Enzymatic and Chemical Footprinting of Anthracycline Antitumor Antibiotics and Related Saccharide Side Chains†

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ABSTRACT: DNase I and three DNA chemical footprinting agents were used to compare the DNA binding properties of the anthracycline antitumor antibiotics daunomycin, aclacinomycin A, and ditrisarubicin B. These anthracyclines contain a tetracyclic chromophore which intercalates into DNA and a monosaccharide, trisaccharide, and two trisaccharide side chains, respectively. These side chains consist of between one and three 2,6-dideoxy, 1,4-diaxially linked sugars. Three chemical probes, fotemustine, dimethyl sulfate, 4-(2′-bromoethyl)phenol, and the enzymic probe DNase I were used in the footprinting experiments. The chemical probes provided a clear picture of the binding pattern at 37 °C and more detailed information than that obtained using the standard DNase I footprinting assay. All three anthracyclines showed preferred binding to 5′-GT-3′ sequences in both the chemical and enzymatic footprinting. DNase I footprinting showed that the number of base pairs of DNA protected from cleavage increased with the number of saccharide groups present at particular sites and is consistent with DNA binding of the saccharide side chains. Alkylation of runs of guanine by fotemustine was inhibited by all three anthracyclines, while alkylation by dimethyl sulfate was enhanced for most guanines. The probe 4-(2′-bromoethyl)phenol showed that all three anthracyclines completely protected all of the adenines in the minor groove from alkylation, and enhanced major groove guanine alkylation was observed with aclacinomycin A, daunomycin, and, to a much lesser extent, ditrisarubicin B. These results are consistent with intercalation of the aglycone ring and binding of the rigid, hydrophobic saccharide side chains in the minor groove. Footprinting of four methyl glycosides related to the anthracyclines showed no evidence of DNA binding with any of the agents studied.

The anthracyclines are a well studied class of antitumor antibiotics that contain a tetracyclic chromophore to which is attached one or two saccharide side chains. A large number of studies have shown that these compounds interact with DNA by intercalation (Lown, 1988, 1993) and that they inhibit topoisomerase II (Jensen et al., 1993). In common with the anthracyclines, other DNA-binding antitumor antibiotics (e.g., chromomycin, calicheamicin, and esperamicin) contain saccharide groups. Recent studies of chromomycin (Keniry et al., 1993; Silva & Kahne, 1993; Gao et al., 1992) and calicheamicin (Kahne, 1995; Walker et al., 1990; Paloma et al., 1994; Li et al., 1994) have shown that the sugars serve an important role in DNA recognition and that they contribute to sequence specificity.

The carbohydrate side chains present in the anthracyclines are structurally related and contain between one and five sugars which have the 2,6-dideoxy-a-L configuration and are coupled via 1−4 axial linkages. The number of these rigid, hydrophobic sugars and their substitution pattern has a profound effect on biological activity, and removal of the sugar side chain results in biological inactivity (Lown, 1988, 1993). X-ray crystal structures (Frederick et al., 1990; Gallois et al., 1993; Wang et al., 1987; Moore et al., 1989, Williams et al., 1990) of the binding of daunomycin and other related monosaccharide anthracyclines, with short oligonucleotide sequences, have provided a detailed picture of the nature of the drug−DNA complex. These crystal structures show DNA intercalation of the tetracyclic chromophore and the sugar resting in the minor groove.

Williams et al. (1989) have proposed that the anthracyclines have evolved with carbohydrate side chains as a natural DNA minor groove binding motif. However, while daunomycin and its derivatives have been well-characterized, there is only one crystal structure of a semisynthetic anthracycline containing a disaccharide (MAR70) complexed to DNA (Gao et al., 1991) and no crystal structures of trisaccharide-containing anthracyclines. The first solution structure of an anthracycline containing three sugars complexed to an oligonucleotide sequence was recently reported (Yang & Wang, 1994) and suggested that the trisaccharide lies in the minor groove of DNA. Further evidence for the groove binding of the sugar side chains of anthracyclines comes from a comparison of the association constants of doxorubicin, betaclamycin A, and ditrisarubicin B with DNA (Kunimoto et al., 1988). These molecules are all members of the anthracycline family and contain a monosaccharide, trisaccharide, and two trisaccharides, respectively, attached to the chromophore. The higher affinity constant of ditrisarubicin B for DNA, compared with betaclamycin A and doxorubicin,

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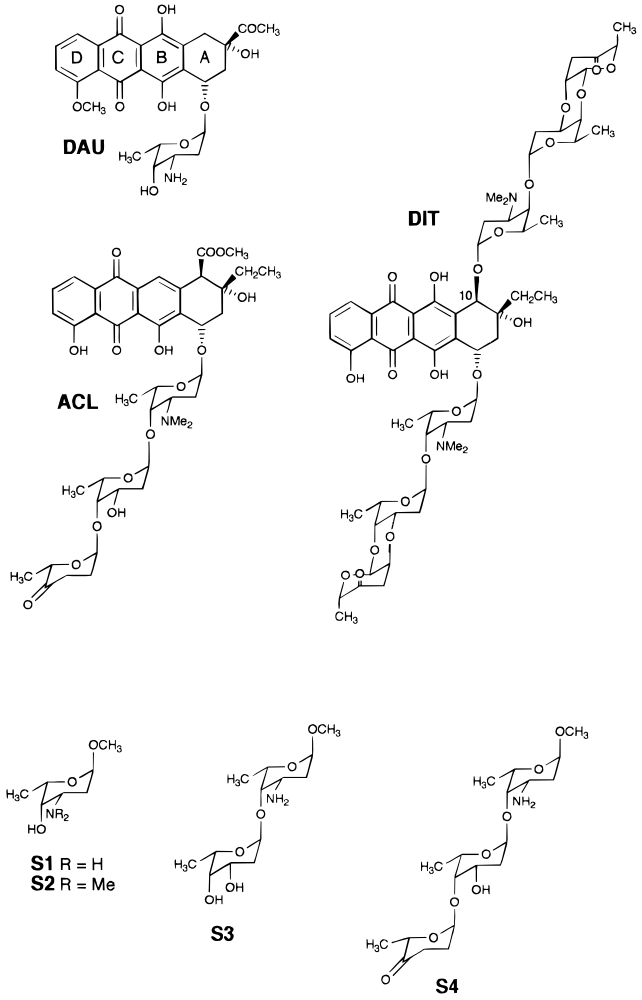

FIGURE 1: Constitutional formulae, numbering scheme, and abbreviations for daunomycin (DAU), aclacinomycin A (ACL), ditrisarubicin B (DIT), and methyl glycosides (S1−S4).

was attributed to the presence of the additional trisaccharide chain located at C10.

