Preferential Intercalation at AT Sequences in DNA by Lucanthone, Hycanthone, and Indazole Analogs. A Footprinting Study†

Christian Bailly‡ and Michael J. Waring*

Department of Pharmacology, University of Cambridge, Tennis Court Road, CB2 1QJ Cambridge, U.K.

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ABSTRACT: DNAase I footprinting has been used to probe the DNA sequence selectivity of the antitumor intercalating agents lucanthone (**1**), hycanthone (**2**), 6-chlorolucanthone (**7**), and four indazole analogs (IA-**3**–IA-**6**). The latter have a benzothiopyranoindazole chromophore substituted with a diethylaminoethyl side chain identical to that attached to the thioxanthenone chromophore of compounds **1**, **2**, and **7**. IA-**3** and IA-**5** are lucanthone analogs bearing a methyl group at position 4, whereas IA-**4** and IA-**6** are hycanthone analogs bearing a hydroxymethyl group. IA-**3** and IA-**4** have an additional chloro group at position 6. Studies employing the 160-bp *tyrT* DNA fragment as substrate to assay inhibition of DNAase I-mediated cleavage show that both lucanthone and hycanthone bind preferentially to AT sites. They discriminate against GC-rich sequences as well as short runs of a single base, which are often cut more readily in the presence of the drugs compared to the control. The indazole analogs exhibit more pronounced selectivity of binding to AT sequences and promote enhanced DNAase I cleavage both at GC-rich sequences and at homooligomeric runs of adenines or thymines. The results of further DNAase I cleavage inhibition assays, performed with three more restriction fragments having different base pair arrangements, are fully consistent with those obtained with the *tyrT* fragment. They reveal that the preferred binding sequences for lucanthone, hycanthone, and the indazole analogs are predominantly composed of alternating A and T residues. A detailed comparison between the seven compounds indicates that (i) the presence of a hydroxyl substituent on the methyl group at position 4 has no effect on the sequence selectivity of binding and (ii) the presence of a chlorine atom at position 6 does not affect the capacity of the drug to recognize AT sequences selectively in DNA. Lucanthone, hycanthone, and their indazole analogs go for a peculiar pattern of binding sites, different from those of the large majority of intercalators. Using osmium tetroxide as a probe for chemical reactivity, it appears that binding of the thioxanthenone compounds **1**, **2**, and **7** distorts the structure of DNA differently from binding of the benzothiopyranoindazole derivatives **3**-**6**, although all seven substances are known to unwind the DNA double helix to a similar extent. Results from enzymic and chemical probing experiments are compared and discussed with respect to the intercalative mode of binding of these compounds to DNA.

DNA intercalating agents have a long history in the treatment of human disease and of cancer in particular (Wakelin & Waring, 1990). Since the early proposal of the intercalation model for proflavin, systematic development has led to clinically useful agents such as amsacrine and mitoxantrone. As such, intercalating drugs continue to be the subject of intensive efforts aimed at developing more efficient antitumor compounds (Baguley, 1991). Even if a direct correlation between sequence specificity of binding to DNA and antitumor activity has not yet been established, it is important to develop drugs that can be targeted to defined sites in DNA. Sequence selectivity must play some role in the mechanism of action of cytotoxic DNA-binding substances. The majority of intercalators exhibit no significant sequence preference of binding. Others, such as actinomycin (Rill *et al.*, 1989; Chen, 1992), daunomycin (Chaires *et al.*, 1990), and amsacrine-4-carboxamide derivatives (Bailly *et al.*, 1992), display some more or less pronounced sequence selectivity and bind better to GC than to AT sequences. In contrast, there exist only a few potential intercalating agents which bind preferentially to AT-containing sequences (Wilson *et al.*, 1985).

Among the large diversity of intercalating structures, lucanthone (Miracil D) and its hydroxylated metabolite hycanthone represent a particular structural class. On the one hand, they bear a structural resemblance to chlorpromazine and methylene blue because they contain a divalent sulfur atom in the middle ring of their tricyclic ring system, but on the other hand, they differ from these drugs in that there is no nitrogen atom in their chromophore. Albeit less obvious, a structural analogy with anthracycline derivatives such as daunomycin and mitoxantrone (Lown *et al.*, 1987) can be seen, not only because of the keto group at position 9 but because all these compounds have in common an uncharged chromophore. The diethylaminoethyl side chain at position 1 of the thioxanthenone aromatic structure is, without doubt, positively charged under physiological conditions and plays a prominent role in allowing the drug to contact DNA and stabilizing the drug-DNA complex (Hirschberg *et al.*, 1968). Lucanthone and hycanthone exhibit significant antitumor properties, but their mutagenicity proscribes their use in cancer chemotherapy. They are, nevertheless, still used as a one-dose treatment for schistosomal infections. Different series of analogs have been developed principally in an effort to confer higher antitumor activity (Archer *et al.*, 1983, 1988; Croisy-Delcey & Bisagni, 1983).

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‡ Permanent address: Laboratoire d'Oncohématologie Moléculaire, INSERM Unité 124, Institut de Recherches sur le Cancer, Place de Verdun, 59045 Lille, France.

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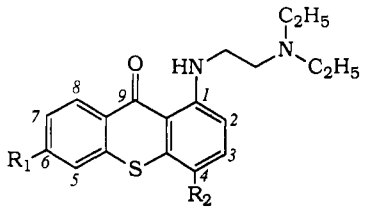
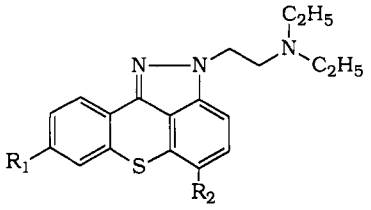
Table I: Chemical Structures and Unwinding Angles of Drugs Used in This Study

Thioxanthenones



compd	R_1	R_2	ϕ^a
lucanthone (**1**)	H	CH_3	15°
hycanthone (**2**)	H	CH_2OH	15°
6-chlorolucanthone (**7**)	Cl	CH3	

Benzothiopyranoindazoles



compd	R_1	R_2	ϕ^a
IA-**3**	Cl	CH_3	10°
IA-**4**	Cl	CH_2OH	15°
IA-**5**	H	CH_3	14°
IA-**6**	H	CH_2OH	15°

[a] Unwinding angles (ϕ, degrees) were determined by Waring (1970, 1973) and corrected to refer to an assumed unwinding angle of 26° for ethidium as is now known to be the correct average value applicable in studies with naturally occurring circular DNA molecules (Wakelin & Waring, 1990).

