Covalent Binding of Antitumor Antibiotics in the Minor Groove of DNA. Mechanism of Action of CC-1065 and the Pyrrolo(1,4)benzodiazepines

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"We dance round a ring and suppose
But the secret sits in the middle and knows"
Robert Frost
"The Secret Sits"

DNA is believed to be the molecular target of a number of clinically important antitumor antibiotics, including adriamycin, actinomycin D, bleomycin, and mitomycin C. Based upon their diverse structural types, it is not surprising that these compounds have each been found to react in quite different ways with DNA and that the biochemical consequences of DNA damage are equally diverse, although all can ultimately produce cell death. For example, while the planar actinomine moiety of actinomycin D can intercalate between base pairs in DNA and is a very potent inhibitor of RNA polymerase, bleomycin can form a unique molecular complex with DNA which in the presence of a divalent cation and molecular oxyen can result in a complex degradation of DNA.[1] Other antitumor drugs such as mitomycin C and *cis*-Pt cross-link DNA[1], and compounds such as adriamycin and AMSA[2] can form a ternary complex with DNA, and DNA binding proteins such as topoisomerases, which may mediate the potent biological effects of these drugs.

Understanding the interactions between drugs such as those listed above and DNA is a necessary first step in elucidating the molecular basis for the potent antitumor activities of these compounds. The availability of a number of techniques such as oligodeoxynucleotide synthesis, high-field NMR, and DNA sequencing has provided great impetus to the field of drug–DNA interactions. For example, optimum binding sequences for DNA-reactive drugs can be determined by DNA-sequencing technology,[3] and these sequences can then be synthesized in large enough amounts by oligodeoxynucleotide synthesis technology so that their drug complexes can be studied by high-field NMR. More recently, site-directed DNA adducts have been made in much longer DNA sequences, including circular plasmids, so that these can be subsequently used to evaluate the biochemical and biological consequences of structurally defined lesions on DNA.[4] When these construction methods are used in combination with purified proteins such as those involved in DNA transcription, replication, or repair, then correlations between DNA modification and biochemical and biological consequences seem entirely possible. This is a great improvement upon previous experimental strategy, where the type and site of modification was unknown and whole cell preparations were used to evaluate biochemical and biological consequences.

For some years we have been studying the mechanism of action of the pyrrolo(1,4)benzodiazepines [P(1,4)Bs] (Figure 1) and CC-1065. These compounds bind covalently to the minor groove of DNA and exert extremely potent biological effects. Recent reviews on various aspects of the P(1,4)Bs[5] and CC-1065[6] have appeared, and the reader is referred to these for information concerning chemistry, including synthesis and biosynthesis, and pharmacological and toxicological data.[6,7] This Account will consider the mechanisms by which CC-1065 and the P(1,4)Bs associate and react covalently with DNA. In addition, stereochemical aspects of drug binding in the minor groove, modes of DNA sequence recognition and selectivity, and possible reasons for their extremely high drug potencies will be described.

Several classes of DNA-reactive antineoplastic agents interact in some manner with the minor groove of B-form DNA. Adriamycin and actinomycin are stabilized upon intercalation by secondary noncovalent associations within the minor groove.[7] Noncovalent DNA ligands such as distamycin and netropsin bind entirely

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(1) Gale, E. F.; Cundliffe, E.; Reynolds, P. E.; Richmond, M. H.; Waring, M. J. *The Molecular Basis of Antibiotic Action*, 2nd ed.; Wiley: London, 1981.

(2) For a recent review on DNA topoisomerases as targets for cancer therapy, see: Ross, W. E. *Biochem. Pharmacol.* **1985**, *34*, 4191.

(3) For reviews on DNA sequence specificity of DNA binding drugs, see: Dobrowiak, J. C. *Life Sci.* **1983**, *32*, 2915. Wilkins, R. J. *Mol. Cell. Biochem.* **1984**, *64*, 111. Dervan, P. B. *Science* **1986**, *232*, 464.

(4) (a) Essigmann, J. M.; Green, C. L.; Croy, R. G.; Fowler, K. W.; Buchi, G. H.; Wogen, G. N. *Cold Springs Harbor Symp. Quant. Biol.* **1982**, *47*, 327. (b) Johnson, D. L.; Reid, T. H.; Lee, M. S.; King, C. M.; Romano, L. J. *Biochemistry* **1986**, *25*, 449.

(5) For reviews on the P(1,4)Bs, see for biosynthesis: (a) Hurley, L. H. *Acc. Chem. Res.* **1980**, *13*, 263. For synthesis: (b) Thurston, D. E.; Hurley, L. H. *CIPS* **1983**, *8*, 957. More recent original papers: Kaneko, T.; Wong, H.; Doyle, T. W.; Rose, W. C.; Bradner, W. T. *J. Org. Chem.* **1985**, *28*, 388. (d) Thurston, D. E.; Langley, D. L. *J. Org. Chem.* **1986**, *51*, 705. (e) Suggs, J. W.; Wang, Y.-S.; Lee, K. S. *Tetrahedron Lett.* **1985**, *26*, 4871. For pharmacological and toxicological aspects: (f) Hurley, L. H. *J. Antibiot.* **1977**, *30*, 349. (g) Kohn, K. W. In *Antibiotics III. Mechanism of Action of Antimicrobial and Antitumor Agents*; Cocoran, J. W.; Hahn, F. E. Eds.; Springer: New York, 1975; pp 3–11.

(6) For a recent review on all aspects including chemistry, biosynthesis, synthesis, pharmacology, and toxicology of CC-1065, see: Reynolds, V. L.; McGovern, P. J.; Hurley, L. H. *J. Antibiot.* **1986**, *39*, 319.

(7) (a) Quigley, G. L.; Wang, A. M. J.; Ughetto, G.; van der Marel; G., von Boom, J. H.; Rich, A. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 7204. (b) Takusagawa, F.; Dabrow, M.; Neidle, S.; Berman, H. M. *Nature* **1982**, *296*, 466.