While there is good evidence for the binding of the sugar side chains of the anthracyclines to DNA, their role, if any, in sequence specificity has not been established. The role of the sugar side chains in the antitumor activity of the anthracyclines could involve a contribution to the DNA sequence selectivity of the anthracycline molecule and/or protection from the activity of particular DNA replication enzymes. Topoisomerase II has been shown to be inhibited by the anthracyclines (Jensen et al., 1993), but the nature of this inhibition is yet to be identified. This paper reports enzymatic and chemical footprinting of three anthracyclines, daunomycin (DAU), aclacinomycin A (ACL), and ditrisarubicin B (DIT) which contain a single saccharide, a trisaccharide, and two trisaccharides, respectively. The footprinting of four methyl glycosides (S1−S4) which are related to the anthracycline side chains (Figure 1) is also reported. Chemical probes which are sensitive to DNA secondary structure were used to complement the results obtained using DNase I. Chemical probes have been used previously to study structural changes associated with binding of the DNA-bisintercalating agent echinomycin (McLean & Waring, 1988) with limited applications in DNA-footprinting (Coley et al., 1993; Chaires et al., 1987; Bailly & Waring, 1995). Footprinting studies using methidiumpropyl-EDTA·Fe(II) probe which cleaves DNA via hydroxy radicals failed

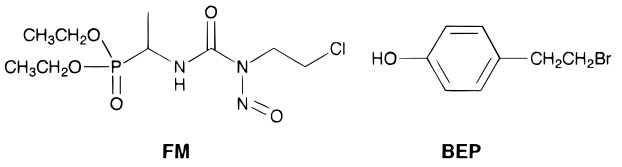

FIGURE 2: Structures of alkylating agents fotemustine (FM) and 4-(2′-bromoethyl)phenol (BEP).

to show any footprinting for DAU (Dyke et al., 1982). The chemical probes used in this study were chosen such that (i) they do not alter the binding of antibiotics to DNA unlike in the case of MPE·Fe(II) and echinomycin (Bailly & Waring, 1995), (ii) they are sensitive to DNA secondary structure, and (iii) they react with DNA under physiological conditions. These chemical probes allowed a more detailed picture of DNA recognition and binding to be obtained.

MATERIALS AND METHODS

Materials. Ditrisarubicin B (DIT) was a gift from Professor Uchida (Pharmaceutical Research Laboratory, Kirin Brewery, Japan). Aclacinomycin A (ACL) was obtained from the National Cancer Institute. Daunomycin (DAU) was obtained from the Sigma Chemical Co. Fotemustine (FM) was supplied by Servier Laboratories. 4-(2′-Bromoethyl)-phenol (BEP) was a gift from Dr. David Young (University of Queensland). Methyl glycosides S1, S3, and S4 were prepared as previously reported (Shelton & Harding, 1995), and the final deprotection was carried out in aqueous sodium hydroxide and dried without readjustment of the pH to 7; any trace of acid led to cleavage of the glycosidic linkages between the first and second sugar and the second and third sugar. The dried products were dissolved in methanol and purified by a preequilibrated silica column chromatography (80% dichloromethane/19.5% methanol/0.5% triethylamine). Reductive *N*-methylation of S1 was carried out according to the method of Monneret et al. (1988) to give S2. The structures of the antibiotics and the chemical agents used are shown in Figures 1 and 2.

DNA Fragment. A 375 base pair *Eco*RI to *Bam*HI fragment of pBR322 DNA was 3′-end-labeled at the *Eco*RI site using Klenow fragment and [α-^{32}P]dATP according to published procedures (Prakash et al., 1990). The fragment was isolated on a 4% nondenaturing polyacrylamide gel.

Sample Preparation. Stock solutions were as follows: Anthracyclines (1 mM in methanol), methyl glycosides (50 mM in methanol), DNase I (10 units/mg) in DNase I buffer (1×), FM (100 mM in methanol), DMS [2% (v/v) in ethanol], BEP (50 mM in dimethyl sulfoxide), and piperidine (1 M in water). TE buffer contained 10 mM Tris-HCl and 1 mM EDTA, pH 7.4; phosphate buffer: 10 mM, pH 7.1; DNase I buffer stock (5× final concentration): 100 mM NaCl, 10 mM $MgCl_2$, and 10 mM $MnCl_2$.

Footprinting Methods. In all footprinting, appropriate controls were run with the test samples. Labeled DNA (at 10 000 cpm) samples were preincubated with one of the anthracycline antibiotics (1 mL) or one of the methyl glycosides (2 μL) for 30 min at room temperature in an appropriate buffer adjusted so that the total reaction volume was 100 μL. DNase I footprinting was carried out in DNase1 buffer (1×) in the presence of 2 μL of 10 units/mg DNase I and incubated at room temperature for 30 min. The samples were then precipitated twice in ethanol. Foot-

