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Drug-Induced Stereochemical Changes in Psoralen Photoaddition to DNA

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The photoaddition of HMT (4′-hydroxymethyl-4′,5′,8-trimethylpsoralen) to DNA gives, as the major monoadducts, *cis–syn* photodimers formed between the psoralen 4′–5′ furan double bond and the thymidine 5–6 double bond. Enzymatic hydrolysis liberates the HMT-dT nucleoside as a mixture of diastereomers, which arise from addition to the 3′ face (diastereomer **I**) or 5′ face (diastereomer **II**) of dT in DNA. For calf thymus DNA, the **I/II** ratio is 66/34. DNA-binding drugs, specifically cisplatin, distamycin A, and dipyrandium, change the **I/II** ratio. In each case relatively more of the 5′ adduct results.

INTRODUCTION

The study of conformational variations in DNA structure has used numerous reagents as kinetic probes for altered DNA conformation. An unusual DNA structure will in general be either much more or much less reactive than normal, B-form DNA. The enzyme DNase I, for example, preferentially hydrolyzes chromatin near actively transcribed genes (*1*). These nuclease-sensitive regions may have altered DNA conformations (*2*). Hydroxylamine and dimethylpyrocarbonate rapidly react with DNA bases at B–Z junctions but only slowly react with native DNA (*3*). Mung bean nuclease cuts regions of DNA in the alternating B conformation much more rapidly than in the normal B conformation (*4*). However, certain reagents appear capable of recognizing altered DNA conformations not only via altered rates of reaction but also through alterations in the stereochemistry of DNA modification. This change in conformational specificity has not been used previously as a probe for alterations in DNA structure. The conformational analogy with nuclease enzymes, such as DNase I, would be if the protein reacted not only at different rates with regions of the phosphodiester backbone, but also gave, depending upon the backbone conformation, either 3′- or 5′-OH groups.

Molecules which interact with DNA can be classified as reacting covalently or noncovalently. Among the covalent DNA modifiers are antitumor antibiotics such as mitomycin C, cisplatin, and anthramycin; alkylating agents including the nitrogen mustards and N-nitrosoamides; and photoactivated species in the psoralen

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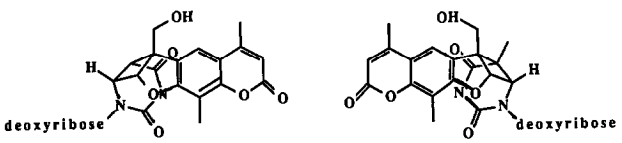
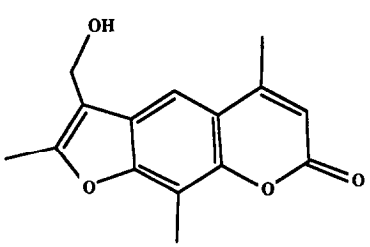


FIG. 1. The diastereomeric adducts formed from HMT photoaddition to dT.

family (*5*). An additional subdivision within the covalent DNA modifiers depends upon the creation of new asymmetric centers. A reagent may covalently bind to DNA to produce no new asymmetric centers, as when dimethyl sulfate alkylates purine's N-7 (*6*). Alternately, covalent modification can generate a new center in the small molecule without generating one on DNA. Alkylation of DNA by tomaymycin belongs to this class (*7*). A third possibility involves covalent modification which introduces a new stereocenter only on the DNA. For example, methylation of the phosphodiester backbone will produce an R or S phosphotriester (*8*). OsO_4 glycosolation at a pyrimidine 5–6 double bond will add two new asymmetric centers in DNA (*9*). Finally, a small molecule can react with DNA to give new asymmetric centers on both the DNA and the reagent. Such is the case of the photomonoadduct formed between psoralens and thymidine in double-stranded DNA which produces four new asymmetric centers (*10*).

In the example of psoralen addition to DNA, the groups of Hearst and Rapoport have shown that the predominant covalent monoadduct between 4′-hydroxymethyl-4′,5′,8-trimethylpsoralen (HMT) and DNA is the *cis–syn* photodimer formed between the psoralen 4′–5′ double bond and the thymidine 5–6 double bond (*11*). Two diastereomeric adducts are observed, arising from psoralen addition to the 3′ face (compound **I**) or the 5′ face (compound **II**) of dT (Fig. 1). Conditions were described for the isolation and quantification of these diastereomers. Furthermore, the major isomer formed upon addition of HMT to calf thymus DNA (at the level of 5% total modification of base pairs) was established to be the 3′ product, **I** (ratio **I/II** 63 : 37) (*12*). All of the presently available evidence is consistent with the 3′ to 5′ photoadduct ratio being determined at the stage of intercalation of HMT into DNA (*13*).