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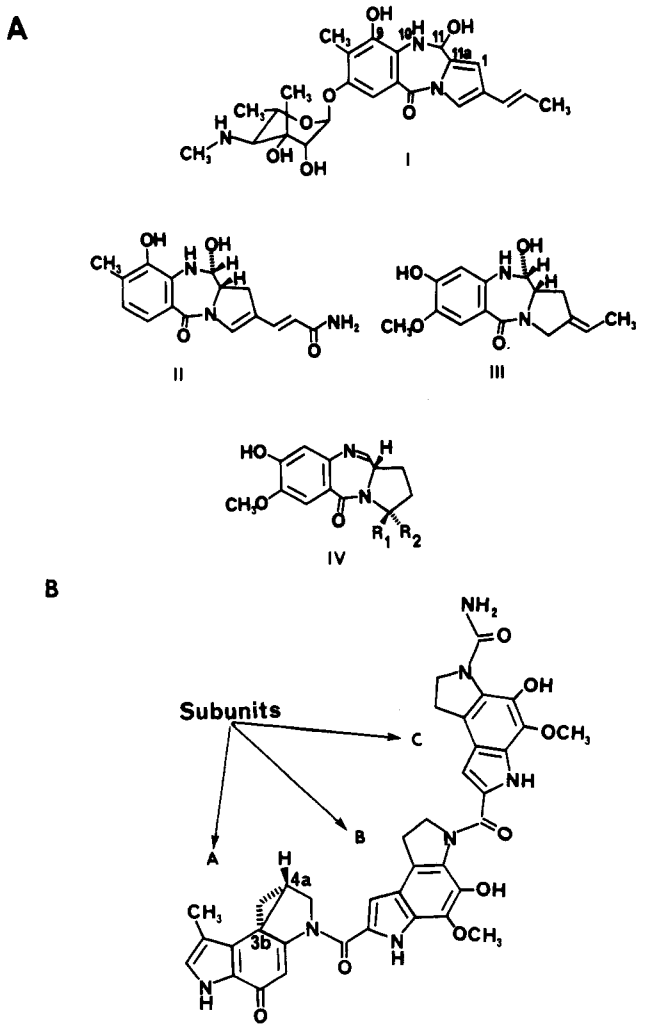


Figure 1. Structures of the P(1,4)Bs; sibiromycin (I), anthramycin (II), tomaymycin (III), and the neothramycins A (IVa) and B(IVb) (A) and CC-1065 (B).

within the minor groove.[8] A third class of antineoplastics (that with which this Account is concerned) bind covalently within the minor groove of B-form DNA. Members of this latter group of compounds include CC-1065, the saframycins,[9] naphthridinomycin,[10] and the P(1,4)Bs. The latter three groups contain the DNA-reactive carbinolamine or its chemical equivalent. The covalent minor groove binders have been observed to be 10^2 to 10^3 times more potent as antitumor agents than those of the intercalating class, while the latter agents possess significantly greater potency than the noncovalent minor groove binders.[11]

Elucidation of the Covalent Drug-DNA Adduct Structures

A vigorous demonstration of the points of covalent attachment between both the P(1,4)Bs and CC-1065 and DNA was an essential first step in investigating the structural and biological consequences of DNA modification. For both groups of drugs, a series of steps were undertaken to first establish that DNA was the primary target and then gain circumstantial and finally unambiguous structural information on the DNA adduct structures.

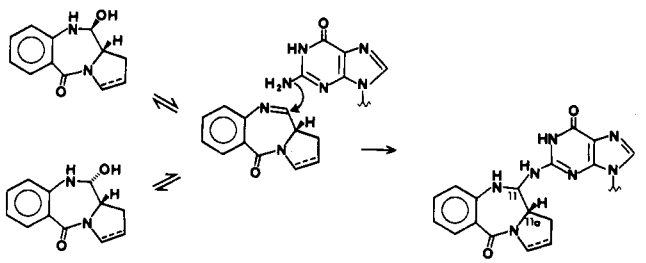


Figure 2. Proposed mechanism for alkylation of DNA by the P(1,4)Bs to form the P(1,4)B–(N2-guanine)DNA adduct.

Structures of the P(1,4)B-DNA Adducts

Covalent binding of the P(1,4)Bs to duplex DNA was first suggested by the stability of drug–DNA complexes to ethanol precipitation or dialysis.[12] In contrast, similar stability experiments following drug incubations with RNA, protein, or denatured DNA did not show evidence of covalent binding.[12] Subsequent experiments revealed that the P(1,4)Bs form DNA adducts which significantly increase the DNA helix melting temperature, do not cause unwinding of supercoiled DNA, produce shifts in circular dichroism spectra, and induce bathochromic shifts in the absorbance maxima of the drug chromophores.[5f,13]

Indirect evidence for the structures of the P(1,4)B–DNA adducts was obtained by reaction of these agents with synthetic DNA polymers.[13,14] In particular, the inability of anthramycin to form a covalent adduct with poly(dI)-poly(dC) contrasted with its avid binding to poly(dG)-poly(dC).[13] Denaturing alkaline cesium sulfate gradient centrifugation of the latter species demonstrated that tritiated drug chromophore was only associated with the poly(dG) strand.[14] Taken together, these results suggested that anthramycin binds covalently in the minor groove of DNA through N2 of guanine.

Further evidence for covalent *minor* groove DNA binding was obtained from experiments in which it was demonstrated that anthramycin binds covalently to coliphage T7 DNA, which has extensive glucosylation in the major groove.[15] The possibility of covalent binding through N-7 or C-8 of guanine was further eliminated by experiments in which incubation of (8-^{3}H)-guanine-labeled DNA with anthramycin did not show loss of tritium.[14] Finally, melting of the DNA strands in P(1,4)B–DNA adducts by heat (100 °C for 5 min) released chemically unchanged drug from the DNA.[12a]

Collectively, these results suggest that the P(1,4)Bs bind covalently to guanine, and furthermore specifically through the exocyclic 2-amino group, which resides within the minor groove of B form DNA.[14,15] The stability of the *N*-glycosidic linkage of the covalent mod-