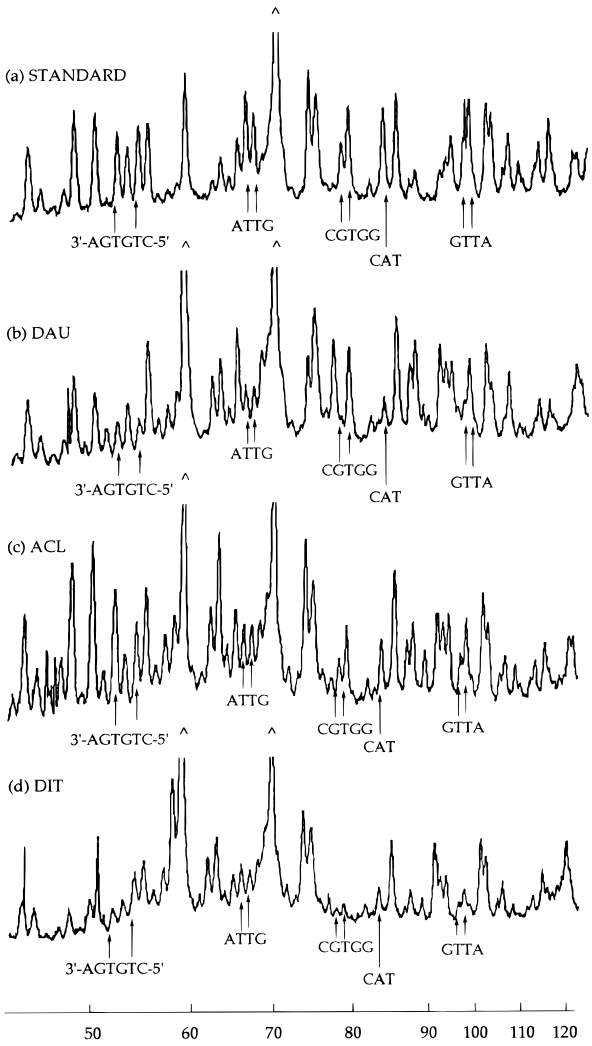

FIGURE 3: Densitometer scans from a 3′-end-labeled *Eco*RI−*Bam*HI pBR322 fragment of DNA cleavage patterns obtained with (a) DNase I alone and in the presence of (b) DAU, (c) ACL, and (d) DIT; arrows on trace b highlight bases reduced in intensity by >80% compared to the standard trace a; the symbol "^" indicates peaks which are off-scale. Note that the highlighted base sequences listed below individual traces are slightly out of alignment due to compressed nature of the numerical scale at the bottom of the figure.

printing in the presence of 1 mM FM and 0.02% DMS were carried out in TE buffer and incubated for 1 h at 37 °C. The samples were precipitated followed by piperidine treatment in which the samples were heated for 10 min at 90 °C in the presence of 1 M piperidine followed by lyophilization to remove the solvent. BEP footprinting was carried out in phosphate buffer in the presence of 0.5 mM BEP and incubated for 1 h at 37 °C. The precipitated samples were then redissolved in TE buffer (100 μL) and heated at 90 °C for 10 min, reprecipitated, and then subjected to the piperidine treatment (White et al., 1995). The samples and sequencing lanes were run on a denaturing 6% polyacrylamide gel. Densitometer scans (Personal Densitometer 68−272 and Molecular Dynamics Software) were used to analyse the DNase I autoradiograms. The DNase I footprints were carried out six times, varying the anthracycline and enzyme concentrations. The DNase I results were reproduced in two independent experiments using very similar drug and enzyme concentrations. The chemical footprinting experiments were repeated six times altering the drug concentration only. All of these gels gave similar footprinting patterns.

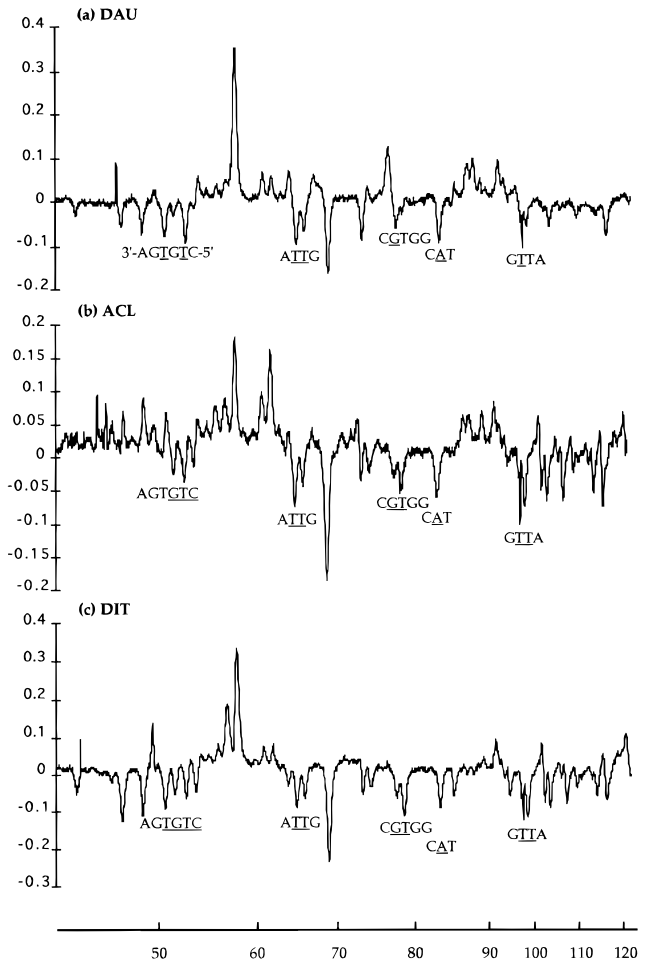

FIGURE 4: Difference spectra obtained from densitometer scans shown in Figure 3. Spectra were obtained by subtracting the standard DNase I digestion band intensities from the digestion carried out in the presence of each anthracycline. Each spectrum was normalized to the biggest positive peak; the *Y* scale indicates the raw intensity difference of each spectra. Positive peaks are sites where DNA cleavage was enhanced, and the negative peaks are sites where DNA cleavage was inhibited.

RESULTS

Studies were carried out with DAU, ACL, and DIT and with monosaccharides S1 and S2, disaccharide S3, and trisaccharide S4, which are saccharides related to the anthracycline sugar side chains. Footprinting studies were carried out enzymatically using DNase I and the chemical probes FM, DMS, and BEP.

