Interaction of echinomycin with $A_n.T_n.$ and (AT)$_n$ regions flanking its CG binding site

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ABSTRACT

We have prepared DNA fragments containing the sequences $A_{15}CGT_{15}$, $T_{15}CGA_{15}$ and $(AT)_8CG(AT)_{15}$ cloned within the Smal site of the pUC19 polylinker. These have been used as substrates in footprinting experiments with DNase I and diethylpyrocarbonate probing the effects of echinomycin, binding to the central CG, on the structure of the surrounding sequences. No clear DNase I footprints are seen with $T_{15}CGA_{15}$ though alterations in the nuclease susceptibility of surrounding regions suggest that the ligand is binding, albeit weakly at this site. All the other fragments show the expected footprints around the CG site. Regions of $A_n$ and $T_n$ are rendered much more reactive to DNase I and adenines on the 3'-side of the CG become hyperreactive to diethylpyrocarbonate. Regions of alternating AT show unusual changes in the presence of the ligand. At low concentrations (5 mM) cleavage of TpA is enhanced, whereas at higher concentrations a cleavage pattern with a four base pair repeat is evident. A similar pattern is seen with micrococcal nuclease. Modification by diethylpyrocarbonate is strongest at alternate adenines which are staggered in the 5'-direction across the two strands. We interpret these changes by suggesting secondary drug binding within regions of alternating AT, possibly to the dinucleotide ApT. DNase I footprinting experiments performed at 4°C revealed neither enhancements nor footprints for flanking regions of homopolymeric A and T suggesting that the conformational changes are a necessary consequence of drug binding.

INTRODUCTION

The antitumour antibiotic echinomycin binds to DNA by the mechanism of bifunctional intercalation, unwinding the DNA helix by 48° [1]. It has long been known to interact preferentially with GC-rich DNAs [2] and has been shown to recognise the dinucleotide step CG [3,4]. Several studies have shown that this drug, in common with other sequence selective ligands, can modify the DNA structure in regions surrounding its binding site [4—12].

Footprinting studies on natural [4,13] and synthetic [5] DNA fragments have shown that echinomycin causes enhancements in DNase I cleavage in regions surrounding some of its binding sites. These are especially pronounced in adjacent regions of homopolymeric A and T and are explained by an increase in the local DNA minor groove width caused by the ligand. When regions of alternating A and T are sandwiched between two echinomycin binding sites DNase I cleavage of TpA, which is normally poor, is greatly enhanced so that it is now similar to that of ApT. Other structural changes have been detected by the sensitivity of adjacent purines (especially adenine) to modification by diethylpyrocarbonate. B-DNA is relatively unreactive to this probe yet in the presence of echinomycin modification of certain bases is greatly enhanced [6—8], especially at the sequence CGA [13]. This is now generally understood to reflect a local unwinding of the DNA helix. In this study we have studied the effects of echinomycin on the structure of regions of $A_n$. $T_n$, and (AT)$_n$ by preparing DNA fragments containing these sequences surrounding canonical echinomycin binding CpG sites. These fragments have been used in footprinting studies with DNase I and diethylpyrocarbonate.

MATERIALS AND METHODS

Drugs and enzymes

Echinomycin was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Since this drug is only sparingly soluble in aqueous systems it was stored as a 1mM stock solution in dimethylsulphoxide and diluted to working concentrations in 10mM Tris-HCl pH 8.0 containing 10mM NaCl immediately before use. The final dimethylsulphoxide concentration was always less than 10% (v/v); this had no effect the digestion patterns. DNase I and micrococcal nuclease were purchased from Sigma and stored as previously described [4,14]. The oligonucleotides $T_{15}CGA_{15}$, $A_{15}CGT_{15}$ and (AT)$_{15}CG(AT)_{15}$ were prepared on an Applied Biosystems DNA synthesiser and used without further purification.