(8) Kopka, M.; Yoon, C.; Goodsell, D.; Pjura, P.; Dickerson, R. E. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 1376.
(9) Lown, J. W.; Joshua, A. V.; Lee, J. S. *Biochemistry* **1982**, *21*, 419.
(10) Zmijewski, M. J., Jr.; Miller-Hatch, K.; Mikolajczak, M. *Chem. Biol. Interact.* **1985**, *52*, 361.
(11) In the stem cell assay system CC-1065 is 600 to 1000x more potent than adriamycin. P(1,4)Bs are about 10x (sibiromycin) to 100x (tomaymycin) less potent than CC-1065. Bhuyan, B. K.; Newell, K. A.; Crampton, S. L.; Von Hoff, D. D. *Cancer Res.* **1982**, *42*, 3532.
(12) (a) Hurley, L. H.; Gairola, C.; Zmijewski, M. *Biochim. Biophys. Acta* **1977**, *475*, 521. (b) Kohn, K. W.; Spears, C. L. *J. Mol. Biol.* **1970**, *51*, 551.
(13) Kohn, K. W.; Glaubiger, D.; Spears, C. L. *Biochim. Biophys. Acta* **1974**, *361*, 288.
(14) Petrusek, R. L.; Anderson, G. L.; Garner, T. F.; Fannin, Q. L.; Kaplan, D. J.; Zimmer, S. G.; Hurley, L. H. *Biochemistry* **1981**, *20*, 1111.
(15) Hurley, L. H.; Petrusek, R. L. *Nature* **1979**, *282*, 529.

ified guanine to acid and alkaline hydrolysis is consistent with alkylation at N2 of guanine, which does not disturb the purine aromaticity. DNA-binding experiments using structural analogues of the P(1,4)Bs which lack the carbinolamine or its chemical equivalent (N10,C11 imine or 11-alkyl ethers) failed to yield covalent DNA adducts,[16] providing the first evidence that this functionality is the DNA-reactive moiety. Several reaction mechanisms which would lead to formation of an aminal linkage between C11 of the P(1,4)B and N2 of guanine were proposed,[5f] including nucleophilic attack on the carbinolamine or the imine. As described later, the imine intermediate is favored as the ultimate DNA-reactive species[17] (Figure 2). Last, the facile hydrolysis of P(1,4)B–DNA adducts following DNA melting under neutral or acidic conditions, but not under basic conditions, is consistent with an aminal linkage (N–C–N) from the drug carbinolamine carbon (C-11) to N2 of guanine.

^{1}H and ^{13}C NMR experiments provided direct evidence for the covalent linkage sites between the P-(1,4)Bs and DNA. Anthramycin specifically enriched with carbon-13 at C-11 was bound to calf thymus DNA and analyzed by ^{13}C NMR.[18] Upon covalent binding to DNA, a 16 ppm upfield shift was observed for C-11 of anthramycin relative to anthramycin-11-methyl ether, which is consistent with the carbinolamine carbon of anthramycin being the point of covalent attachment to DNA. Additionally, the carbon-13 chemical shift observed for C-11 upon binding to DNA agreed with model compounds for the formation of an aminal linkage at C-11 from the drug carbinolamine to N2 of guanine. Confirmation that N2 of guanine was the point at which DNA was covalently bound to the drug was obtained from ^{1}H NMR studies on the anthramycin-5′(ATGCAT)$_2$ oligomer adduct.[18]

Structure of the CC-1065–DNA Adduct

Similar studies to those described for the P(1,4)Bs employing various cellular macromolecules such as RNA, protein, and synthetic or natural DNAs were used to demonstrate that covalent binding occurred exclusively to DNA and subsequently to pinpoint the location of CC-1065 binding to DNA.[19] CC-1065 was shown to bind most avidly to synthetic poly(dA)-poly(dT), less avidly to poly(dA-dT)-poly(dA-dT), and did not bind covalently to poly(dG)-poly(dC) or poly(dG-dC)-poly-(dG-dC).[20] CC-1065 was able to prevent binding of netropsin to calf thymus DNA and could also displace prebound netropsin from DNA.[20] CC-1065, like anthramycin, binds strongly to phage T7 DNA. These results suggested that CC-1065 binds covalently in the minor groove of B-form DNA to AT-rich regions.[20] When CC-1065 calf thymus DNA adducts were heated and butanol extracted, the organic phase was found to

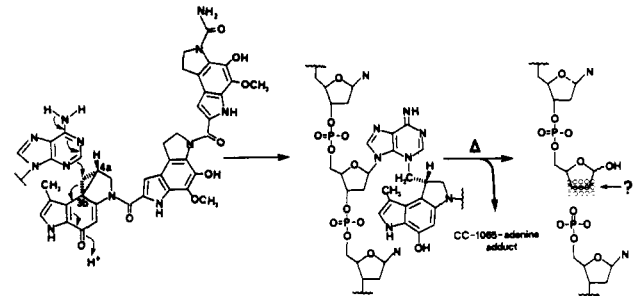


Figure 3. Reaction of CC-1065 to form the CC-1065–(N3-adenine)DNA adduct and the products of thermal cleavage of the CC-1065–DNA adduct.

contain a chromophore similar to CC-1065 which could be isolated by RP-HPLC, but which did not comigrate with CC-1065.[21] In similar experiments, but using DNA which was prelabeled with tritiated adenine, guanine, cytosine, or thymine, only butanol extraction of the CC-1065-[(^{3}H)-adenine]DNA produced fractions containing excess tritum. The excess radioactivity was found to comigrate with the modified CC-1065 chromopore in the RP-HPLC system.[21] Thus adenine seemed a likely candidate for the base modified by CC-1065. The sole adenine nucleophile in the minor groove is N-3. Since alkylation of N-3 of adenine alters the aromaticity of the base, this would explain the base loss observed upon thermal treatment of CC-1065–DNA adducts. These observations allowed tentative identification of N-3 of adenine as the CC-1065 binding site on DNA. The identity of the CC-1065–DNA adduct linkage was determined by ^{1}H and ^{13}C NMR studies on the CC-1065–adenine adduct, which was isolated as described before.[21] The isolated species proved to be adenine alkylated at the N-3 position by the methylene carbon (C-4) of the cyclopropyl ring of the "A-subunit" of CC-1065 (Figure 3).