Lucanthone and hycanthone have for some time been known to intercalate into DNA (Hirschberg *et al.*, 1968; Miller *et al.*, 1980), as judged from the measured unwinding angle of 15° (Waring, 1970; see Table I), but the sequence selectivity of binding, if any, has never been examined in detail. On screening the overall sequence preferences of a vast number of intercalating agents by NMR, Feigon *et al.* (1984) concluded that a weak GC preference characterized hycanthone binding to DNA. However, using electric linear dichroism spectroscopy, a marked preference for binding to AT-rich DNA was recently evidenced (Bailly *et al.*, 1993b). To resolve this discrepancy, we report a study employing DNAase I footprinting to investigate sequence preferences in the binding of lucanthone (**1**) and hycanthone (**2**) to restriction fragments derived from plasmid DNA. Moreover, in an effort to identify the structural features of the drugs responsible for DNA recognition, we have extended our investigations to four indazole analogs, hereafter referred to as IA-**3** to IA-**6** (Table I), which differ from the thioxanthenones by their chromophore and also by the presence or absence of a chloro group at position 6. These drugs bind to DNA by intercalation, and the helix unwinding angle associated with their intercalative binding is close to 15° (Waring, 1973), i.e., very similar to the angle measured with lucanthone and hycanthone (Waring, 1970) (Table I). But the benzothiopyranoindazole derivatives exhibit antitumor properties often superior to those of lucanthone and hycanthone (Leopold *et al.*, 1985a; Werber *et al.*, 1985). Moreover, the same benzothiopyranoindazole chromophore occurs in the compound CI-958 and its derivatives (Fry & Besserer, 1988; Hopfinger & Kawakami, 1992; Kawakami & Hopfinger, 1992), which have a broad spectrum of antitumor activity and are considerably less cardiotoxic than the clinically used anthracyclines. It is also worth mentioning that the indazole analogs resemble the anthrapyrazole (Showalter *et al.*, 1987; Leopold *et al.*, 1985b; Hartley *et al.*, 1988) and the pyrazoloacridine (Sebolt *et al.*, 1987) series of anticancer agents. Consequently, elucidation of the sequence preferences for binding of these indazoles may furnish useful information concerning the likely DNA binding properties of several structurally related antitumor drugs.

MATERIALS AND METHODS

Chemicals and Biochemicals. Hycanthone, lucanthone, 6-chlorolucanthone, and the indazole compounds were kindly supplied by Dr. Edward F. Elslager of Parke, Davis & Company, Ann Arbor, Michigan; drug solutions were prepared in water. Electrophoretic reagents (Tris, acrylamide, bis(acrylamide), urea, *N,N,N′,N′*,-tetramethylethylenediamine (TEMED), and ammonium persulfate) were from BDH. Osmium tetroxide was purchased from Sigma Chemical Co. DNAase I (Sigma Chemical Co.) was stored as a 7200 units/mL solution in 20 mM NaCl, 2 mM $MgCl_2$, and 2 mM $MnCl_2$, pH 8.0, at -20 °C and was freshly diluted to the desired concentration immediately prior to use. Restriction enzymes *Ava*I, *Bst*EII, and *Eco*RI were from New England Biolabs, and reverse transcriptase was from Pharmacia. [α-^{32}P]dATP and [α-^{32}P]dCTP (6000 Ci/mmol) were purchased from New England Nuclear. The plasmid pBS was from Stratagene (La Jolla, CA). The plasmids pKMΔ-98 and pMLB1048 (Lamond & Travers, 1983) were prepared from *Escherichia coli* according to standard procedures employing sodium dodecyl sulfate–sodium hydroxide lysis followed by purification using Qiagen columns.

DNA Restriction Fragments. The 160-bp *tyrT* DNA was obtained by digestion of plasmid pKMΔ-98 with *Eco*RI and *Ava*I in order to generate sticky ends of unique sequence. It was labeled (i) at the *Eco*RI site on the lower strand (the Crick strand) with [α-^{32}P]dATP and (ii) at the *Ava*I site on the upper strand (the Watson strand) with [α-^{32}P]dCTP and reverse transcriptase, so as to give specific 3′-end labeling of the chosen strand (Low *et al.*, 1984). The 166-bp *pTyr2* DNA fragment was obtained by cleavage of pMLB1048 with *Eco*RI and *Bst*EII endonucleases and was radiolabeled at the *Eco*RI site with [α-^{32}P]dATP. The 117-mer and the 253-mer were obtained from the plasmid pBS digested with *Pvu*II/*Eco*RI and *Pvu*II/*Ava*I, respectively. These digestions also yielded fragments suitable for 3′-end labeling by the reverse transcriptase. The detailed procedures for isolation, purification, and labeling of these duplex DNA fragments have been described previously (Bailly *et al.*, 1990, 1992). Electrophoresis on a non-denaturing 6.5% (w/v) polyacrylamide gel served to remove excess radioactive nucleotide, with the desired 3′-end-labeled product being cut out of the gel and eluted overnight in 500 mM ammonium acetate and 10 mM magnesium acetate. The purifed DNA was then precipitated twice with 70% ethanol prior to being resuspended in 10 mM Tris and 10 mM NaCl buffer, pH 7.0.

DNAase I Footprinting, Gel Electrophoresis, and Data Processing. This was performed essentially according to the published protocols (Low *et al.*, 1984; Fox & Waring, 1984). DNAase I footprinting experiments were performed at 37 °C in 10 mM Tris-HCl buffer, pH 7.0, containing 10 mM NaCl, with aliquots removed from the digestion mixture 1 and 5 min after initiation of the cleavage reaction. DNAase I experiments included approximately 0.02 unit/mL enzyme, 20 mM NaCl, 2 mM $MgCl_2$, and 2 mM $MnCl_2$, pH 7.3. 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. The reactions were stopped by adding 3 μL of dye-containing formamide loading buffer. The chemical identities of the digestion products were assigned by coelectrophoresis of dimethyl sulfate/piperidine G markers (Maxam & Gilbert, 1980).