DNase I Studies. DNase I digestion of a 375 base pair pBR322 fragment, alone and in the presence of DAU, ACL, and DIT, was carried out under standard conditions. A densitometer scan of a section of the DNA sequence showing representative results was obtained (Figure 3) and converted to a series of difference spectra (Figure 4) to allow a more quantitative analysis of the data to be carried out. The difference spectra were obtained by subtracting the standard DNase I digestion band intensities from the digestion carried out in the presence of each anthracycline. Each spectrum was normalized according to the biggest positive peak, and the *Y* scale has been increased in each spectrum shown in Figure 4 to allow direct comparison of the results. The positive peaks are sites where DNA cleavage was enhanced, and the negative peaks are sites where DNA cleavage was inhibited.

Table 1: Summary of the DNA Sequences (5′−3′) Affected by the Anthracyclines in the Presence of Various Footprinting Agents[a]

agent	DAU	ACL	DIT
DNAse I (Figures 3 and 4)	TGT (54−52) GTTA (68−65) GGTGCC (81−76) TACA (86−83) TTG (99−97) GAT (115−113) GGTGC (124−120)[b] CAGGGTG (133−127)[b] CTATGCCTAC (149−140)[b]	CTGT(54−52) GTTA (68−65) GGTGC (81−77) TAC (86−84) TTG (99−97) CAGGGT (133−128)[b] TCC (135−133)[b] CTATGCCTAC (149−140)[b,d]	CGTG (53−51) GTTA (68−65) GTGC (80−77) TAC (86−84) ATTG (100−97) GAT (115−113) TGCCG (122−118) CAGGGT (133−128)[b] CTATGCCTAC (149−140)[b]
FM (Figure 5)	 GGT (81−79)[c] AGA (94−92) AGCGC (105−101)[c] AGGA (117−114)[c] CGGT (125−122)[c] GGGT (131−128)[c]	TGC (71−69) GGT (81−79) AGA (94−92) AGCGC (105−101) TGA (113−111) AGGA (117−114) CGGT (125−122) GGGT (131−128)	 CGGT (82−79) AGCGC (105−101) TGA (113−111) AGGA (117−114) CGGT (125−122) GGGT (131−128)
DMS (Figure 6)	not studied	 GGT (81−79) GGT (124−122) GGGT (131−128)	CGT (69−67) GGT (81−79) GGT (124−122) GGGT (131−128)
BEP (Figure 5)	all adenines inhibited[c] guanines strongly enhanced except: GTG (53−51)[c] TGC (71−69)[c] TGA (75−73)[c] AGGA (117−114)[c] GCA (138−136)[c]	all adenines inhibited guanines strongly enhanced except: GGT (81−79) GGT (124−122) GGGT (131−128)	all adenines inhibited guanines weakly enhanced

[a] Underlined sequences indicate sites of inhibition for DNase I and FM and sites of least enhancement for DMS and BEP results. [b] Sites not shown in Figure 3. [c] Autoradiogram not shown. [d] Site not shown in Figure 7.

From Figure 3, inhibition of DNA cleavage in the presence of DAU occurred at (in 5′ to 3′ direction) TGTG (54−52), GTTA (68−65), GGTGCC (81−76), TACA (86−83), TTG (99−97) and GAT (115−113, off scale). The sites estimated to be reduced in intensity by more than 80% are highlighted by arrows (Figure 3b) compared to the standard sequence (Figure 3a) and have been underlined in the text. These inhibition sites are more clearly seen from the difference spectrum (Figure 4a), where the inhibition sites are shown by negative peaks. Many of these sites occur in regions of alternating purines and pyrimidines, mainly at 5′-GT-3′. The thymine of these sequences was most often reduced in intensity, although the adenine and guanine adjacent to a thymine were also sometimes inhibited (e.g., TACA 86−83). Note that the biggest negative peak around base 70 is actually due to software limitation in obtaining difference in intensity between two off-scale peaks. This does not pose a problem for the positive peak at base 58 because the standard peak (Figure 3a) is within scale.

Inhibition of DNA cleavage in the presence of ACL was similar to that found for DAU (Figures 3c and 4b), but the GGTGCC (81−76) sequence showed inhibition of an extra T base compared with DAU. Inhibition to DNA cleavage in the presence of DIT gave the same sites as observed for DAU (Figures 3d and 4c): TGTG (54−51), GTTA (68−65), GGTGCC (81−76), TACA (86−83), ATTG (99−97), and GAT (115−113). The inhibition sites TGTG (54−51), GGTGCC (81−76), and ATTG (99−97) covered an additional base pair in most sequences compared to DAU (Figure 4c).

Table 1 (first section) summarizes the sequence data presented in Figures 3 and 4 plus other sequence data obtained from the full gel. The 5′-GT-3′ sequences were inhibited most commonly by all three anthracyclines as well as 5′-AC-3′ (85−84), which is complementary to a GT sequence in the opposite strand. Each of the anthracyclines inhibited DNA cleavage at the same sites. However, a trend of increasing protection area was observed with an increasing number of sugar groups present in the drug. Not all of the available 5′-GT-3′ sequences were inhibited by the anthracyclines, consistent with different flanking sequences which affect DNA secondary structure.

DNase I footprinting of the methyl glycosides S1, S2, S3, and S4 was carried out using identical conditions to that described for the anthracycline antibiotics. The experiments were carried out twice and showed that the dimethylated monosaccharide S2 and the trisaccharide S4 increased the activity of DNase I. The methyl glycosides S2 and S3 did not alter the DNase I footprinting pattern.

Chemical Footprinting Studies. Chemical footprinting of DAU, ACL, and DIT and the methyl glycosides was carried out with the chemical probes FM, DMS, and BEP using the same 375 base pair pBR322 fragment as used in the DNase I experiments.