Stereochemistry at the Linkage Site and Orientation of Drug Molecules in the Minor Groove of DNA

Two possible P(1,4)B–DNA adducts, which differ in the stereochemistry at the anthramycin C11-N2 guanine linkage site (i.e., 11*R* or 11*S*) and also have opposite drug orientations in the minor groove relative to the covalently modified guanine, can theoretically result from alkylation of DNA by the P(1,4)Bs. The adduct orientation in which the aromatic ring of the drug lies to the 3′ side of the modified guanine is associated with a linkage stereochemistry of "*S*" at C-11, while the opposite drug orientation produces the reverse configuration at C-11. Molecular modeling studies carried out by Remers et al.[22] using the united atom force-field parameters of AMBER (Assisted Model Building with Energy Refinement) on anthramycin and tomaymycin–d(ATGCAT)$_2$ adducts suggested that these drugs might differ in their abilities to orient themselves in one or both possible adduct orientations in the minor groove. For tomaymycin, there is only a relatively small difference (2.3 kcal/mole) between the energy levels of the two possible orientations, whereas for anthramycin over a four-fold higher energy level difference (10.8

(16) Horwitz, S. B.; Chang, S. C.; Grollman, A. P.; Borbovec, A. B. *Science* **1981**, *147*, 159.

(17) We have previously suggested (5f) that at least in the case of sibiromycin the N10,C11 imine cannot be the ultimate DNA reactive species. However, recent ^{1}H NMR experiments on natural sibiromycin lead us to seriously question this postulate.

(18) Graves, D. E.; Pattaroni, C.; Balakrishnan, C.; Ostrander, J. M.; Hurley, L. H.; Krugh, T. R. *J. Biol. Chem.* **1984**, *259*, 8202.

(19) Li, L. H.; Swenson, D. H.; Schpok, S. L. F.; Kuentzel, S. L.; Dayton, B. D.; Krueger, W. C. *Cancer Res.* **1982**, *42*, 999.

(20) Swenson, D. H.; Li, L. H.; Hurley, L. H.; Rokem, J. S.; Petzold, G. L.; Dayton, B. D.; Wallace, T. L.; Lin, A. H.; Krueger, W. C. *Cancer Res.* **1982**, *42*, 2821.

(21) Hurley, L. H.,; Reynolds, V. L.; Swenson, D. H.; Petzold, G. L.; Scahill, T. A. *Science* **1984**, *226*, 843.

(22) Remers, W. A.; Mabilia, M.; Hopfinger, A. C. *J. Med. Chem.*, in revision.

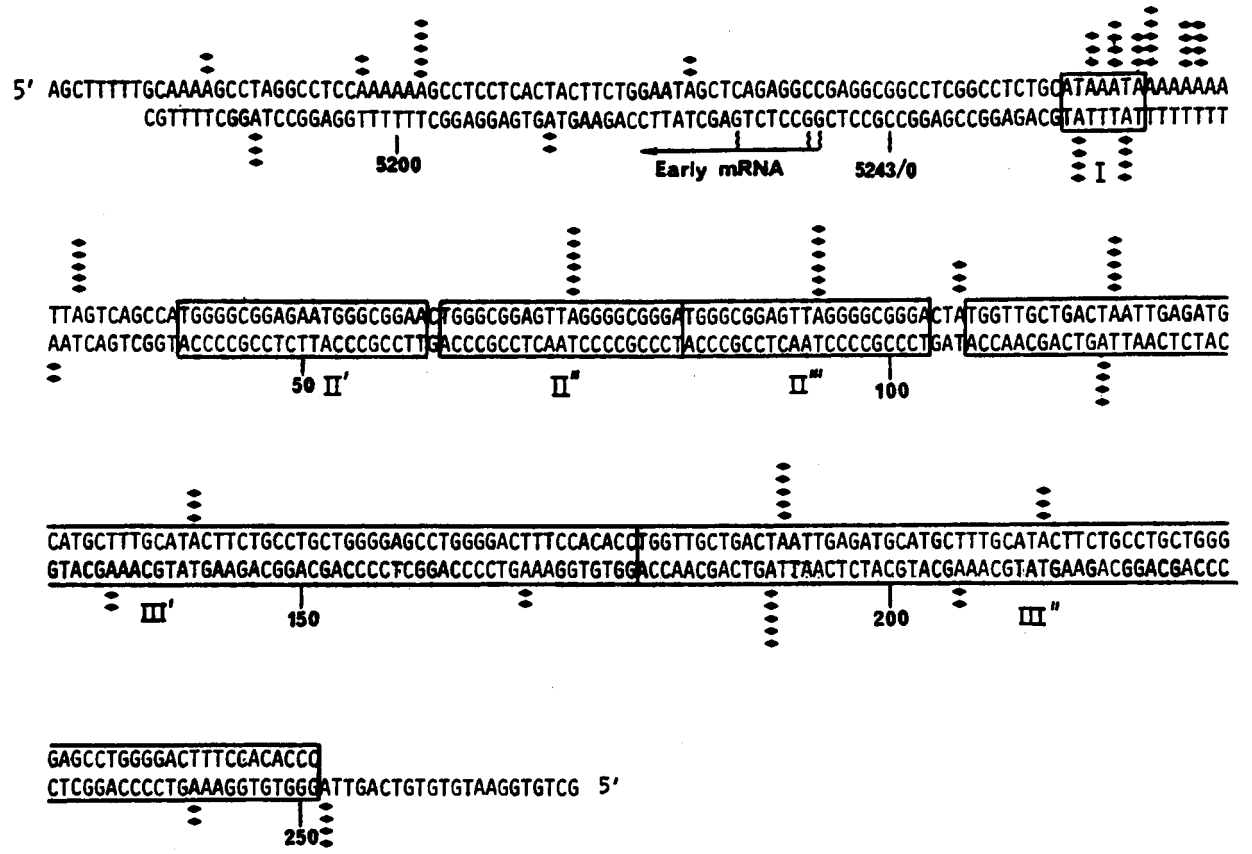


Figure 4. DNA sequence specificity of CC-1065 within the early promoter region of SV40. Restriction enzyme fragments were single end-labeled with ^{32}P and incubated with CC-1065 and then heated under standard conditions to produce DNA strand breakage. The ♦s show the CC-1065–adenine covalent binding sites and the number of indicate the relative sensitivity to strand breakage by CC-1065, e.g., ♦ ♦ and ♦ ♦ ♦ are $1:10^2$ and $1:10^3$ dilutions, respectively, of a stock solution of CC-1065 which are the maximum dilutions at which strand breakage is still observed. I-TATA box; II′, II″ and III‴-21 base repeat regions; III′ and III″-72 base repeat regions.[26]