Osmium Tetroxide Modification. The procedure of McLean and Waring (1988) was closely followed. Briefly, the reaction was conducted at 0 °C by mixing 35 μL of the preequilibrated drug-DNA solution with 5 μL of a freshly prepared OsO_4/pyridine (4/1, v/v) solution to adjust the final OsO_4 concentration to 2.5 mM. After 15 min at room temperature, the reaction was stopped by extracting the reagent twice with diethyl ether and the modified DNA was recovered by precipitation with ethanol. After two successive washes with 70% ethanol and vacuum drying, the DNA pellet was resuspended in 40 μL of 1 M piperidine, heated at 90 °C for 15 min, lyophilized, and resuspended in 5 μL of gel loading buffer.

Gel Electrophoresis. Products of the enzymic and chemical DNA cleavage reactions were taken up in 80% formamide containing 10 mM EDTA and 0.1% bromophenol blue–xylene cyanol as tracking dyes. Samples were heated to 90 °C for 4 min and then chilled in an ice bath just before being loaded on a sequencing gel capable of resolving DNA fragments differing in length by one nucleotide. Cleavage products were separated on 0.3-mm-thick, 8% (w/v) polyacrylamide gels containing 8M urea. After a 2-h electrophoresis in TBE buffer (89 mM Tris base, 89 mM boric acid, and 2.5 mM Na_2EDTA, pH 8.3) at 60 W (1700-1800 V; BRL Model S2 sequencer), the 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 were adjusted according to the number of counts per lane loaded on each individual gel.

Densitometry and Data Processing. Autoradiographs were scanned using a computer-operated gel scanner at the Medical Research Council Laboratory of Molecular Biology, Cambridge. Gel profiles were plotted and displayed on a raster graphics screen. Quantitative analysis of the gel electrophoresis profiles was limited to regions where peaks were sufficiently well resolved to permit accurate analysis and was performed by integration of the area under each peak, using the computer program GELTRAK developed specially for the purpose (Smith & Thomas, 1990). The area under each peak was integrated by simple addition of the pixels under the curve. Data are presented in the form $\ln(f_a) - \ln(f_c)$ representing the differential cleavage at each bond relative to that in the control (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; positive values indicate enhanced cleavage, whereas negative values indicate blockage.

RESULTS

DNAase I Footprinting. The *tyrT* fragment containing the *E. coli* tyrosine tRNA promoter region has been used extensively as a model system for studying DNA structural variation in solution (Lamond & Travers, 1983; Drew & Travers, 1984), to investigate the structural parameters of DNA which determine DNAase I sequence selectivity (Worrall & Connolly, 1990; Doherty *et al.*, 1991), and in our laboratory as the substrate for a large number of footprinting studies of intercalating agents [for examples, see Low *et al.* (1984), Fox and Waring (1984), Fox *et al.* (1986), Chaires *et al.* (1990), Bailly and Waring (1993), and Bailly *et al.* (1990, 1992, 1993a)]. Therefore, we began by investigating the binding of the compounds shown in Table I to this DNA duplex in order to compare their sequence selectivity with that of other DNA-binding drugs. In separate experiments, the 160-bp *tyrT* fragment was prepared with one of its complementary strands labeled at the 3′-end with ^{32}P. DNAase I was allowed to cleave this fragment alone or after prior equilibration with hycanthone, lucanthone, 6-chlorolucanthone, or one of the four indazole analogs. Typical autoradiograms from such experiments are presented in Figure 1. The pattern of cleavage in the control lanes has been described previously (Drew & Travers, 1984). This irregular distribution of bands is known to arise from the sensitivity of DNAase I cutting to DNA flexibility and helical dimensions such as groove width (Hogan *et al.*, 1989; Weston *et al.*, 1992). As shown by the gels in Figure 1, in the presence of lucanthone the DNAase I cleavage patterns do not differ profoundly from those seen in the control. However, closer inspection of the autoradiograms reveals that relative band intensities in the drug-containing lanes and the drug-free lanes are frequently different, certain bands showing attenuated cleavage while others show relative enhancements. Yet there are no sites

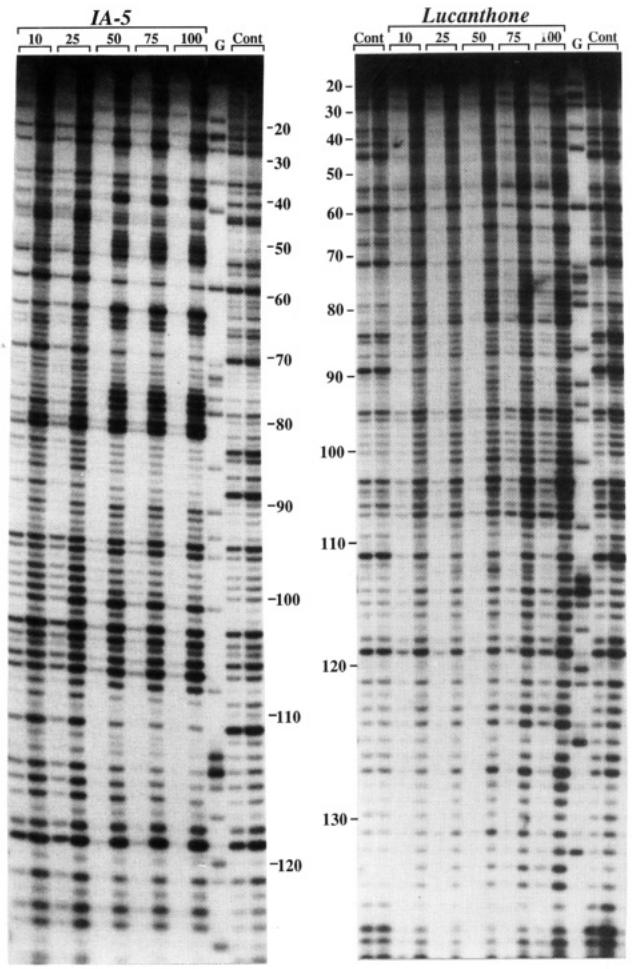


FIGURE 1: DNAase I footprinting of lucanthone and the indazole analog IA-**5** on the 160-bp *tyrT* DNA fragment. The Watson strand of the duplex DNA was 3′-end labeled with [α-^{32}P]dCTP in the presence of AMV reverse transcriptase. The concentration (μM) of the drug is shown at the top of the appropriate gel lanes. Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 min. The tracks labeled "Cont" contained no drug. The track labeled "G" represents a dimethyl sulfate–piperidine marker specific for guanine. Numbers at the side of the gels refer to the numbering scheme of Drew and Travers (1984) as used in Figure 2.