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DNA fragments

The self complementary oligonucleotides were ligated into Smal (CCC/GGG) cut pUC19 and transformed into E.coli TG2. Transformants were picked off X-Gal containing plates in the usual way as white colonies. The sequences of the resulting plasmids were confirmed by dideoxy sequencing using a T7 sequencing kit (Pharmacia). Each of the plasmids was found to contain a single copy of the synthetic insert except the clone for alternating AT surrounding the central CG site which contained the truncated sequence T(AT)gCG(AT)|j. Plasmids were purified using Qiagen columns according to the manufacturers instructions.

Radiolabelled DNA fragments containing the synthetic inserts were prepared by digesting with HindIII, labelling at the 3'-end with [α-32P]dATP using reverse transcriptase and cutting again with EcoR1. The labelled fragments of interest (about 75 base pairs) were separated on 7% polyacrylamide gels. For some experiments the DNA was labelled at the opposite end by reversing the order of addition of HindIII and EcoRI. Each of these fragments contains a single central canonical echinomycin binding site (CpG) within a region of at least twelve AT base pairs. The remainder of the pUC polylinker contains 3 additional CG sites (two at the EcoRI end and one at the HindIII end.

Footprinting and gel electrophoresis

Footprinting experiments with DNase I, micrococcal nuclease and diethylpyrocarbonate were performed as previously described [4,6,14]. The products of digestion were resolved on denaturing polyacrylamide gels (8% for fragments labelled at the HindIII end, 12% for fragments labelled at the EcoRI end) containing 8M urea. These were then fixed in 10% (v/v) acetic acid, transferred to Whatman 3MM paper, dried under vacuum at 80°C, and subjected to autoradiography at -70°C with an intensifying screen. Autoradiographs were scanned with a Joyce-Loebl Chromoscan 3 microdensitometer.

RESULTS

Homopolymeric A and T

Figure 1 presents the results of DNase I digestion of fragments containing the inserts T15CGA15 and A15CGT15 in the presence and absence of various concentrations of echinomycin. The drug free control lanes for A15CGT15 show little cleavage within the insert, even the central ACGT is uncut. The pattern changes considerably in the presence of echinomycin although no footprint is evident due to the poor cutting of this region in the control (a footprint can be seen at the sequence TCGG just above the insert). The strong enhancements on either side of the CG site are strongly suggestive of drug binding. These enhancements are greatest at the 5'-ends of the runs of A and T and fade out in the 3'-direction. A different pattern can be seen with T15CGA15. In the control lanes no cleavage is evident in the runs of A and T though four bonds are visible corresponding to cleavage of the central TTTTCG sequence. In the presence of echinomycin no footprint is evident around the central TCGA step, although enhancements on either side suggest that some interaction between the drug and DNA has occurred. Once again a footprint can be seen at the CG site outside the insert. The enhancements vary according to the concentration of echinomycin; at 5μM ligand they are seen at the second and third ApA bond on the 3'-side of the CG step, with 25μM echinomycin the pattern becomes more like that of A15CGT15 with enhancements on either side of the CpG site.
of the CG site which fade out in the 3'-direction. These extend from the second to the seventh adenine on the 3'-side of the drug binding site.

The results of DEPC mediated strand scission for the A₁₅CGT₁₅ and T₁₅CGA₁₅ inserts are presented in Figure 2 in the presence and absence of echinomycin. Cleavage in the controls reveals weak modification of 14 adenines (the one closest to the 3'-end is unreactive for both fragments). In the presence of echinomycin the patterns change. Looking first at T₁₅CGA₁₅ it can be seen that the first adenine on the 3'-side of the CpG (CGA) is especially reactive. Modification of the second adenine is also increased, the other adenines show a reduced susceptibility to DEPC. With A₁₅CGT₁₅ modification of the third adenine on the 5'-side of the CpG is increased. The strongest cleavage products are found at higher concentrations of echinomycin (25µM and above) at the two adenines furthest from the CG site. It is possible that these enhancements arise from secondary drug binding to the CC or CA steps at the junction between the end of the insert and the rest of the fragment.