FM is an example of a new class of chloroethylnitroso ureas currently in clinical trials as an antitumor agent. The compound alkylates N7 of guanines with a preference for the 3′ end of guanine tracts. Alkylation of DNA by FM is slow (>1 h), and the sites of reaction are dependent on DNA structure and conformation (Prakash et al., 1995). FM was

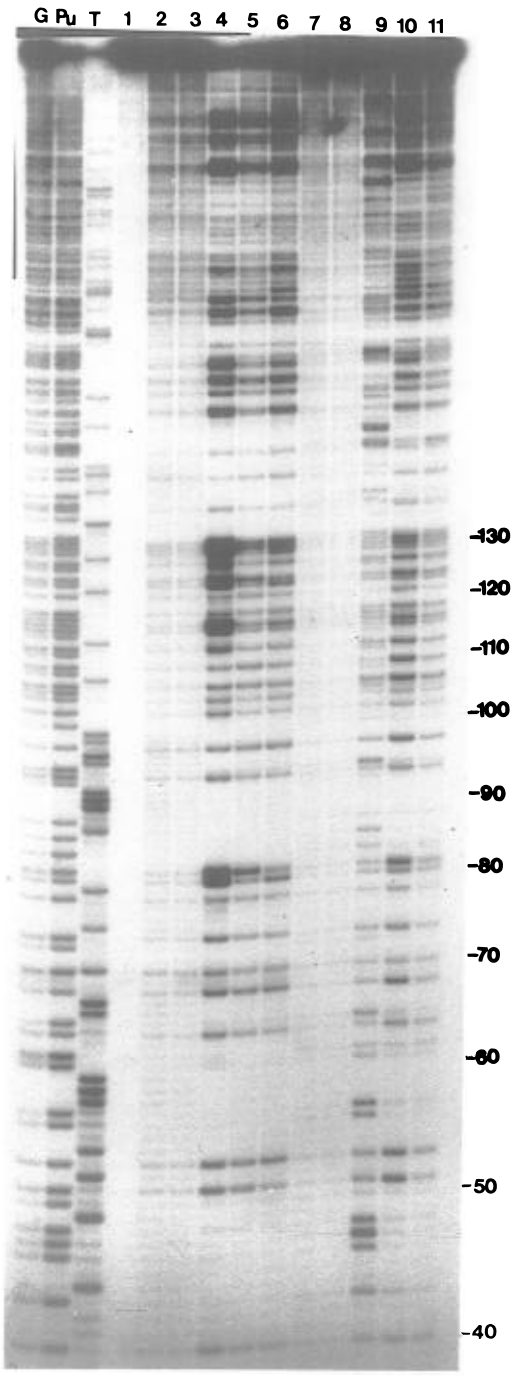

FIGURE 5: Autoradiogram of alkylation patterns of anthracyclines ACL and DIT using FM and BEP. Lane 1, control (TE buffer); lane 2, ACL; lane 3, DIT; lane 4, FM; lane 5, FM + ACL; lane 6, FM + DIT; lane 7, control (phosphate buffer); lane 8 ACL; lane 9 BEP; lane 10 BEP + ACL; lane 11 BEP + DIT. G, Pu, and T are sequencing lanes.

used in this study to investigate the effect of drug binding on the major groove.

The effect of ACL and DIT on the alkylation of DNA by FM is shown in Figure 5 (lanes 1−6). The relative intensities of the bands in lanes 5 and 6, which contain ACL and DIT, respectively, are quite different to the control lane 4 which contains FM alone. Overall, ACL and DIT protected the DNA from FM alkylation as can be seen from the intensity of the unmodified DNA band at the top of the gel (Figure 5, lanes 4−6). The FM footprint pattern in the presence of ACL (lane 5) shows inhibition at the following sites, where the underlined sequences are those estimated to be reduced in intensity by more than 80%: TGC (71−69), GGT (81−79), AGA (94−92), AGCGC (105−101), TGA (113−111), AGGA (117−114), CGGT (125−122), and GGGT (131−128). In the presence of DIT, inhibition was observed at GGT (81−79), AGA (94−92), AGCGC (105−101), TGA (113−111), AGGA (117−114), CGGT (125−122), and GGGT (131−128). Minor variations between ACL and DIT in the footprinting pattern were observed at GGT (81−79) and AGCGC (105−101). As can be seen from the above sequences, runs of guanines were protected strongly by both ACL and DIT with a preference for the final 5′ guanine in the run. The first base after these guanine runs was often a thymine. Some guanines not flanked by a 5′ thymine were also protected to a lesser extent: AGGA (117−114), AGA (94−92), and AGCGCA (105−100).

DAU was observed to inhibit sites similar to that obtained with ACL and DIT (data not shown) but showed much less preference for the 5′ guanine at the end of the run and tended to inhibit all of the guanines in the run: GGT (81−79), AGCGC (105−101), AGGA (117−114), CGGT (125−122), and GGGT (131−128). Two of the inhibition sites found for each of the anthracyclines in both the DNase I footprinting and the FM footprinting were common: GGT (81−79) and GGT (130−128). The methyl glycosides (S1−S4) did not alter the footprinting patterns or alkylating activity of FM (data not shown).

A second alkylating agent, DMS, was also studied. As a chemical probe, DMS is similar to FM in that it alkylates the N7 of guanine in the major groove. However, due to its small size it is relatively insensitive to steric effects. Figure 6 shows the effect of ACL (lane 9) and DIT (lane 10) on the alkylation of DNA by DMS. For comparison, lanes 4−7 again show the effect of ACL (lane 5) and DIT (lane 6) on FM. In contrast to the FM results, DMS alkylation in the presence of ACL and DIT was enhanced. However, certain guanines were not enhanced in the DMS reaction. Despite the differences in the alkylation activity between DMS and FM, in that DMS alkylation was enhanced while FM alkylation was reduced in the presence of the anthracyclines, the sites that were modified were similar in each case. The sites showing least guanine enhancement in the presence of ACL were: GGT (81−79), GGT (124−122), and GGGT (131−128) and in the presence of DIT were CGT (69−67) and GGT (81−79), GGT (124−122), and GGGT (131−128). The guanines not enhanced correspond to the 5′-GGT-3′ sequences in the FM studies (81−79, 124−122, and 131−128), and two correspond to the DNase I footprints (81−79 and 131−128). The footprinting patterns and activity of DMS was not altered by the methyl glycosides (S1−S4).