where the DNAase I cleavage is totally inhibited so as to generate a true footprint, i.e., a gap in the gel: all phosphodiester bonds in the *tyrT* fragment remain cleavable by the enzyme. Very similar patterns were observed with hycanthone and compound **7** (not shown). In order to afford a better comparison on a quantitative basis, intensities from selected gel lanes were measured by densitometry and converted into differential cleavage plots. From Figure 2a, it is readily apparent that the plot generated with lucanthone for both strands of the *tyrT* DNA superimposes quite well upon that obtained with 6-chlorolucanthone (**7**). This establishes that the presence of a chlorine atom at position 6 has little effect, if any, on the sequence selectivity of binding, just as it was found to have no clear-cut influence on biological activity (Hirschberg *et al.*, 1968). There is apparently excellent agreement between sites affected on the two strands of the DNA. For both drugs, the major sites of protection can be identified at the following sequences on the Watson strand (5′ → 3′, top strand) of the fragment: 5′-AGTT (42–45); 5′-TCAACGTAA (54-62); 5′-TTACAG (67-72); 5′-ATTTGATAT (82-90), and 5′-GATAA (108–112). The complementary sequences on the Crick strand (3′ → 5′, bottom strand) are, on the whole, comparably protected from enzymic attack. These regions of attenuated cleavage, presumptive drug binding sites, are essentially composed of adenine and thymine residues. Binding of the thioxanthenone derivatives **1**, **2**, and **7** to GC-rich sequences is clearly disfavored as judged from the enhanced cleavage observed around positions 77 and 100. Similarly, the differential cleavage plots in Figure 2a indicate that the drugs do not bind to homooligomeric runs of A and T residues. The DNAase I cutting is unmistakably enhanced at the five consecutive AT base pairs around position 50, at the run of four Ts (28–31) on the Crick strand, and at the run of four As (127–130) on the Watson strand. Therefore, at first sight lucanthone, hycanthone, and 6-chlorolucanthone bind best to regions composed of alternating A and T residues. However, the two main binding sites around positions 59 and 86 each contain at least one GC base pair, and we cannot exclude on the basis of this set of data alone the possibility that the drugs bind best to a GC base pair embedded in an AT-rich sequence.

The gel obtained in the presence of the indazole analog IA-**5** (Figure 1) shows that this substance has a much more marked effect on the rate of DNAase I cleavage than has its parent compound lucanthone. For example, in the presence of 50 μM IA-**5** the blockages at various positions of the *tyrT* DNA are clearly visible, while with lucanthone, even at higher concentrations, the differences between the control and the drug lanes are weaker. The enzyme cleavage pattern is slightly more radically altered with IA-**5** than with the other indazoles, but all four indazole analogs are superior to the thioxanthenone derivatives. With IA-**5**, binding sites around positions 70, 87, and 110 are seen as light regions in the drug lanes relative to the control lanes, with regions of enhanced endonucleolytic cleavage visible as darker zones near positions 53, 65, and 80 (Figure 1). This convincingly indicates that the indazole analog IA-**5** binds to DNA in a sequence-selective fashion.

Footprinting patterns observed with both strands of the *tyrT* fragment are illustrated for all four indazoles in the form of differential cleavage plots in Figure 2. To compare their effects, we have superimposed the plots for IA-**3** and IA-**5** in Figure 2b and for IA-**4** and IA-**6** in Figure 2c. Here again, the regions of protection and enhancement appear at roughly the same positions on either strand. The footprinting pattern of IA-**5** on *tyrT* DNA is almost the same as that determined for IA-**3** (Figure 2b). This lends credence to the previous suggestion that the chlorine atom at position 6, which distinguishes IA-**3** from IA-**5**, has no effect on the DNA-binding selectivity. The same observation holds true for compounds IA-**4** and IA-**6**, which produce closely similar effects on DNAase I cleavage of the *tyrT* fragment (Figure 2c). It can be seen by comparing the plots for lucanthone and its indazole analog IA-**5** that they both indicate footprints at the AT sites near positions 44, 59, 70, 86, and 111, together with enhanced cleavage at the run of five AT base pairs and at the two GC-rich sequences near positions 50, 77, and 100, respectively.

Thus DNAase I footprinting experiments with *tyrT* DNA lead to the conclusions that (i) the indazole analogs affect nuclease cutting more strongly than do their thioxanthenone counterparts, (ii) the presence of a chlorine atom at position 6 does not hinder the drug from gaining access to its preferred sites, and (iii) the hydroxyl group on the methyl substituent at position 4 has no effect on the sequence selectivity of binding to DNA. Most importantly, the experiments indicate that all seven drugs bind preferentially to AT sequences. This unexpected observation prompted us to investigate the selectivity of binding in more detail by using other restriction fragments.

Footprinting experiments with *pTyr2* DNA are reported in Figure 3. With hycanthone and lucanthone it appears that there are five sites of reduced DNAase I cutting, at the sequences 3′-TATACTA (34–40), 3′-AGAATT (70–75), 3′-CACGTGATAT (79–88), 3′-TTCATGA (91–97), and 3′-AAAT (115–118). Regions of enhanced cleavage are visible, especially in a large tract between nucleotide positions 41 and 69 which does not contain any ApT or TpA steps but is rich in GC base pairs and in the region around positions 105–110 where a GC sequence lies close to an A-tract (Figure 3a). Thus, the preference of lucanthone and hycanthone for binding to AT sequences is confirmed. It is interesting to note also that the differential cleavage plot for lucanthone is not much different from that for hycanthone (and that for 6-chlorolucanthone, not shown), again attesting that the hydroxyl group which distinguishes the two drugs has no significant effect on the DNA sequence recognition properties. To illustrate further the lack of influence of the chloro group, the differential cleavage plots for the indazole analogs IA-**3** and IA-**5** are superimposed in Figure 3b. Closely similar plots were obtained with compounds IA-**4** and IA-**6** (not shown). Overall, these analogs exhibit the same sequence selectivity as their parent compound lucanthone since the regions of attenuated cleavage are also localized at the five AT-rich sites near positions 36, 72, 85, 95, and 117. However, on closer inspection one can perceive interesting differences between lucanthone and the corresponding indazoles. As mentioned above, no binding site is observed in the large tract between nucleotide positions 41 and 69 in the presence of lucanthone and hycanthone, but in the presence of the indazole analogs two discrete footprints (weak binding sites) can be discerned which are both centered around a 3′-TpC step at positions 47 and 61. Therefore, the binding of these drugs to DNA is not restricted to AT sites, but (5′) CpT steps can also provide binding sites. Indeed, it turns out that the binding sites identified on the *tyrT* and *pTyr2* DNA fragments often contain at least one GC base pair flanked by an AT base pair. The enhanced levels of cleavage at the GC-rich sequence around position 43 and at the five consecutive T residues around position 65 are more pronounced with the indazole analogs **3**-**6** than with compounds **1**, **2**, and **7** (Figure 3), an observation which further attests to the more pronounced preference for alternating AT