kcal/mole) is calculated between opposite orientations. These predictions are supported by the results of binding studies of P(1,4)Bs with synthetic oligomers. Fluorescence studies suggest strongly that tomaymycin binds in two orientations to calf thymus DNA.[23] In contrast, anthramycin was shown to bind in only one orientation (11*S*) relative to the covalently modified strand of the oligomer 5′(ATGCAT)$_2$.[24] Significantly, the molecular mechanics calculations by AMBER are predictive of the correct anthramycin orientation on 5′(ATGCAT)$_2$.[22,24]

CC-1065 has two chiral centers which are located at the cyclopropyl ring junctions, C-3b and C-4a. Only the relative configurations at C-3b and C-4a of CC-1065 were determined by X-ray crystallographic analysis.[25] CPK modeling studies showed that nucleophilic attack by N-3 of adenine upon the methylene carbon (C-4) of the CC-1065 cyclopropyl ring must result in a drug–DNA adduct with the bulk of the CC-1065 molecule lying either to the 5′ or the 3′ side of the covalently modified adenine, dependent upon the absolute stereochemistry at C-3b. It was proposed that if CC-1065 exhibits a sequence selectivity in binding, the directionality of this specificity would reveal the absolute configuration of the adduct, and by inference, the absolute stereochemistry of CC-1065 (see later).

(23) Barkley, M.; Cheatham, S.; Thurston, D. E.; Hurley, L. H. *Biochemistry*, **1986**, *25*, 3021.
(24) Graves, D. E.; Stone, M. P.; Krugh, T. R. *Biochemistry* **1985**, *24*, 7573.
(25) Chidester, C. G.; Krueger, W. C.; Mizak, S. A.; Duchamp, D. J.; Martin, D. G. *J. Am. Chem. Soc.* **1981**, *103*, 7629.

Determination of Binding Sequence Specificity for CC-1065 and the P(1,4)Bs

CPK model building studies with both CC-1065 and the P(1,4)Bs showed close van der Waals contacts between the floor of the minor groove of B-form DNA and the concave edges of the drug molecules. These studies were suggestive of a DNA sequence specificity extending for the length of the overlap between these minor groove binding agents and DNA. By necessity, two different methods were used to investigate the sequence specificity of CC-1065 and the P(1,4)Bs, but both involved the use of a modified Maxam–Gilbert DNA sequencing method.

For CC-1065, supercoiled circular DNA molecules (FI) were shown to unwind to relaxed circular molecules (FII) following thermal treatment (90 °C, 30 min) of CC-1065-FI-SV40 DNA adducts.[20] Subsequent experiments using singly 5′ or 3′-^{32}P end-labeled DNA restriction fragments showed that thermal treatment of CC-1065–DNA adducts results in a single strand breakage event at the 3′ side of the adenine covalent attachment site (Figure 3).[26] CC-1065–DNA binding sequences were determined on Maxam–Gilbert sequencing gels by locating CC-1065 thermally induced DNA strand breaks on single 5′-^{32}P end-labeled DNA restriction enzyme fragments isolated from the early promoter region of SV40 DNA[26] (Figure 4). Analysis of the data from Figure 4, as well as fragments from T7 DNA, led to the identification of two subsets of se-

(26) Reynolds, V. L.; Molineux, I. J.; Kaplan, D. J.; Swenson, D. H.; Hurley, L. H. *Biochemistry* **1985**, *24*, 6228.

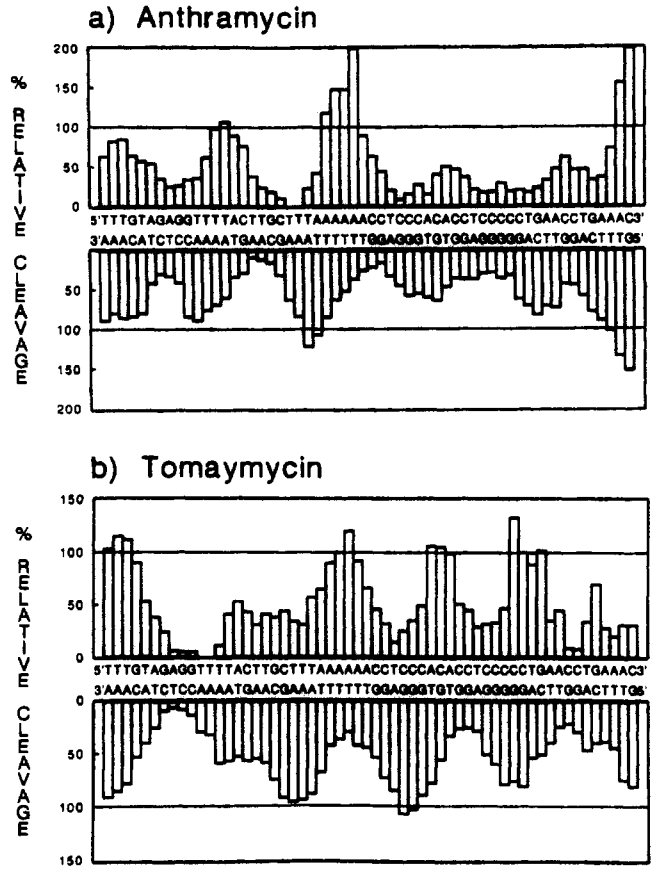


Figure 5. Histograms of MPE-Fe(II) footprints of Anthramycin (a) and Tomaymycin (b) on opposite strands of a 241 base pair DNA fragment. Less than 100% cleavage indicates areas of drug protection whereas greater than 100% cleavage indicates regions of drug-induced enhanced cleavage of DNA.[28]

quences, 5′PuNTTA (Pu = Purine, N = any base) and 5′AAAAA, which are preferred CC-1065 binding regions, with the 3′ terminal adenine of each of the sequences covalently modified by CC-1065. Since this marked sequence specificity lies solely to the 5′ side of the covalently modified adenine, the absolute stereochemistry of CC-1065 must be 3b-*R*, 4a-*S* (see before).