The third chemical agent assayed was BEP which has been shown to preferentially alkylate at N3 of adenine in the minor groove (White et al., 1995) and to some extent N7 of guanine in the major groove. This agent was used in this study to assess the selectivity of the anthracyclines in the minor groove. Figure 5 gives a typical example of the effect of ACL and DIT on the alkylation of DNA with BEP (lanes 7−11). Lanes 10 and 11 show the action of BEP in the presence of ACL and DIT, respectively, while lane 9 shows BEP alone. ACL and DIT completely inhibited the alkylation of adenines in the minor groove, and ACL strongly enhanced guanine alkylation in the major groove in most sequences. DIT enhanced guanine alkylation to a much lesser extent.

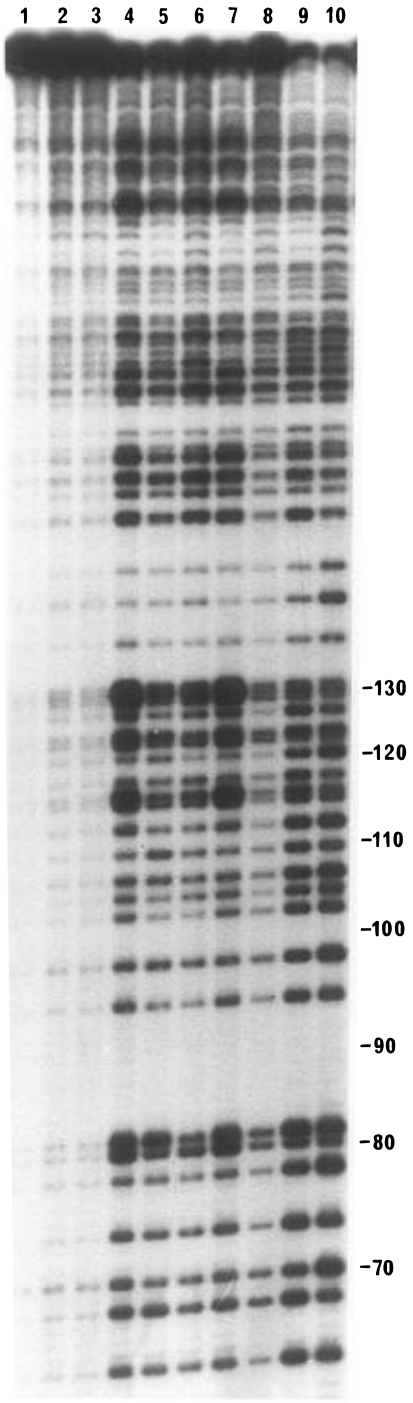

FIGURE 6: Autoradiogram of alkylation patterns of ACL and DIT using FM and DMS; lane 1, control; lane 2, ACL; lane 3, DIT; lane 4, FM; lane 5, FM + ACL; lane 6, FM + DIT; lane 7, FM; lane 8, DMS; lane 9, DMS + ACL; lane 10, DMS + DIT.

DAU behaved in a manner similar to that of ACL (data not shown). The guanines that were not as enhanced by DAU were GTG (53−51), TGC (71−69), TGA (75−73), AGGA (117−114), and GCA (138−136) and those for ACL were GGT (81−79), GGT (124−122), and GGGT (131−128). The sequences where guanine alkylation was not as enhanced correspond to sequences less enhanced in the DMS study (81−79, 124−122, and 131−128). These sequences are also common sites observed in the FM assay and DNase I assay. The methyl glycosides (S1−S4) did not affect the footprinting patterns or alkylating activity of BEP.

Table 1 summarizes the sites affected by all footprinting agents in the presence of DAU, ACL, and DIT, respectively. For easier comparison, these data have been shown schematically for ACL in Figure 7. Overall, the most common sites selected by the anthracyclines using any of the DNA cleaving agents were GGT (81−79), GGT (124−122), and GGGT (131−128).

DISCUSSION

Analysis of the DNA footprinting data obtained with DNase I and three chemical agents has provided molecular information about the sequence specificity and groove location of the three anthracyclines DAU, ACL, and DIT. While detailed footprinting of DAU has been reported (Chaires et al., 1987) and some data on the sequence selectivity of DIT are available (Fox & Kunimoto, 1989), DNA footprinting of ACL has not been reported. The chemical footprinting results presented here represent an alternative, complementary technique to DNase I footprinting. These agents give sharp footprints at 37 °C and can provide DNA binding information not available from standard enzymatic assays alone.

The two major groove alkylating agents FM and DMS were used in this study in order to distinguish between electronic and steric factors. Both agents are sensitive to changes in the nucleophilicity of N7 in guanine (Klopman et al., 1985; Pearlstein et al., 1980), which is heavily influenced by flanking bases in DNA (Pullman & Pullman, 1981). For example, a cytosine on the 5′ side decreases nucleophilicity while 3′ guanine enhances it. Drug binding to DNA in the vicinity alters the way the flanking bases affect the reactivity at this site. While both FM and DMS alkylate the N7 of guanine, FM is considerably larger than DMS, and hence different molecular information can be obtained from footprints with these reagents. DMS, due to its small size, can access sterically hindered sites in the major groove (Pearlstein et al., 1980) and hence assist in the identification of steric factors associated with drug−DNA complexes. Binding of agents in the minor groove has been shown to have long-range effects on alkylation in the major groove, and hence assignment of explicit drug binding sites is difficult. By contrasting the behavior of FM, BEP, and DMS along with DNase I results, we have attempted to identify or rule out long-range effects. It is important to emphasize that this is not possible by looking at the results of any one footprinting agent in isolation. Hydroxyl radical is also commonly used as a footprinting agent and has been shown to give well-defined footprints. This sequence neutral probe, due to its small size, can give different information to DNase I. However, hydroxyl radical alone also cannot distinguish between steric and electronic factors.

