should be potentially capable of participating in a binding site—provided that its purine–pyrimidine (R–Y) or pyrimidine–purine (Y–R) orientation is correct. So for echinomycin, which recognizes CpG steps,[8,9] all YpR sequences could constitute potential binding sites, and, conversely, for actinomycin (which recognizes GpC[10–12]) all RpY steps could be available.

Figure 1 shows that this prediction is essentially true, but there

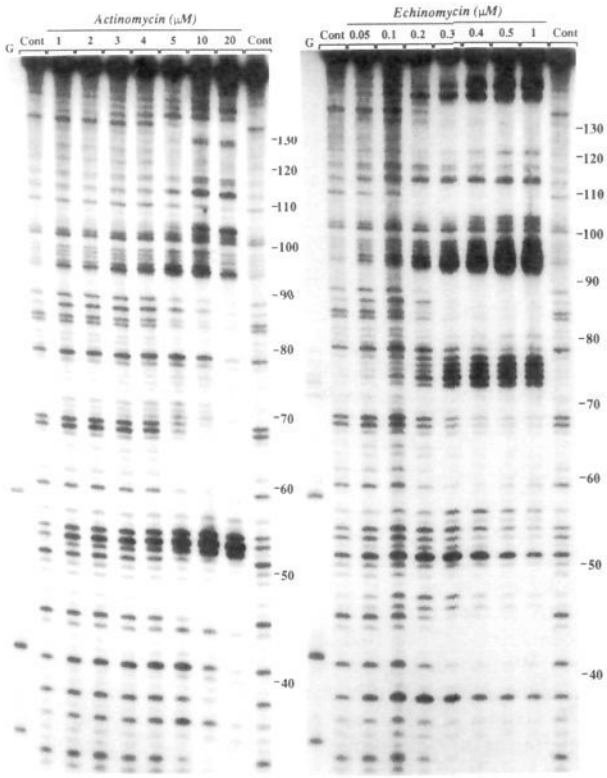

Figure 1. DNAase I footprinting of echinomycin and actinomycin on the *tyr*TDNA fragment from *Escherichia coli*[17] containing 2,6-diaminopurine residues in place of adenine. DAP residues were introduced by PCR amplification using primers specifically designed to ensure selective labeling of the antisense strand.[16] Chemical identities of the digestion products were assigned by reference to a Maxam–Gilbert G-specific sequencing reaction[18] (lane G), taking into account the differences in mobility of the fragments due to the presence or absence of a 3′-phosphate group. Lanes marked Cont refer to the control DNAase I digests of the DAP-containing DNA fragment in the absence of antibiotic.

are some surprises. With both antibiotics strong footprints are evident as the concentration is raised, but the sites of protection from DNAase I cleavage are clearly different for the two ligands. Nor are those sites the same as occur in natural (adenine-containing) DNA. Most striking is the appearance of a substantial region of enhanced cleavage extending five or more base pairs to either side of position 100, in a markedly GC-rich region which is normally strongly protected by both antibiotics.[8,10] With echinomycin there is another heavily cut GC-rich region between positions 70 and 80 where a second cluster of strong binding sites occurs in natural *tyr*T DNA.[8]

The observed differences in sequence selectivity are emphasized in Figure 2, where a quantitative comparison with natural DNA is presented. It is obvious that for echinomycin the sites of binding (protection) and enhanced cutting are practically reversed in the DAP-containing DNA. For actinomycin they are also radically altered. The chief reason seems to be that neither antibiotic now provides protection against nuclease cleavage at its canonical guanine-containing sites, though they should be unaffected by the substitution with DAP. Rather, those sites (CpG or GpC steps) now frequently lie in the regions of enhanced cleavage which commonly appear flanking strong binding sites or clusters of sites in footprinting experiments.[8–12] A search for the common denominator of the new binding sites for each antibiotic suggests that, with one exception, echinomycin is binding preferentially to YpR steps but not CpG (i.e., TpA, CpA, and TpG), whereas actinomycin binds to RpY steps but not GpC (i.e., ApT, ApC, and GpT). In each case the exception is a single YpR or RpY step at the 5′ end of the conspicuously GC-rich run stretching

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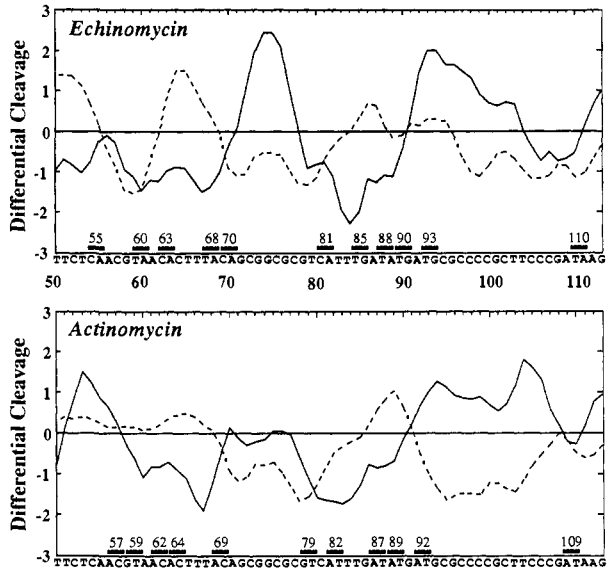