**Alternating AT**

Figure 3 presents the results of DNase I digestion of the fragment containing the sequence T(AT)₈CG(AT)₁₅ labelled on both strands in the presence of echinomycin. Corresponding densitometer traces are shown in Figure 4. An alternating pattern of bands can be seen in the control lanes in which cleavage of ApT is much better than TpA as previously noted by other workers [15,16]. In the presence of echinomycin a clear footprint is evident around the central CG extending over 6–8 bases. More bases are protected towards the 3'(lower)-side of the CG on account of the staggered cleavage of the enzyme. In the presence of low concentrations of echinomycin cleavage of TpA is enhanced while that of ApT remains unchanged. This is similar to the changes reported for a DNA fragment containing alternating AT flanked by two echinomycin binding sites [5]. At higher concentrations of echinomycin (25µM and above) an unusual cleavage pattern is evident with a four base repeat. This is more clearly seen in the densitometer traces shown in Figure 4. The first TpA step on the 3'-side of the CG is cut poorly, this is followed by an unchanged ApT step, a TpA step which is strongly enhanced (though slightly weaker than the preceding ApT) and an enhanced ApT. This pattern, in which every other ApT bond is cut best, repeats itself throughout the (AT)ₙ stretches on both sides of the central CG and can be seen when the DNA fragment is labelled at either end. Comparison of the

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**Figure 3.** DNase I footprinting patterns for the effect of echinomycin on the fragment containing the insert T(AT)₈CG(AT)₁₅ at 37°C. The DNA is labelled at the 3'-end of (a) the HindIII site (b) The EcoRI site. Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 minutes. Echinomycin concentrations (µM) are shown at the top of each lanes. Echinomycin controls (µM) are shown at the top of each lanes. The drug-free controls are labelled CON. The tracks labelled ‘G’ are dimethylsulphate-piperidine markers specific for guanine. The square brackets indicate the position and length of the inserts; arrows show the CpG sites.

**Figure 4.** Densitometer scans of the data presented in Figure 3 for DNase I digestion of T(AT)₈CG(AT)₁₅ in the presence of 50µM echinomycin. The DNA is labelled at the 3'-end of the EcoRI site (A) and the HindIII site (B). Boxes show the CpG binding sites, vertical lines indicate cleavage of the ApT steps. c) summarises the susceptibility of ApT bonds to cleavage by DNase I in the presence of echinomycin. S and w indicate bonds which are cut strongly and weakly respectively.
results for the two strands reveals that the most sensitive ApT bonds are found at identical, complementary steps (Figure 4c). A similar four base pair repeat pattern is also detected with micrococcal nuclease (not shown). The simplest explanation for this phenomenon is that secondary echinomycin binding has occurred within the (AT)ₙ region, but that this is not strong enough to yield a footprint presumably because the drug dissociates too quickly. This will be considered further in the Discussion.

Figure 5 presents the results of DEPC modification of the T(AT)₈CG(AT)₁₅ fragment, labelled on both strands, in the presence and absence of echinomycin. No cleavage products are visible in the control lanes; in the presence of echinomycin adenines throughout the insert become hyperreactive to DEPC. On inspection an alternating pattern of cleavage products is evident in which every other adenine is susceptible to modification. This strict alternation fades with increasing distance from the CG site. Reactive adenines are staggered in the 5'-direction across the two strands (Figure 5c). In this instance modification of the adenine immediately distal to the drug binding site CGA is no stronger than that of the other adenines in the sequence.

**DISCUSSION**

**Sequence selectivity**

The results presented above confirm the selectivity of echinomycin for the dinucleotide step CpG. However not all such potential binding sites yield clear footprints; T₁₅CGA₁₅ showed enhancements only. It seems reasonable to suppose that this is a weaker binding site and that the drug is resident on the DNA for a shorter period of time, insufficient to protect from nuclease attack. The enhanced modification of the distal adenine by DEPC and the flanking enhancements in DNase I activity indicate some interaction between the CpG site and the drug. This does not necessarily mean that TCGA is a poor echinomycin binding site since the fragment containing flanking regions of alternating A and T also contains a central TCGA and reveals a clear echinomycin footprint. However by comparison with A₁₅CGT₁₅ it does appear that TCGA is a weaker binding site. It may be relevant that NMR studies reveal the presence of Hoogsteen base pairs for AGCT but not TCGA [9].