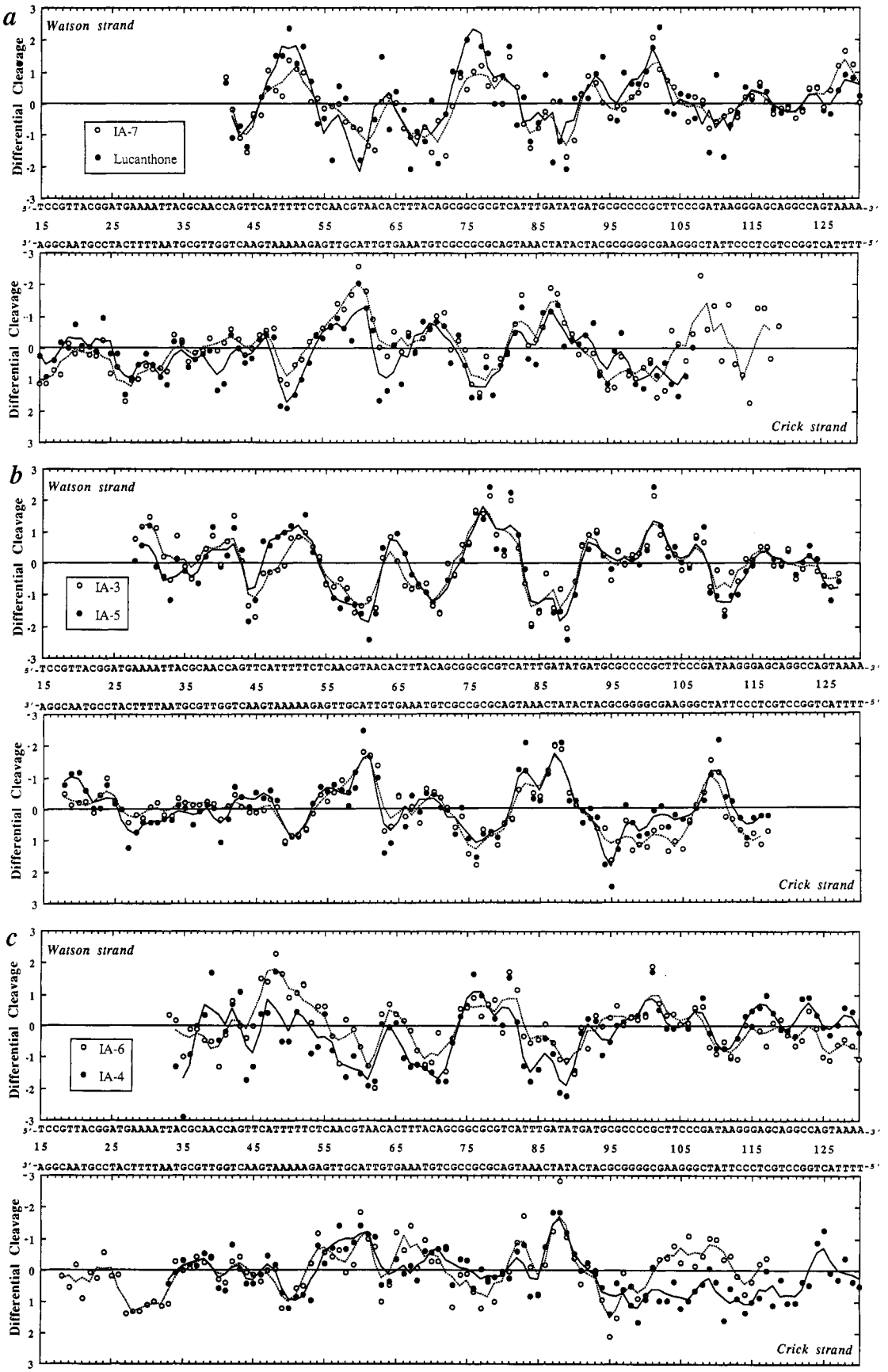


FIGURE 2: Differential cleavage plots comparing the susceptibility of the *tyrT* DNA fragment to DNAase I cutting in the presence of (a) lucanthone and 6-chlorolucanthone (100 μM each), (b) IA-3 and IA-5, and (c) IA-4 and IA-6 (50 μM each). The upper panel shows differential cleavage of the upper Watson strand; the lower, of the complementary Crick strand. The ordinate scales for the two strands are inverted, so that the deviation of the points *toward* the lettered sequence (negative values) corresponds to a ligand-protected site and the deviation *away* (positive values) represents enhanced cleavage. Vertical scales are 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, given closely similar extents of overall digestion. Each line drawn represents a 3-bond running average of individual data points, calculated by averaging the value of $\ln(f_a) - \ln(f_c)$ at any bond with those of its two nearest neighbors.