More indirect methods for determination of the P-(1,4)B preferred binding sequences were required, because these drugs do not generate DNA strand breaks under alkaline or thermal conditions.[14] A DNA footprinting method using methidium propyl-EDTA-Fe(II) [MPE-Fe(II)][27] on singly 5′-^{32}P end-labeled DNA restriction fragments was used to identify the P(1,4)B consensus binding sequences.[28] MPE-Fe(II) cuts unmodified DNA fairly randomly, but the presence of drug molecules inhibits MPE-Fe(II) cutting, and a "footprint" appears on electrophoretic gels where drug molecules are bound. The footprinting results shown in Figure 5 are derived from a portion of a 241 base pair fragment which was subjected to MPE-Fe(II) footprinting analysis on singly 5′ end-labeled DNA molecules to which either anthramycin or tomaymycin had been bound. The 3′ offset of the footprints on opposite strands has been previously observed and can be rationalized on the basis of asymmetric protection of the DNA from MPE-Fe(II) digestion.[29] Footprinting experiments showed that the P(1,4)B antibiotics bind most avidly to 5′PuGPu (Pu = Purine) sequences, and least readily to 5′PyGPy (Py = Pyrimidine) sequences.[28]

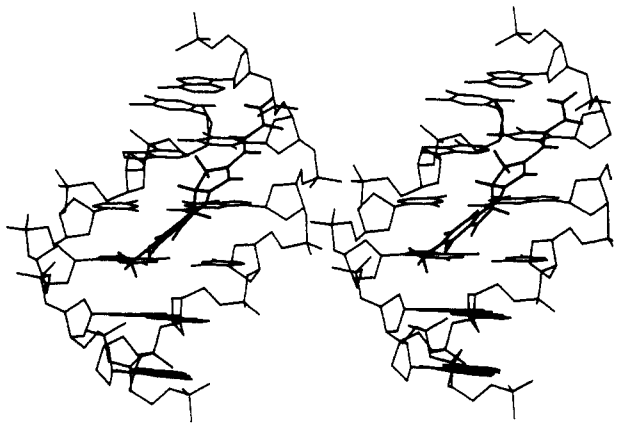


Figure 6. Stereodiagrams of the anthramycin–d(ATGCAT)$_2$ duplex adduct.

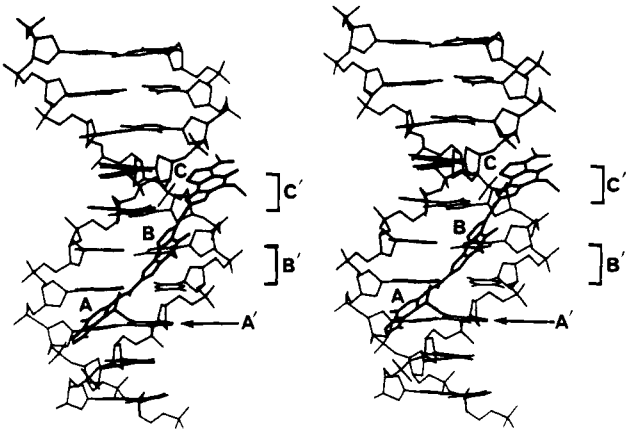


Figure 7. Stereodrawings of the CC-1065–DNA adduct. Key: A, B, and C are the subunits of CC-1065; A′ is the adenine covalent binding site for CC-1065; B′ is a pair of highly conserved bases immediately to the 5′ side of adenine covalent binding site; C′ is a pair of less well conserved bases at the 5′ end of CC-1065 binding site. The base sequence from top to bottom is 5′ CGGAGTTAGG 3′.

Preparation of Stereodiagrams for CC-1065 and the P(1,4)B-DNA Adducts

The investigation described above allowed identification of the following critical parameters for the reaction of CC-1065 and the P(1,4)Bs with DNA: (a) the chemical identities of the covalent linkages, (b) drug orientations and stereochemistries relative to the covalent linkage, and (c) the preferred binding sequences of the drugs. This information, combined with the X-ray crystallographic coordinates of B-form DNA, anthramycin,[30] and CC-1065[25] was utilized to generate stereodiagrams of the CC-1065 and anthramycin–DNA adducts using molecular graphics (Figures 6 and 7). Molecular modeling has allowed us to refine our understanding of these drug-DNA interactions, and to postulate mechanisms for DNA sequence "recognition" and covalent binding to DNA by CC-1065 and the P-(1,4)Bs.

(27) Van Dyke, M. W., Hertzberg, R. P., Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 5470.
(28) Hertzberg, R. P.; Hecht, S. M.; Reynolds, V. L.; Molineux, I. J.; Hurley, L. H. *Biochemistry* **1986**, *25*, 1249.
(29) Van Dyke, M. W. and Dervan, P. B. *Cold Spring Harbor Symp. Quant. Biol.* **1982**, *47*, 347.
(30) (a) Mostad, A.; Romming, C.; Storm, B. *Acta Chem. Scand. Ser. B.* **1978**, *32*, 639. (b) Arora, S. K. *Acta Cryst.* **1979**, *B35*, 2945.

Mechanisms for Covalent Binding of CC-1065 and the P(1,4)Bs in the Minor Groove of DNA.