Although DNase I footprints are only found around the sequence CpG some of our results indicate weaker drug binding at CpC or CpA steps. Enhancements in DNase I activity are clearly visible at the 3'-ends of both T₁₅CGA₁₅ and A₁₅CGT₁₅, furthest from the CG site which fade out towards the centre of the inserts. If we assume that enhancements should be stronger close to the ligand binding site then this would suggest an echinomycin binding site at the 3'-end of the inserts, i.e. CT, CA or CC. The enhancement in DEPC modification of the first two adenines in A₁₅CGT₁₅ lends weight to this suggestion.

**Echinomycin binding to alternating AT?**

At concentrations of 25µM and above echinomycin induces unusual cleavage patterns for DNase I, micrococcal nuclease and DEPC based on a four base pair repeat. The observation that this pattern can be detected with all three probes argues against this being an artefact. It seems unlikely that this four base pair repeat reflects the formation of a novel DNA structure induced by the binding of the ligand. A more likely explanation is that echinomycin has

**Effect of temperature**

DNase I footprinting experiments were also performed at 4°C for two reasons. Firstly we reasoned that at this lower temperature the DNA should be more rigid and less easy to distort. Secondly it has been shown that, for some drugs which are in rapid exchange with DNA, lowering the temperature generates clearer footprinting patterns [17]. We were therefore interested to see how this might affect the pattern seen with adjacent regions of alternating AT. The results for T(AT)₈CG(AT)₁₅ (not shown) were identical to those obtained at 37°C with a footprint around the central CG and a four base pair repeat in the remainder of the (AT)ₙ region. In contrast temperature has a profound effect on T₁₅CGA₁₅ and A₁₅CGA₁₅. At the lower temperature both inserts are uncut by the enzyme; it is therefore impossible to detect echinomycin footprints. However the enhancements which were found at 37°C are no longer present. The lack of any changes suggests that the drug has failed to bind to DNA at this lower temperature. This is similar to results for actinomycin binding adjacent to regions of homopolymeric AT and suggests that the enhancements are a necessary consequence of drug binding. Restricting the DNA conformation prevents drug binding.
interacted directly with these regions of alternating AT. It has long
been known that echinomycin binds well to poly(dA-dT) with an
intrinsic binding constant of $3 \times 10^3 M^{-1}$, not much less than that
of $5 \times 10^3 M^{-1}$ for poly(dG-dC) [2] and that this binding is highly
cooperative. We therefore propose that the unusual cleavage
patterns arise from drug binding to the (AT)$_n$ regions. This
interaction must be too weak (fast dissociation) to yield clear
footprints, consistent with the very different dissociation rates
measured for echinomycin from poly(dG-dC) and poly(dA-dT)
[18]. The repeat structure is the same size as the echinomycin
binding site size of four base pairs [3].

However, if echinomycin does bind to ApT (or TpA) we would
expect still an even ladder of cleavage products for each identical
dinucleotide step. The unusual pattern suggests that not all ApT
(or TpA) steps are equally occupied. We explain this as follows.
T(AT)$_3$CG(AT)$_3$ contains a central strong echinomycin binding
site (CG) which we can assume will be preferentially occupied
by the ligand. This then will exclude drug binding at the adjacent
AT (or TA) steps due to neighbour exclusion. If there is any
cooperativity in the interaction then binding will be strongest at
the next available site (i.e. the second AT or TA). This in turn
should improve drug binding at the next available step (the fourth
TA or AT since binding to the third will be forbidden due to
neighbour exclusion). This process will be repeated along the
(AT)$_n$ region and give rise to a four base pair repeat pattern.
As we move away from the central drug binding site the probability
that the ligand will bind to the ‘wrong’ step, out of phase,
will increase. This is indeed what we observe—the repeat
structure fades out as we move away from the central drug
binding site. This explanation for the unusual cleavage pattern
is totally dependant on the presence of the central strong
echinomycin site. It is this that forces the drug molecules to line
up in phase; without it echinomycin should interact randomly
with each of the binding steps in the (AT)$_n$ region.