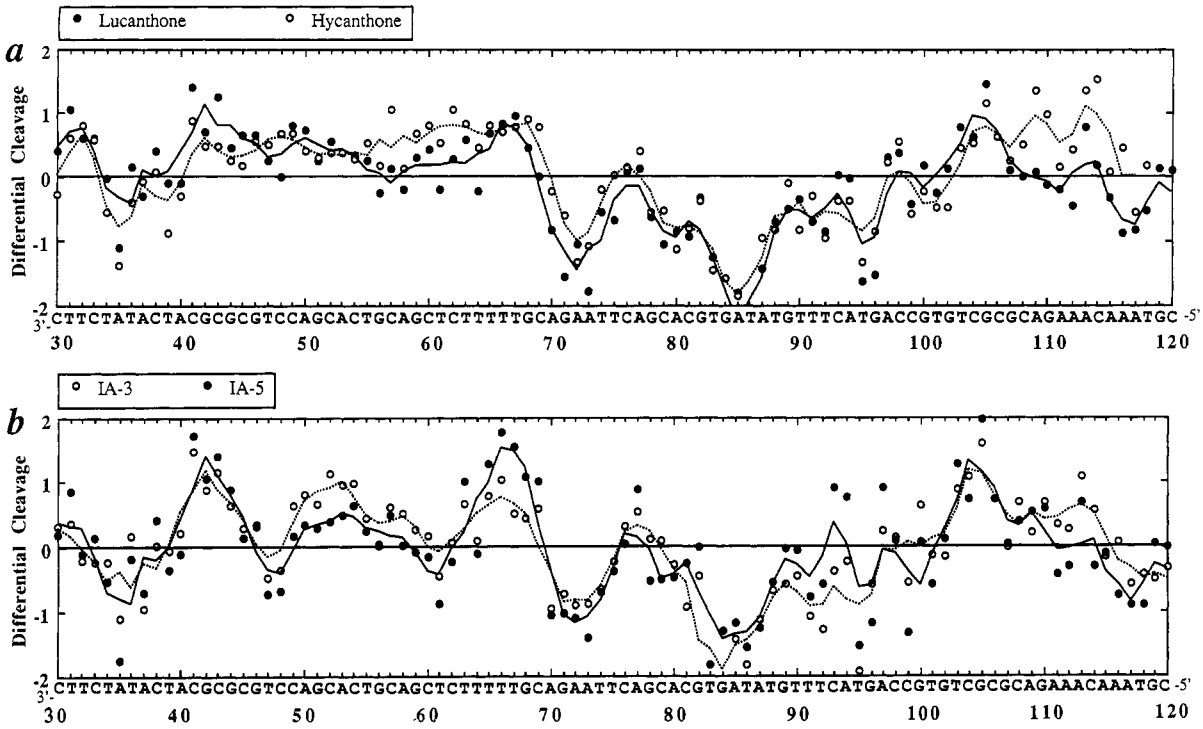


FIGURE 3: Differential cleavage plot showing differences in susceptibility of the Crick strand of the *pTyr2* DNA fragment to DNAase I in the presence of (a) lucanthone and hycanthone (100 μM) and (b) IA-3 and IA-5 (50 μM). Other details are as for Figure 2.

Table II: Sequences of the Two pBS DNA Fragments Showing Protection and Enhanced DNAase I Cleavage in the Presence of the Indazole Analogs of Lucanthone and Hycanthone, Inferred from Differential Cleavage Plots[a]

protection	enhancement
117-mer from pBS	
3′-AATGTTA (22–28)	3′-ACCGGCAGCA (33–42)
3′-AATGTT (44–49)	3′-GCAGC (50–54)
3′-CAAT (74–77)	3′-ACCCTTTTGGG (59–69)
3′-TGAATT (82–87)	3′-AGCGGAACG (88–96)
	3′-GTAGGGGG (101–108)
253-mer from pBS	
3′-CGTAC (27–31)	3′-TCG (34–36)
3′-AAATC (47–51)	3′-TCCC (54–57)
3′-CATT (75–78)	3′-AAAGCT (62–67)
3′-AGTAT (85–89)	3′-AACC (70–73)
3′-GACACACTT (98–106)	3′-AAG (95–97)
3′-ATT (111–113)	3′-AGGCGA (114–119)

[a] The position of the sequence is indicated in parentheses.

Table III: Comparative Protection of Different Dinucleotides against DNAase I Cleavage by IA-5[a]

dinucleotide (5′ → 3′)	no. of occurrences	no. protected	no. enhanced	comparative protection[b]
TpA–TpA	28	22	2	0.78
ApT–ApT	30	21	5	0.70
TpT–ApA	84	39	31	0.46
TpC–GpA	48	19	17	0.40
TpG–CpA	62	27	19	0.43
ApC–GpT	61	31	21	0.51
ApG–CpT	45	19	15	0.42
CpG–CpG	37	8	20	0.22
GpC–GpC	37	7	20	0.19
GpG–CpC	46	3	28	0.07

[a] A total of 483 bases were examined, comprising 202 in *tyrT* DNA, 91 in *pTyr2* DNA, 89 in the 117-mer from pBS, and 101 in the 253-mer from pBS. For each dinucleotide the number of times it appeared in a region protected from DNAase I cleavage was determined; likewise its appearance in a region of enhanced cutting was estimated. [b] Comparative protection shows how frequently the particular type of dinucleotide occurred in a protected region, as a fraction of the total number of occurrences.

sequences of the indazoles compared to lucanthone and hycanthone.

Further footprinting experiments were carried out with the 117-bp and 253-bp restriction fragments obtained by digesting plasmid pBS with *Pvu*II/*Eco*RI and *Pvu*II/*Ava*I, respectively. Again the preferred binding sites of the indazole analogs are composed principally of A and T residues. Sequences protected from DNAase I cleavage and sequences where the enzymatic cleavage is enhanced are listed in Table II. Nearly all the sequences protected contain a TpA or an ApT step, and conversely, none of the sequences of enhanced cutting contain such a step. The only binding site that does not contain an AT dinucleotide step is the sequence 3′-GACACACTT (98–106) of the 253-mer, thus showing that binding can also occur at 3′-CpA (5′-GpT) steps. Judging from the variety of DNA sequences protected from DNAase I cleavage, it seems that the binding specificities of both the thioxanthenone and the benzothiopyranoindazole derivatives can be rationalized most easily by preferential intercalation at any TpX site, with TpA steps preferred over TpT (ApA), TpC (GpA), and TpG (CpA) sites (Table III).

In summary, the DNAase I footprinting experiments performed on four DNA fragments with very different base pair arrangements concur to show that (i) hycanthone and lucanthone intercalate preferentially at AT sequences in DNA, (ii) substitution of the oxygen atom at position 9 by a nitrogen atom and cyclization to form a benzothiopyranoindazole aromatic ring leads to compounds endowed with a more pronounced AT selectivity of binding to DNA, (iii) the hydroxyl substituent on the methyl group at position 4 has little effect on the DNA recognition properties, and (iv) substitution of the chromophore by a bulky chloro group at position 6 does not inhibit the capacity of any drug to form stable complexes with AT sequences.