HMT

This study is concerned with changes in the diastereomer ratio of **I** to **II** brought about by drug binding to DNA. Its purpose is to establish how conditions which can alter DNA conformation affect the stereochemical outcome of psoralen pho-

tobinding. Other work has shown that binding of certain drugs to DNA can cause short- and long-range modulations of DNA conformation (*14*). While such changes could be expected to influence the accessibility of the 3′ vs 5′ face of dT residues, the extent of such influences was unknown prior to our studies.

EXPERIMENTAL METHODS

Materials

Calf thymus DNA was isolated from natural sources and sonicated to an average size of 200 base pairs. Hydrolytic enzymes and trioxsalen were from Sigma, as was distamycin A. Cisplatin and dipyrandium were prepared following the literature, and HMT was made from trioxsalen (*15*). [^{3}H]HMT was made by oxidation of HMT with pyridinium chlorochromate and reduction with $NaBT_4$ (New England Nuclear). Purification by TLC gave [^{3}H]HMT with a specific activity of 9.25 Ci/mol.

Isolation of Psoralen Photoadducts

Three milligrams of HMT in 1 ml MeOH (~4 mM) was stirred with a solution of 20 mg calf thymus DNA in 40 ml TE buffer (10 mM Tris–HCl, pH 7.6, 1 mM Na_2EDTA) for 30 min in the dark. Photolysis took place in an ice-packed illumination apparatus consisting of an inner chamber surrounded by a glass sleeve containing cobaltous nitrate (40% w/w) acting as a 340 to 380-nm transmission filter. The light intensity of the 1000-W mercury-xenon Oriel lamp was 100 mW/cm^2 at the solution surface. Following photolysis the DNA was ethanol-precipitated and redissolved in 15 ml hydrolysis buffer (0.1 mM $ZnCl_2$ in 50 mM NH_4OAc at pH 5.3). Liquid scintillation counting using a Packard Model 3330 TriCarb spectrometer determined the net binding of HMT to DNA. Nuclease digestion was as follows: 5 mg P1 nuclease for 48 h at 45°C, 25 units phosphodiesterase II for 24 h at 37°C, and 500 units alkaline phosphatase at pH 8 for 24 h at 37°C. The enzymes were denatured by heating at 95°C for 2 min and pelleted by centrifugation. The sample was concentrated and then purified by applying to a Waters C_{18} Sep-Pak and eluting with 1.5 ml water, 2 ml 50 mM NaH_2PO_4, and 1 ml methanol. The methanol fraction was analyzed with HPLC (Perkin–Elmer Series 400 injector, Waters Model 440 absorbance detector, Houston Instrument Series 4500 chart recorder) using a Waters Radial-Pak C_{18} cartridge in a RCM-100 radial compression module and a flow rate of 1 ml/min. Absorption was recorded at 254 nm. Elution was with a 35% methanol solution. The identity of the peaks with the appropriate retention times on the HPLC trace as **I** and **II** was confirmed by isolation of the peaks off the HPLC using multiple injections. ^{1}H NMR spectra were identical to published spectra. The ratio of **I** to **II** determined by NMR integration was the same as the ratio of the peak areas in the HPLC trace.

To determine the amount of psoralen incorporated during photolysis a known amount of [^{3}H]HMT was added to the photolysis mixture. After photolysis the DNA was ethanol-precipitated and redissolved in 10 ml of TE buffer; 200 μl of this solution was mixed with 5 ml of a 5% trichloroacetic acid solution, 10 mM in

sodium pyrophosphate and incubated for 5 min at 0°C. The acid-insoluble fraction was collected on a glass filter and washed five times with 5 ml acid solution and two times with 95% ethanol, followed by scintillation counting.