Granted that echinomycin interacts within the AT region does
it bind to ApT or TpA? We suggest ApT on the basis of the results
obtained for DEPC modification. This revealed that the most
hyperreactive adenines were staggered in the 5' direction across
the two strands. This is what we predict for a ligand sandwiching
the dinucleotide step ApT. In contrast for drug binding to TpA
the intermediate adenines would be staggered in the 3'-direction
across the two strands. In addition, in all previous studies the
bases most reactive to DEPC have been located on the 3'-side
of echinomycin (CG) binding sites. This is still so if the ligand
binds at ApT; if the drug is binding to TpA then the reactive
bases are in the proximal position. The binding of echinomycin
to (AT)$_n$ sequences is explored in more detail in the following
paper.

Structural changes
The results presented in this paper confirm that echinomycin can
alter the susceptibility of regions surrounding its binding sites
to both nuclease and chemical attack. The simplest explanation
for these changes is that the drug has altered the local DNA
structure, possibly by virtue of its ability to unwind the DNA
helix, rendering it more susceptible to nuclease attack. The other
plausible explanation for these changes is that they arise as a
necessary consequence of changes in the ratio of enzyme to free
DNA substrate [19,20]. Is this sufficient to explain the results
presented in this paper? We think not for several reasons. Firstly
this can not account for the observation that bonds within the
insert are enhanced to the same extent. This is especially relevant
when we consider changes in the cleavage of adjacent bonds.
For example within regions of homopolymeric A and T all ApA
(and TpT) bonds should experience the same increase in relative
free enzyme concentration and therefore be enhanced to the same
extent. This is not what we observe. Secondly this theory applies
to changes in susceptibility to nuclease attack and does not account
for the increased modification by diethylpyrocatecarbonate.

$T(AC)CG(AC)$ and $T(AC)CG(AC)$
PolydA.polydT is known to adopt an
unusual structure with 10.0 bases per turn in which the bases
are highly propeller twisted and which is resistant to nuclease
attack and intercalative drug binding [21–23]. It appears that
when echinomycin binds adjacent to these regions their structure
is modified so that it more closely resembles that of B-DNA.
This most likely results from a combination of the local unwinding
of the DNA helix and the flattening of the propeller twist caused
by inserting the rigid quinoxaline chromophore. The results
presented in Figure 1 suggest that for DNase I enhancements are
propagated in both directions for $T(AC)CG(AC)$ but are much
stronger on the 3'-side of $A(AC)CG(AC)$. This is consistent with
the observation that enhancements in DEPC modification are found
in adenines on the 3'- (not 5')-side of CG. A similar effect has
been shown for actinomycin binding to A(AC)CG(AC) and TpGCAG(AC)
[24]. It appears that conformational changes are transmitted
differently across purine-purine and purine-pyrimidine steps.

$A(AC)CG(AC)$ and $T(AC)CG(AC)$
Regions of alternating AT are known to adopt
an unusual B-DNA structure in which the poor stacking of TpA
is sacrificed to improve the ApT interaction [15,16,25]. As a
result this structure is easy to deform since large conformational
changes have only small effects on the energetics, especially at
the TpA steps. Low concentrations of echinomycin appear to
increase cleavage of ApT by DNase I though the interpretation
is complicated by the four-base pair pattern induced at higher
ligand concentrations as a result of secondary drug binding.
However it should be noted that within this unusual pattern
cleavage of alternate TpA bonds (which were barely evident in
the control) is now similar to the adjacent ApT bonds. We
postulate that echinomycin alters the local helix conformation of
(AAT)$_n$ so that it more closely resembles an unwound B-DNA
helix with similar conformations at the ApT and TpA steps.

Effect of temperature
Echinomycin binding sites flanked by homopolymeric A and T
display a different temperature dependence to those flanked
by regions of alternating A and T. We suggest that this is the result
of the rigid DNA conformation adopted by the former which
prevents the propagation of conformational changes and prevents
opening of the intercalation sites to accept the quinoxaline
chromophores. Indeed this may also explain why $T(AC)CG(AC)$
presents a poor echinomycin binding site, even at $37^\circ C$. These
results serve to emphasise that flanking sequences affect the ability
of echinomycin to bind to its selective sites, and that DNA
flexibility plays an important role in sequence selectivity.

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