DNase I Footprints. The results of the DNase I digests show several patterns. First, with increasing length of the sugar chains going from DAU to ACL to DIT, the footprinting patterns are clearly different, as can be seen from the difference spectra (Figure 4). For example, the comparison of the difference spectra in the region 54−48 obtained with DAU (Figure 4a) showed inhibition of two thymines. ACL, which contains an additional two sugars compared with DAU, showed inhibition of adjacent bases (GTC), while DIT which contains two trisaccharides showed inhibition of additional flanking bases TGTC. This overall

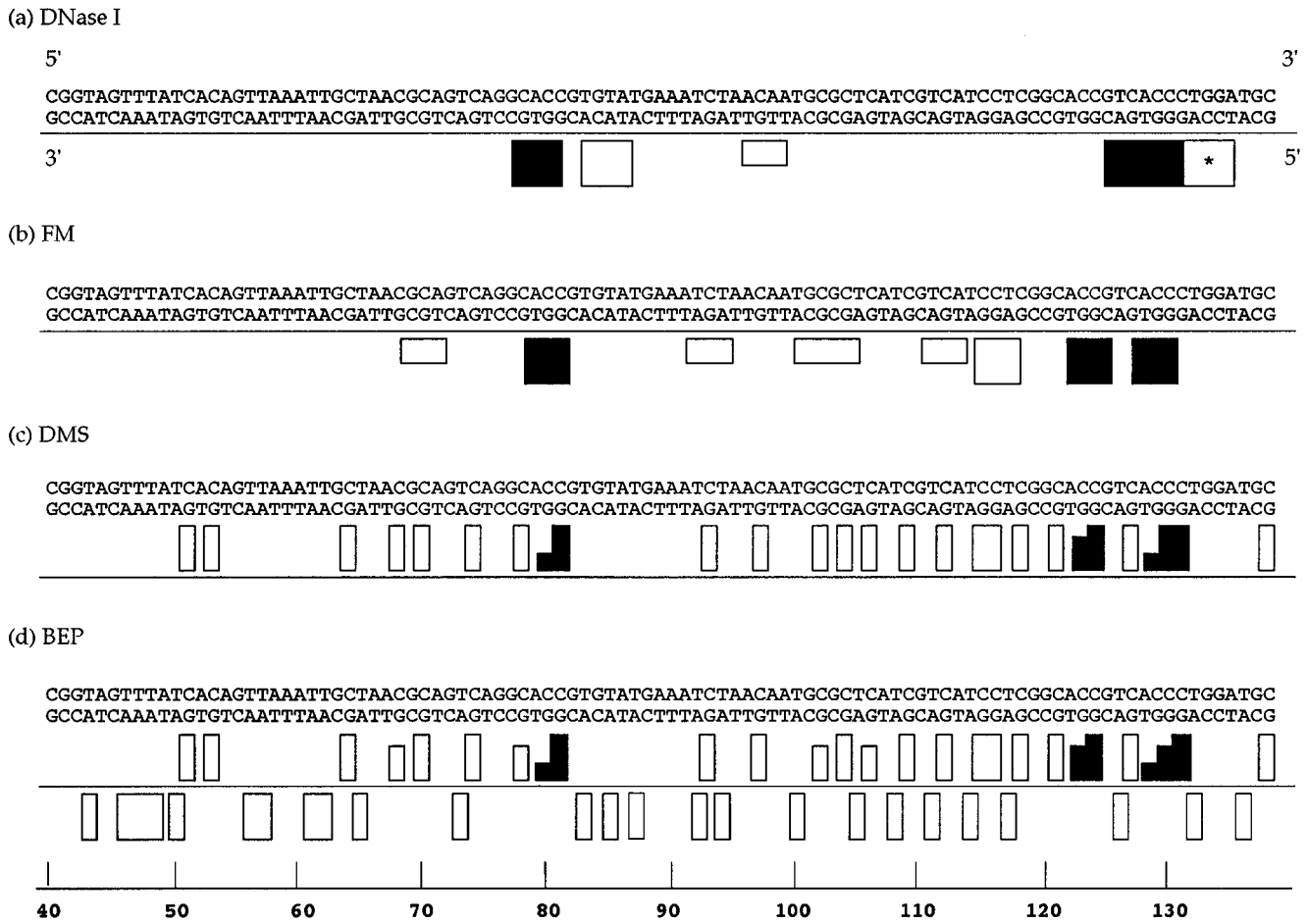

FIGURE 7: Schematic diagram showing the cleavage patterns for DNA in the presence of ACL with DNase I (a), FM (b), DMS (c), and BEP (d). Blocks above the line indicate the major sites of increased cleavage (height is proportional to intensity), and blocks below the line indicate major sites of protection. Asterisks indicates the site not shown in Figure 3. Dark boxes highlight common sequences observed with all four agents.

pattern is consistent with the saccharide chains lying in the minor groove and supports model building and literature data on related compounds, which suggest that DAU, ACL, and DIT cover approximately 3, 4, and 6−8 base pairs, respectively, when bound to DNA.

Assessment of the contribution of the sugar side chains to the sequence specificity observed is difficult, as the chromophores in the three drugs, while similar, contain subtle differences in the substitution patterns in rings A and C. Furthermore, while DAU contains a free amino group, both ACL and DIT exist as the *N*,*N*-dimethylated derivatives. Overall, there was little difference in sequence selectivity. We conclude that the dominant interaction that determines the preferred binding sequences of the anthracyclines is intercalation of the tetracyclic chromophore into DNA. Fox (1988) has compared the results of footprinting of nogalamycin, an anthracycline consisting of two monosaccharide side chains, and arugomycin, an anthracycline which has a tetrasaccharide and a pentasaccharide side chain attached to the aglycone. Similar binding sites were observed which suggests that the sugars do not play a significant role in the DNA sequence specificity.