RESULTS

Changes in the I/II Ratio Produced by DNA-Binding Drugs: Cisplatin

cis-Dichlorodiammineplatinum (**II**) (cisplatin) is one of the best understood DNA-binding antitumor drugs. In aqueous solution it can hydrolyze to the *bis*-aquo dication and bind to N-7 of purines. Its favored binding mode is intrastrand chelation at two adjacent dG sites. This crosslink substantially perturbs DNA base stacking, producing at least local changes in the DNA's conformation (*16*).

Intercalators are known to affect the binding of cisplatin to DNA. For example, addition of ethidium bromide switches the preferred binding sites of cisplatin between various dG-rich sequences in a 165-base-pair restriction fragment (*17*). Conversely, addition of cisplatin to DNA changes the DNA-nicking selectivity of bleomycin, a drug that may have an intercalative component to its DNA binding (*18*). The ability of one drug to modify the binding of another, especially drugs with different binding geometries, is thought to work through the medium of drug-induced DNA conformational change (*19*). Thus, the influence of cisplatin on the **I/II** diastereomer ratio was investigated as a specific example of one drug influencing the binding stereochemistry of another.

Figure 2 summarizes the influence of cisplatin on the extent of photobinding of tritium-labeled HMT to sonicated calf thymus DNA. Small amounts of cisplatin (0.01 mol/1 base pair) actually enhance the efficacy of photobinding. This may arise via cisplatin inducing local melting at its binding site and these regions prompting increased flexibility in adjacent sequences. At a saturating level of cisplatin, the amount of HMT bound rapidly levels off, to a value below 0.02 mol of HMT per base pair. More importantly, the **I/II** HMT–dT monoadduct ratio at all levels of cisplatin changes significantly from the **I/II** ratio in the absence of the platinum complex. Because the **I/II** ratio was not sensitive to the degree of psoralen modification, as summarized in Table 1, only the **I/II** diastereomer ratio after

TABLE 1

Diastereomer Ratio as a Function of HMT Bound

Photolysis time (min)	mol bp/ mol HMT	I/II	mol bp[a]/ mol HMT	I/II[a]
1.5	146	77/23	200	—
6	70	72/28	100	—
15	60	76/24	55	—
25	25	66/34	34	63/37

[a] From Ref. (*11*).

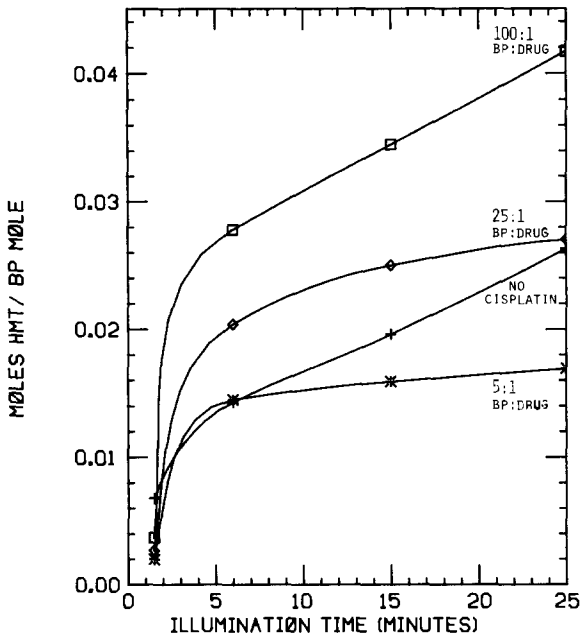


FIG. 2. Time course of covalent binding of [^{3}H]HMT to sonicated calf thymus DNA in the absence and presence of the drug cisplatin.

25 min of irradiation was determined. For the irradiation with the lowest (1 mol%) concentration of cisplatin, the **I/II** ratio was 51 : 49. The two higher concentrations of cisplatin gave **I/II** ratios of 46 : 54 in both cases. Thus, cisplatin caused the major HMT–dT monoadduct recovered to convert to the 5′-side adduct from the 3′-side adduct.