Figure 2. Differential cleavage plots comparing the DNAase I-mediated cleavage of normal and modified *tyr*TDNA in the presence of echinomycin and actinomycin. The plots represented by continuous lines refer to the modified *tyr*TDNA fragment containing DAP residues and were obtained by densitometry of the lanes containing 10 μM actinomycin and 0.5 μM echinomycin shown in Figure 1. The plots indicated by dashed lines refer to normal *tyr*TDNA containing adenine residues in the presence of actinomycin[10] and echinomycin,[8] both at 10 μM. Positive and negative values correspond, respectively, to enhanced or decreased DNAase I cutting efficiency at each internucleotide bond. The values plotted compare measured probabilities of cleavage expressed in logarithmic units and are smoothed by taking a three-bond running average. Thick bars over the indicated dinucleotide steps show, for echinomycin, the positions of the YpR steps with the exception of CpG and, for actinomycin, the positions of the RpY steps with the exception of GpC.

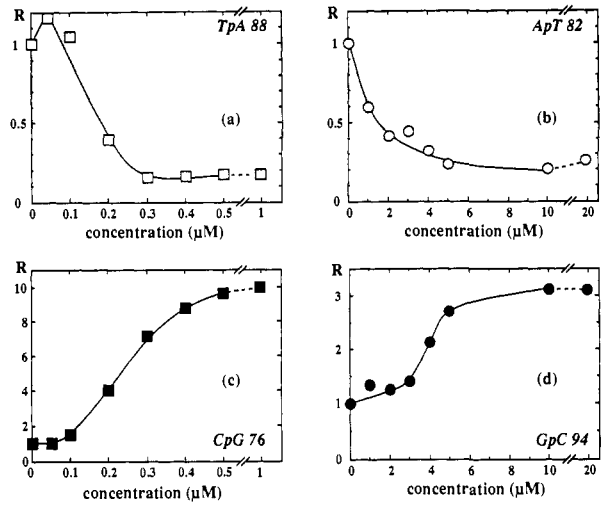

Figure 3. Footprinting plots[12] for selected bonds in the *tyr*T fragment containing DAP residues. The relative band intensity R corresponds to the ratio I_c/I_0 where I_c is the intensity of the band at a ligand concentration c and I_0 is the intensity of the same band in the absence of antibiotic. Data points were obtained from the gels shown in Figure 1. Plots a and b show strong binding at new sites for echinomycin and actinomycin respectively. Plots c and d show enhancement of cleavage occurring at canonical binding sites for echinomycin (c) and actinomycin (d) in normal DNA. Digitized images from selected gel lanes were analyzed by integrating all the densities between two selected boundaries using the interactive program GEL-TRAK[19] developed specifically for quantitative analysis of DNAase I footprinting gels.

from position 94 to 108. Perhaps the juxtaposition of that step, TpG 93 for echinomycin and ApT 92 for actinomycin, to the evidently disfavored GC-rich tract explains why it does not constitute part of a binding site.

The second oddity lies in the dramatically increased sensitivity to echinomycin but not actinomycin. For the latter antibiotic the concentration range over which strong footprints develop is not much different from that seen with natural DNA,[10,12] whereas for echinomycin there is at least a 1 order of magnitude increase in sensitivity. Footprints (and enhancements) are plainly in evidence at 0.2 μM in Figure 1a, and incipient effects can even be spotted at 0.05 μM. It takes at least 1–2 μM echinomycin to produce equivalent effects with natural DNA,[8] suggesting that the affinity of the antibiotic for its newly-created binding sites in DAP-containing DNA must be much higher. In Figure 3 are plotted examples of the concentration dependence of antibiotic effects on DAP-containing DNA. Although the data for protection and enhancement have not been smoothed as would be required for a rigorous thermodynamic study,[12] it can be seen that C_{50}, the concentration for half-maximal effect of actinomycin, is on the order of 3–4 μM, whereas for echinomycin C_{50} is not greater than 0.2 μM. Under the conditions of these footprinting experiments a large fraction of the ligand must be free, such that C_{50} values may approximate to dissociation constants for binding to individual sites.[12] By any comparison with published estimates[13,14] this corresponds to a 1 order of magnitude increase in affinity for echinomycin compared to sites in natural DNA.

Why the increase in potency of effect for echinomycin but not actinomycin? The answer may lie in the greater perturbation of DNA helical structure associated with bis-intercalative binding of echinomycin[3,14] compared to simple intercalation of actinomycin.[4-6] Greater perturbation, perhaps the unwinding, of the helix may allow for greater flexibility in optimizing interactions with the DNA bases. It is also noteworthy that echinomycin forms only three hydrogen bonds to its cognate CpG site in model oligonucleotides,[6] whereas actinomycin forms four,[5] again suggesting that the echinomycin–DNA complex may be intrinsically more flexible and better able to adapt to a novel opportunity for molecular recognition.

The decisive effect of relocating the purine 2-amino group reported here may be compared with the observed lack of specific antibiotic binding to DNA in which the 2-amino group has been deleted altogether by virtue of substitution with inosine in place of guanosine.[15,16] Together, the results leave little room for doubt that the 2-amino group of guanine is the critical determinant for recognition of specific binding sites in DNA.

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