Although CC-1065 and the P(1,4)Bs bind within the minor groove of DNA, they exhibit marked differences in their mechanisms of covalent binding, and consequently form distinctly different covalent adducts. The reaction of the P(1,4)Bs with DNA is postulated to occur by stepwise dehydration of the carbinolamine at C-11 to the imine species, which is then susceptible to nucleophilic attack by N2 of guanine within the minor groove[23] (Figure 2). The evidence for this is indirect and is based upon the ability of the P(1,4)Bs and solvent anions to form addition products at C-11, presumably mediated by imine formation. Primary evidence for solvent interaction with the P(1,4)Bs stems from ^{13}C NMR studies, in which the (11*R*,11a*S*) diastereomer of tomaymycin was observed to undergo epimerization at C-11 over several hours in $^{13}CH_3OH$.[23] Furthermore, dissolution of tomaymycin methyl ether in chloroform yielded the N10-C11 imine species, which was reconverted to the carbinolamine 11-methyl ethers when redissolved in methanol.[23] This suggests that these drugs may also react with biological nucleophiles (i.e. protein, RNA, and other DNA nucleophiles), but in the absence of secondary stabilizing forces (hydrogen bonding, hydrophobic interactions) the conjugate products would not be expected to be stable, and probably undergo either elimination to the imine or hydrolysis to the carbinolamine, the end result in both cases being regeneration of free drug. In contrast to this, the reaction of the P(1,4)Bs with N2 of guanine leads to an aminal linkage product which, according to molecular mechanics calculations, is stabilized by steric and electrostatic interactions and consequently is less likely to undergo hydrolysis unless these noncovalent interactions are reduced.[22] Moreover, experiments show that if P(1,4)B–modified DNA is melted under neutral or acidic conditions, the P(1,4)Bs are released in active form,[12] attesting to the stabilizing effect of the intact duplex minor groove on the N2 guanine adduct. Further evidence for the importance of an intact minor groove for binding of the P(1,4)Bs is that these drugs do not bind covalently to a single-stranded DNA template.[12]

CC-1065 alkylates DNA specifically at N-3 of adenine.[21] Nucleophilic attack by N-3 occurs at the cyclopropyl methylene carbon of the "A subunit" of CC-1065 with concomitant ring opening and reduction of the indole quinone to an indole phenol (Figure 3). This is believed to be an essentially irreversible reaction which results in a net positive charge on the adenine ring system. In contrast to the P(1,4)B carbinolamine or imine, the relative electrophilicity of CC-1065 is rather weak, and we suspect that a proximity effect[31] (see later) is important in activating CC-1065 to the more reactive species.

Sequence Recognition within the Minor Groove of DNA

Binding of CC-1065 and the P(1,4)Bs to DNA is proposed to be a biphasic event. Initially, there is probably a rapid noncovalent association of the ligands within the minor groove. The right-handed twist of the molecules and the relatively hydrophobic nature of their concave "inside edges" is highly compatible with the environment within the minor groove. Several forces have been identified as important in noncovalent association of ligands within the minor groove.[8,32] Dehydration energies for the minor groove and the ligand significantly affect binding affinities, with more hydrophobic ligands providing a greater driving force for association.[32b] Electrostatic interactions between the receptor and ligand stabilize associations,[32b] and hydrophobic interactions between ligand protons and base protons (specifically, H-2 of adenine) increase binding affinities.[32c] The above considerations, combined with steric interactions generated from close van der Waals contacts between ligands and the floor of the minor groove (most notably, N2 of guanine), determine which sequences within the minor groove have the highest affinities for a given ligand. Interestingly, this "sequence selectivity," or "recognition" within the minor groove does not seem to be mediated to any great extent by hydrogen bonding, although such bonding may subsequently stabilize bound species.[9,32b] This is in sharp contrast to the more precise DNA sequence recognition of polypeptide DNA ligands such as repressor proteins and restriction enzymes.[33] In these cases, recognition and binding to specific DNA sequences is proposed to occur largely through discrete hydrogen bonding interactions between ligands and the DNA and generally takes place within the major groove of DNA.

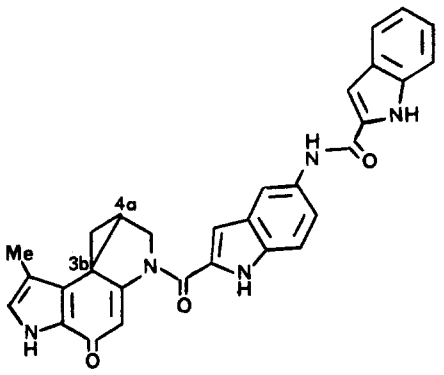
The P(1,4)Bs bind most efficiently to DNA at 5′PuGPu sequences, and least efficiently at 5′PyGPy sequences.[28] However, anthramycin, sibiromycin, and tomaymycin have been shown to prefer different subsets of the consensus 5′PuGPu binding sequence,[28] suggesting local interactions differ to some extent between each drug molecule and its preferred binding sequence in DNA. Since the concave side of the drug molecules (i.e., positions 9,10,11,11a, and 1 in structure I of Figure 1) follows the floor of the minor groove of DNA, the overall characteristic twist of each molecule probably is of major importance in the determination of sequence recognition. Both the dihedral angle between the aromatic ring the five-membered ring and the pucker of the pyrrole ring contribute to the overall twist (and therefore to the sequence recognition properties) of each drug.

CC-1065 binds covalently to the minor groove of DNA through N-3 of adenine. In addition, it is highly selective for AT rich sequences in DNA, binding with greatest affinities to the 3′-terminal adenine of the sequences 5′PuNTTA and 5′AAAAA.[26] As was the case with the P(1,4)Bs, the proximity of specific regions of the CC-1065 molecule to groups within the chemically interactive range on the DNA molecule can be used to rationalize the extent and overall nature of the DNA recognition sequences. The sequence specificity data reveals that there is an overwhelming preference for AT pairs over GC pairs. This is similar to the data for netropsin and distamycin, which also bind in the minor

(31) Menger, F. M.; Venkataram, U. V. *J. Am. Chem. Soc.* **1985**, *107*, 4706.

(32) (a) Zimmer, C. *Comments Mol. Cell. Biophys.* **1983**, *1*, 399. (b) Pullman, B. In *Specificity in Biological Interactions*; Chagas, C; Pullman; B., Eds.; The Vatican Press and Reidel Publishing Company: Vatican City and Dordrecht, The Netherlands, 1984. (c) Zakrzewska, K.; Lavery, R.; Pullman, B. *Nucleic Acids Res.* **1984**, *12*, 6559.

(33) (a) Matthews, B. W.; Ohlendord, D. H.; Anderson, W. F.; Fisher, R. G.; Takeda Y. *Trends Biochem. Sci. (Pers. Ed.)* **1983**, *8*, 25. (b) Rosenberg, J. M.; Greene, P. *DNA* **1982**, *1*, 117.