The amount of monoadduct to crosslinked dT–HMT–dT diadduct was not significantly perturbed by the addition of cisplatin, since the A_{320} units of DNA after photobinding was essentially the same with and without cisplatin (for the irradiation with 4 mol% cisplatin). Unlike the HMT–dT monoadducts, the crosslinked dT–HMT–dT diadducts do not absorb at 320 nm (*20*). The results from the photolysis with 1 and 20% cisplatin further suggest that cisplatin is not changing the **I/II** ratio by changing the crosslinking probability of one diastereomer relative to the other. With 20% cisplatin, essentially all of the HMT is bound after 5 min. Thus, during most of the photolysis only crosslinking is taking place (*21*). With photolysis in the presence of 1% cisplatin, new adducts are forming throughout the course of irradiation. Nevertheless, the diastereomer ratios for photolysis carried out under the two conditions are nearly the same. If the change in the **I/II** ratio from 63 : 37 with no cisplatin present, to 46 : 54 in the presence of the drug, were only being caused by cisplatin increasing the rate of destruction of **I** through crosslinking (or through some other destructive process), then the ratio of **I/II** in the 20% cisplatin photolysis would have been much lower than in the 1% cisplatin photolysis. Instead, the ratios were almost the same. The

HMT–dT monoadducts formed via addition to the 3′ or 5′ face of thymidine bases in a random sequence DNA-like calf thymus DNA thus appear to have approximately equal crosslinking rates.

Effects of Distamycin A and Dipyrandium on the I/II Ratio

Psoralens prefer to bind at AT-rich domains in DNA (22). We therefore briefly investigated the influence of two DNA-binding drugs with AT selectivity on the dT–HMT diastereomer ratio. Distamycin A is a pyrrololpeptide antibiotic which binds in the DNA minor groove through a combination of electrostatic forces and hydrogen bonds (*23*). As with virtually all minor groove DNA-binding drugs, the guanosine NH_2 interferes with hydrogen bond formation, causing distamycin A to avoid GC base pairs. The binding of distamycin A causes relatively minor structural perturbations in DNA. The bound drug neither winds nor unwinds closed circular DNA.

Binding of distamycin A to sonicated calf thymus DNA affected the **I/II** diastereomer ratio, but to a lesser extent than seen with cisplatin (Table 2). Like cisplatin, distamycin A decreased the proportion of the 3′-dT monoadduct. However, even at the highest drug-to-base pair ratio tested, the 3′-dT monoadduct remained the major diastereomer.

Dipyrandium is essentially a rigid dication which binds across the DNA minor groove (*24*). The steroid nucleus is too thick to fully intercalate between the DNA base pairs, but its binding kinks the DNA helix and disrupts its local structure. This kind of binding is sometimes called partial intercalation, since the DNA helix distorts to expose base pair faces to the hydrophobic steroid nucleus. Unlike true intercalation, however, stacking interactions between the aromatic bases of DNA and the aliphatic drug are absent, so that binding is driven by electrostatic interactions and entropically by release of bound water. The thick steroid acts as a

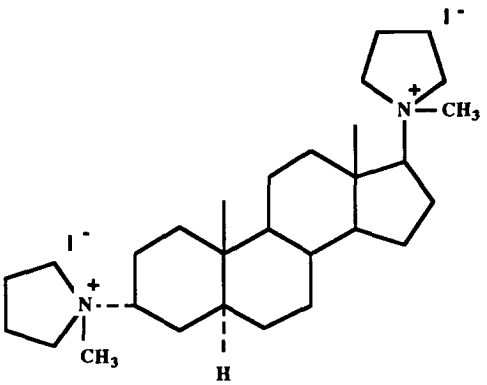
TABLE 2

Diastereomer Ratio **I/II** in the Presence of Different DNA-Binding Drugs

Drug	mol drug/ mol bp[a]	I/II
None	0	66/33
Cisplatin	0.01	53/47
Cisplatin	0.04	45/55
Cisplatin	0.20	47/53
Distamycin A	0.05	53/47
Distamycin A	0.15	52/48
Dipyrandium	0.20	38/62

[a] Ratio of drug to DNA base pairs at the start of photolysis. The photolysis time was in each case 25 min. The diastereomer ratio is the relative area under the HPLC peaks after digestion of the photolysis mixture.

wedge, generating a kink in the DNA at the dipyrandium binding site. From what is known about DNA sequence selectivity of tetraalkylammonium cations, dipyrandium should bind preferentially at AT-rich sequences (*25*).



DIPYRANDIUM

Dipyrandium caused the largest change in the **I/II** diastereomer ratio (Table 2). With 20 mol% dipyrandium, the dT–HMT monoadduct ratio inverted from 2 : 1 favoring the 3′ adduct to 2 : 1 favoring the 5′ adduct. It is interesting that all three drugs tested, a major groove covalent binder, a minor groove binder, and a kinker, lead to lowering of the proportion of **I** in the diastereomer mixture.