The sequence selectivity of DAU has been studied previously by several groups using footprinting, spectroscopic (Remeta et al., 1993), and computational techniques (Lown, 1988). These studies have suggested multiple binding sites and a consensus sequence has yet to be established. DNase footprinting of DAU at 4 °C identified preferred triplet binding sites containing adjacent GC base pairs of variable sequence flanked by an AT base pair (Chaires et al., 1987). Using DNase I footprinting and an *Escherichia coli* RNA polymerase transcription-inhibition assay Skorobogaty et al. (1988) identified the 5′-CA sequence as the highest binding affinity site of DAU although modest affinity for five other dinucleotide sequences was noted. Our results are in broad agreement with those of previous reports, with a preferred alternating purine/pyrimidine (GT) binding site, often preceded by runs of guanines. While these sites were the most preferred binding sites, not all of these sequences were selected by the anthracyclines which, not surprisingly, indicates some sensitivity to sequence context and DNA conformation.

Preliminary DNase I footprinting of DIT has also been reported (Fox & Kunimoto, 1989). At 4 °C and low drug concentration a footprinting pattern was observed in which the best binding sites contained the dinucleotide step 5′-GT-3′ located in regions of alternating purines and pyrimidines. Our results agree overall with this report, with the minor sequence variations most probably due to the different DNA sequences used in each study. While the previous report (Fox & Kunimoto, 1989) observed no footprints at 37 °C, we obtained good patterns with DNase I at 25 and 37 °C with the chemical agents studied.

Chemical Footprints. The chemical footprinting assays were carried out at 37 °C and at a pH of 7.1 or 7.4. This is in contrast to standard enzymatic assays (Chaires et al., 1987; Fox & Kunimoto, 1989) which need to be carried out at lower temperatures and high ionic strength and often in the presence of divalent metal ions which are known to affect DNA conformation (Prakash et al., 1990).

As the effect of DAU on the DMS-piperidine cleavage of the *tyrT* fragment has been reported (Chaires et al., 1987), this study focused on the sequence-specific alkylation of ACL and DIT. The major groove alkylating agents FM and DMS gave contrasting results. Overall, alkylation by FM was inhibited, while DMS alkylation was enhanced for most guanines. Previously, it has been reported that minor groove binding can result in significant changes in the reactivity of the major groove towards chemical agents (Chaires et al., 1987). In the case of the anthracyclines, the dominant effect on the major groove that is expected on DNA-binding is steric hindrance as a result of ring D which projects into the major groove. In addition, NMR studies of ACL complexed to a hexanucleotide showed that trisaccharide binding forced the DNA to kink toward the major groove with opening of the minor groove (Yang & Wang, 1994). The apparent inhibition of FM alkylation by the anthracyclines suggests that either the drugs are blocking access to the nucleophilic sites in the major groove due to the protruding D ring or they are reducing the reactivity of these sites. Clear evidence for the former was obtained by comparison of the FM results with those of DMS. Enhanced alkylation observed with DMS shows that N7 nucleophilicity has in fact increased in the presence of the drugs. Thus the reduced alkylation reactions in the case of FM is consistent with the more bulky FM which is unable to access sites in the major groove due to steric effects, from both ring D of the antibiotics and DNA conformational changes that result on binding of the antibiotics.

The results obtained with BEP were as expected and showed complete protection of all adenines by the anthracyclines from alkylation. This result is consistent with binding of the sugar side chains in the minor groove on a time scale that prevents attack by the alkylating agent. Additional guanine alkylation was observed in most sequences with ACL and DAU and, to a much lesser extent, DIT. The increase in guanine alkylation may be explained by the increased reactivity of the N7-guanine as shown by DMS and/or the increased availability of reagent that is prevented from alkylating in the minor groove.

The minor differences between the guanine alkylation patterns observed for ACL and DIT at particular sites in each of the chemical footprinting assays could be attributed to differences in major groove perturbation. The changes observed with the DNase I footprints with increasing length of the sugar side chain were not duplicated in the chemical footprinting experiments, although the BEP footprints showed complete minor groove adenine protection. This is to be expected as FM and DMS are major groove alkylators and may only be subtly affected by the presence of the minor groove binding sugar side chains. The data indicate that the preferred sites of drug binding are at runs of guanines, especially at 5′-GGT-3′ sequences. Many of these sequences were found to be protected by both the enzymatic and chemical footprinting techniques.

Footprinting of Sugar Side Chains. Footprinting of the methyl glycosides S1−S4 showed no evidence of binding to DNA with any of the agents studied. It appears that intercalation of the chromophore at C1 is important to anchor the drug to the DNA and position the sugar side chain close to the minor groove. Calicheamicin, an enediyne antibiotic which also interacts with DNA by intercalation and contains a saccharide side chain, gave contrasting results. The sugar side chain of calicheamicin was cleaved from the aglycone and shown to bind specifically to DNA, with different specificity from the parent drug (Nicolaou et al., 1992). This system has recently led to the design and synthesis of an eight-base sequence-selective DNA-binding oligosaccharide (Nicolaou et al., 1995).

Conclusions. The combined results of our DNA footprinting using enzymatic and chemical agents show that the anthracyclines most commonly protect 5′-GT-3′ sequences, although other sequences were also observed to be protected. Our results with chemical probes show that anthracyclines bound to DNA modifies the major groove behavior, which suggests that the DNA template activities would be affected in cells. Although the sugar side chains do not appear to contribute to DNA selectivity, our results are consistent with the saccharide side chains in the anthracyclines acting as minor groove binding agents. The binding in the minor groove of the sugar side chains may contribute to DNA perturbation surrounding the intercalation site and/or the blocking of particular replication enzymes. Further comparative studies on the biological activity of DIT, ACL, and DAU and their role in topisomerase II and RNA inhibition may provide further insight into the biological role of the saccharide side chains in the anthracyclines.

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