Osmium Tetroxide Modification. To address the question of structural changes in DNA emanating from the intercalative drug binding, we have taken advantage of osmium tetroxide

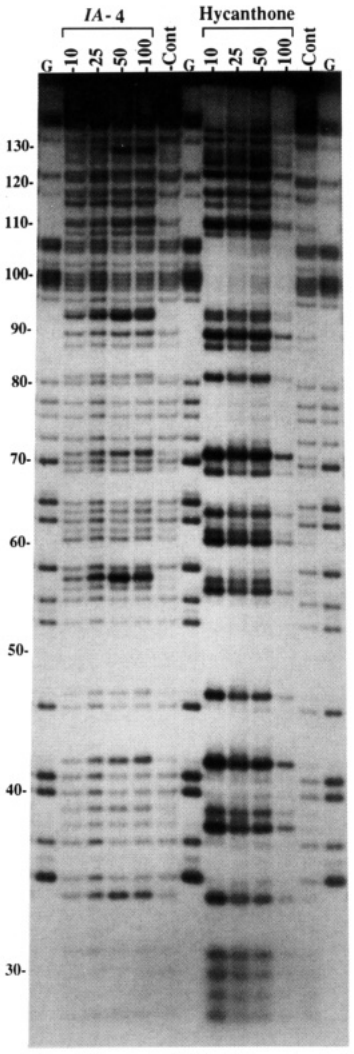


FIGURE 4: Reaction of the *tyrT* DNA fragment with the osmium tetroxide–pyridine complex in the presence of hycanthone and its indazole analog IA-**4**. Specific strand cleavages are shown at pyridine–OsO_4 modified bases on the Crick strand. The concentration (μM) of drugs is indicated at the top of the appropriate gel lanes. Control tracks labeled "Cont" contained no drug. Tracks labeled "G" are markers specific for guanines. Numbers at the side refer to the numbering scheme used in Figure 2.

(OsO_4) because this reagent is particularly well adapted for probing variation in base stacking. It has been widely used to detect unusual DNA structures (Johnston & Rich, 1985; McLean & Wells, 1988; Palecek *et al.*, 1989). In pharmacology, it has been applied with success to examine the structural changes in DNA induced by binding of mono- and bis-intercalating agents (Mclean & Waring, 1988; McLean *et al.*, 1989; Bailly & Waring, 1993). The osmium tetroxide–bis(pyridine) complex attacks the 5,6 double bond of pyrimidines in the major groove, most particularly at thymine residues. We have examined the reactivity of *tyrT* DNA toward OsO_4 in the presence of drugs and compared the data across the two strands. The experiments reveal that hycanthone, lucanthone, and 6-chlorolucanthone affect the structure of DNA differently from their indazole analogs (Figure 4). Cleavage in the control lanes is weak, restricted to the guanine residues, and reflects the sensitivity of guanine residues to the piperidine treatment. It is immediately apparent that numerous bases throughout the molecule are rendered hypersensitive to OsO_4 modification, but the reaction is not enhanced to the same extent and at the same sites in the presence of hycanthone and IA-**4**. With hycanthone the general level of reactivity toward OsO_4 is much more pronounced and affects many bases along the DNA fragment. By contrast, in the presence of IA-**4** the hyperreactivity is restricted to only a few bases. Evidently access to AT base pairs in the DNA is better facilitated by hycanthone than by IA-**4**. These observations directly imply that the indazole analogs (all four of which produce the same pattern of OsO_4 modification) perturb the structure of the double helix to a lesser extent than do the thioxanthenone compounds (no difference could be detected between hycanthone and lucanthone). To analyze the effects of OsO_4 in more detail, autoradiographs were scanned to produce a summary map of reaction sites for both strands of the DNA (Figure 5). In the presence of either drug OsO_4 reacts almost exclusively at thymine residues. Just one cytosine residue, at position 91, is rendered hyperreactive. This cytosine lies immediately adjacent to the main drug binding site and is more intensely modified by OsO_4 in the presence of IA-**4** than in the presence of hycanthone. No significant sequence preference can be deduced from the T-specific OsO_4 reaction because hycanthone causes thymine residues to react all along the *tyrT* fragment, either within or distal to a binding site. However, in the presence of IA-**4** the two main OsO_4 hyperreactive sites occur at T57 and C91, which are both associated with a strong binding site. Other T residues situated within or proximal to a site of attenuated DNAase I cleavage remain only feebly reactive toward the probe. Although the molecular basis of the hyperreactivity of certain thymine residues upon drug binding cannot be precisely defined, it is most likely connected with the helix extension and unwinding induced by intercalation (see below).

DISCUSSION

Lucanthone and hycanthone belong to a unique structural class of thioxanthenone intercalating agents. They also represent a unique class in terms of DNA sequence recognition. Indeed, so far as we are aware, this is the first demonstration using the footprinting technique that a group of polycyclic intercalating agents bind preferentially to AT sequences in DNA. Intercalating drugs such as methylene blue (OhUigin *et al.*, 1987), daunomycin (Chaires *et al.*, 1990), and mitoxantrone (Fox *et al.*, 1986), which bear a structural resemblance to lucanthone and hycanthone, all exhibit a more or less pronounced preference for binding to GC sequences, in common with the large majority of known intercalating agents. The peculiar binding behavior of the thioxanthenone drugs reported here prompts the questions of why lucanthone and analogs should bind best to AT sequences and what may be the critical determinants in their structures.

The only compounds hitherto believed to be capable of intercalation and to exhibit a significant preference for AT sites were the antiviral agent tilorone, certain naphthothiophene and phenanthrene derivatives, the chelate bis(1,10-phenanthroline)copper(I) (Veal & Rill, 1991), and most recently the diuretic drug amiloride. For tilorone, the AT preference initially proposed on the basis of theoretical calculations (Chen *et al.*, 1988) and spectroscopic measurements (Chandra & Woltersdorf, 1976) did not hold up when footprinting experiments were performed. In agreement with Sturm (1982), we found that tilorone binds best to alternating purine–pyrimidine sequences which can contain both AT and GC base pairs (Bailly & Waring, 1993). For amiloride, the cleavage inhibition assays clearly evidenced the AT preference (Bailly *et al.*, 1993a), but the chemical structure of this drug, a pyrazine derivative, is totally different from that of the thioxanthenones, not least in the lack of a polycyclic aromatic ring system. The AT-selective intercalating drugs designed by Wilson *et al.* (1985) consist of an uncharged chromophore