DISCUSSION

Psoralens exhibit sequence specificity in their photobinding to DNA. At the dinucleotide level, (TpA) base pair steps are the most reactive (*26*). The context of these steps strongly influences their reactivity, since isolated (TpA) steps embedded in GC-rich DNA domains are much less reactive than (TpA) steps in AT-rich domains. A study that measured the reactivity of longer AT sequences in a DNA restriction fragment toward psoralen addition found that of the sites studied, reactivity increased in the order of TAT < ATA < ATAT < ATATA. The ratio of 3′ to 5′ adducts at these sites was not reported (*27*).

In calf thymus DNA, 6.1% of the base pair steps are (TpA) steps (*28*). Monoadduct formation at this most favored site would give solely the 3′ dT–HMT adduct **I**. One reason that the 3′ to 5′ ratio is only 2 : 1 for calf thymus DNA under the irradiation conditions used (which result in only 4% of the base pairs reacting) is that the (TpA) step is a crosslinkable binding site. Crosslinks occur from photoaddition to thymidine across the psoralen pyrone double bond as well as across the furan double bond (*20*). Much of the monoadduct formed is lost to crosslinks. In general, the **I/II** diastereomer ratio will be a function of the DNA sequence, the binding constant of HMT at each dT-containing base pair step, the quantum yield for monoadduct formation at each site, and the rate at which each monoadduct

site can be converted to crosslink. Molecules which change DNA conformation can change any or all of the last three reaction variables. This work clearly establishes that DNA-binding drugs do change the **I/II** diastereomer ratio. The mechanism by which each drug produces the change will require more extensive studies with oligonucleotides of defined sequence. The results with cisplatin, however, are most consistent with the changes in the diastereomer ratio arising at the state of monoadduct formation.

The three different drugs investigated in this study have different binding modes, but each caused the **I/II** ratio to change in favor of the 5′ dT–HMT adduct, **II**. Current ideas about the influence of sequence on DNA structure may be able to rationalize this trend. Intercalators, such as HMT, prefer to bind to 5′ (pyrimidine–purine) 3′ base pair steps (*29*). This gives rise to 3′ monoadducts. Heightened binding to (pyrimidine–purine) steps arise, at least in part, from propeller twisting of the base pairs, which in turn leads to interstrand purine–purine steric clashes in the minor groove (*30*). Ultimately, to relieve this clash, the base pair step can decrease its helical twist (unwind) which distorts the (pyrimidine–purine) step toward a destacked geometry favorable to intercalation. Thus, (TpA), and to a lesser extent, (TpG) base pair steps adopt a conformation different from the archetypal B DNA conformation. In addition, the (pyrimidine–purine) steps may have several, closely spaced minimum energy structures. Calladine and Drew have suggested that (TpA) and (TpG) dinucleotide steps are conformationally bistable (*31*). Depending upon the environment, these bistable base pairs can show interstrand or intrastrand base stacking. Further evidence for flexibility at (TpA) steps comes from nuclease digestion studies on alternating AT sequences in closed circular plasmids. Conditions which increase helical twist change the (TpA) phosphodiester bond from a conformation that is resistant to nuclease-catalyzed hydrolysis to one that is normally reactive (*32*).

These results suggest that 5′ (pyrimidine–purine) 3′ base pair steps are the most easily distorted DNA sequences. In their unperturbed ground state conformation they are high-affinity binding sites for intercalators. In the case of HMT this binding leads, upon photolysis, to diastereomer **I**. However, conditions which cause regional perturbations in DNA structure, such as drug binding, may localize the changes at 5′ (pyrimidine–purine) 3′ steps, which may be the most deformable sites in DNA. Conditions which stabilize the double helix would obviously hinder the unwinding at 5′ (pyrimidine–purine) 3′ steps, and thus HMT intercalation. However, conditions which unwind the double helix could also prevent HMT from binding at the (TpA) site, and possibly (TpG) site, since this already underwound base pair step could adopt a conformation close to melted DNA. An intact double helix is required for intercalation. Either of these changes would lead to an increase in the relative binding affinity of other base pair steps toward HMT and thus a change in the **I/II** ratio in favor of **II**.

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