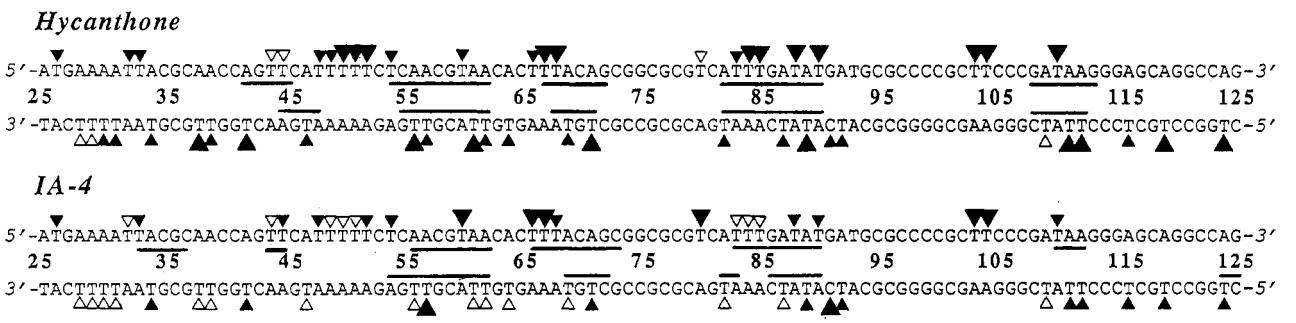


FIGURE 5: Representation of the sites of reactivity toward osmium tetroxide–pyridine on each strand of *tyrT* DNA in the presence of 50 μM hycanthone and the indazole analog IA-4. Symbols denote weak (Δ), moderate (▲), and intense (▲) reactive sites. Regions of diminished DNAase I cleavage in the presence of the drugs (presumptive binding sites inferred from differential cleavage plots) are underlined.

(naphthothiophene or phenanthrene) monosubstituted by a bis(hydroxyethyl)amino hydroxyethyl side chain comparable to the side chain appended to the drugs studied here. But it is very likely that the side chain serves only to stabilize the drug-DNA complex, probably by making electrostatic interaction with the phosphates of DNA. Dialkylaminoalkyl side chains similar to that of lucanthone are present in many other intercalating drugs, such as some acridine derivatives (Wakelin *et al.*, 1990; Bailly *et al.*, 1992) which exhibit a marked preference for GC sites. In the model of Wilson *et al.* (1985), the α-hydroxyl substituent of the side chain would play a prominent role in hydrogen bonding with the thymine C-2 carbonyl oxygen (mediated sometimes via a water molecule). In our case, the side chain has no hydroxyl group, and the hydroxyl on the methyl substituent at position 4 of the chromophore of hycanthone and compounds **IA-4** and **IA-6** clearly has no effect on the sequence selectivity. Therefore, we believe that the structural elements responsible for the AT preference of the thioxanthenone and benzothiopyranoindazole derivatives probably reside in the chromophore.

Recent molecular dynamics simulations of the binding to DNA of several benzothiopyranoindazole derivatives (Hopfinger & Kawakami, 1992; Kawakami & Hopfinger, 1992) structurally very close to the indazole analogs **3-6** have suggested that the most stable intercalation geometry corresponds to insertion of the ligand via the major groove. Although the calculations were carried out with the implicit assumption that GC base pair sequences compose the receptor sites, it is reasonable to suppose that the same geometrical constraints apply to intercalation complexes of the indazole analogs with AT base pairs. Therefore, by analogy we elaborated molecular models assuming that the diethylaminoethyl side chain of the indazoles also lies in the major groove of DNA. These models explain the observed differences of reactivity toward DNAase I and osmium tetroxide between lucanthone and the indazole analogs. In the lucanthone-DNA complex the three-ring thioxanthenone chromophore is probably well overlapped with the plane of the adjacent AT base pair to optimize stacking interactions. Consequently, the methyl group at position 4 would not penetrate far into the minor groove and hence the nuclease would have fairly easy access to the minor groove to cleave the DNA (explaining the weak footprints). Moreover, because of its large size, the sulfur atom would be expected to cause some distortion of the stacking interactions, allowing the osmium probe to gain access more easily to the 5,6 double bond of thymines in the major groove. The structural consequences of intercalation of lucanthone need not be confined to the targeted AT base pairs; witness the hyperreactivity toward OsO_4 spread out over the entire DNA fragment, which is unsurprising because it is known that structural changes can distribute over several nucleotide residues around the intercalation site (Herzyk *et al.*, 1992). It is also worth noting that AT base pairs unstack more easily than GC base pairs, especially at low ionic strength, as used here, which may contribute to the favored intercalation of thioxanthenones at AT sites.

By contrast, in the benzothiopyranoindazole–DNA complex the sulfur atom would not necessarily overlap with the base pairs when the tetracyclic chromophore is optimally stacked within the intercalation site. Accordingly, the R_2 substituent projecting deeply into the minor groove could more efficiently impede the access of DNAase I, explaining the more intense footprints observed with compounds **3-6** than with compounds **1** and **2**. At the same time, the stacking deformation specifically probed by osmium tetroxide would be minimized, thus explaining the lower reactivity toward OsO_4–pyridine. Finally, either mode of placement of the drug would satisfactorily account for the lack of effect of the 6-chloro group, for if R_2 is oriented toward the minor groove, then R_1 is directed toward the sugar–phosphate backbone; i.e., R_1 is in a position where it cannot significantly interfere with DNAase I.

While these drug–DNA intercalation models are as yet largely conjectural, they are fully compatible with the footprinting data and provide a sound basis for further molecular modeling which will aid the design of new ligands. More detailed study of the indazole analogs and similar molecules may provide a general route to the design of AT-selective intercalators. Finally, it remains to be demonstrated whether the higher propensity of the benzothiopyranoindazole derivatives for binding to AT sequences contributes to their superior antitumor activity compared to the thioxanthenones. It has been shown that benzothiopyranoindazoles produce a high level of protein-associated single- and double-strand breaks (Fry & Besserer, 1988), whereas lucanthone is less potent in this respect (Ross *et al.*, 1979). Therefore, it is conceivable that the indazole analogs are able to direct DNA cleavages to defined AT sequences of vital importance for cancer cells. If so, their sequence selectivity would be reflected in their antitumor properties.

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