U-71184 - 3bR,4aS

U-71185 - 3bS,4aR

Figure 8. Structures of U-71,184 and U-71,185.[35]

groove but without a formal covalent linkage.[32a] The snug fit of the CC-1065 molecule along the floor of the minor groove is suggestive that the bulky 2-amino group of guanine may discourage binding via steric interference.

The discovery of two different consensus sequences for CC-1065 binding sites was unexpected.[26] The consensus analysis for CC-1065 reveals that if the base on the 5′ side of the covalently modified adenine is an adenine, than the next base will most likely be another adenine, and similarly for pairs of thymines. The specificity for the 5′-proximal pair of bases in both consensus sequences is less well-defined. Therefore, it is useful to consider the consensus sequence for both subclasses of binding sites as consisting of three basic units. The first unit (A′) at the 3′ end consists of the alkylation site for CC-1065; the second unit (B′) is a pair of highly conserved bases (AA or TT); the third unit (C′) is a pair of less well conserved bases (AA or PuN). Examination of the stereo drawings of the CC-1065–DNA adduct (Figure 7) reveals that the three units (A′, B′, and C′) making up the consensus sequence overlap with subunits A, B, and C of CC-1065, respectively. Since the subunits of CC-1065 are connected by amide linkages, there is some flexibility in the conformational angles between subunits A and B and between subunits B and C. Moreover, the rigidity within subunit B of CC-1065 which overlaps with the second consensus unit (B′) will specify one of a pair of homoduplexes (AA or TT), depending upon the conformational angle between subunits A and B and between subunits B and C. Presumably, the steric interactions presented by a heteroduplex consisting of 5′AT or 5′TA are less favorable. From the sequence specificity data, the order of preference for unit B′ for interaction with CC-1065 is 5′TT greater that 5′AA. The less strict preference for consensus unit C′ makes a similar analysis as carried out for unit B′ less informative.

The importance of proximity or drug juxtaposition to N-3 of adenine is illustrated by the results of experiments where attempts were made to bind the two optical antipodes (U71,184 and U71,185 of Figure 8) of a synthetic CC-1065 analogue to a 118 DNA base pair restriction fragment from SV40 DNA.[34] Thermal DNA strand break analysis of binding of these two diastereomers to DNA demonstrated that only the isomer (U71,184) with the same absolute stereochemistry at the cyclopropyl ring junctions as CC-1065 (3bR,4aS) binds covalently to DNA in a manner analogous to CC-1065, although a somewhat restricted sequence specificity and a temperature dependence between 4 and 37 °C not evident with CC-1065 was noted.[34] In contrast, the diastereomer with the opposite stereochemistry to CC-1065 (U71,185) did not bind covalently to DNA at temperatures up to 37 °C. Modeling studies suggest that the best noncovalent fit of both diastereomers in the minor groove of DNA is in the same location, with the concave edges of the subunits of U-71,184 and U-71,185 interacting through close van der Waals contacts with the floor of the minor groove of DNA. The diastereomer with the same absolute stereochemistry as CC-1065 lies in the minor groove with its electrophilic center (C-4) in proximity to N-3 of adenine, while the cyclopropyl methylene carbon of the opposite isomer resides between base pairs and is not in proximity to N-3 of adenine. These results suggest that the alignment of CC-1065-like molecules in the minor groove of DNA, which is dictated by noncovalent interactions, restricts the number of possible covalent adducts subsequently formed. Significantly, only the isomer which binds covalently to DNA (U71,184) is biologically active.[35]

Molecular Basis for Biological Activity

Molecular modeling studies on the P(1,4)Bs have provided compelling evidence through predictive structure–activity relationships that covalent modification of DNA is necessary to maintain cytotoxic potency.[5b] Although far less extensive evidence is available for CC-1065 and its analogues, structure–activity relationships can also be rationalized on the basis that covalent modification of DNA is the critical event.[34] In spite of our considerable insights into the structures of the drug–receptor complexes for the P(1,4)Bs and CC-1065, the mechanism(s) by which these drugs exert their potent biological effect is far from clear. Nevertheless, some clues are available from studies in which the biochemical and biological effects of these drugs on intact cells has been evaluated.

As noted previously, CC-1065 and the P(1,4)Bs are much more potent than their counterparts which interact with DNA noncovalently.[11] Moreover, individual members of each series show considerable variation in biological potencies. Of the P(1,4)Bs, sibiromycin is by far the most potent, followed in decreasing order of potency by anthramycin, tomaymycin, and the neothramycins.[5f] This rank order mirrors the in vitro stability of the adducts on DNA,[12a] with sibiromycin producing the most stable DNA adduct. The problem, however, is more complex, because although N2 of guanine appears to be the alkylation target on DNA, it is probably also crucial to define both the structural characteristics of the particular modified guanine-containing sequence and its location within the genomic target. In particular, the importance of the location of DNA damage by aflatoxins in actively vs. nonactively transcribed sequences has been previously noted,[36] and

(34) Lee, C-S; Hurley, L. H. *Proc. Am. Assoc. Cancer Res.* **1986**, *27*, 962.

(35) Warpehoski, M. A.; Kelly, K. C.; McGovren, J. P.; Wierenga, W. *Proc. Am. Assoc. Cancer Res.* **1985**, *26*, 870.

(36) Irvine, R.; Wogen, G. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *81*, 664.

may provide a valuable corollary to P(1,4)Bs and CC-1065 activities.

Covalent modification of DNA does not automatically lead to cytotoxic potency. More likely, the charge and steric characteristics of the DNA-adducts determine the resulting reaction processes which can lead to either an innocuous adduct (e.g. N7-methylguanine) or a possible mutagenic lesion (e.g., 06-methylguanine). Indeed the data from DNA repair experiments (see later) with both the P(1,4)Bs and CC-1065 suggest that the minor groove covalent binding agents described in this Account may well represent structurally problematic lesions to either recognized or repair in an error-free and effective manner. The nondistortive nature of the DNA lesions, in which the covalent linkage site is between the concave edge of the drug molecule and the floor of the minor groove of DNA, may require a complex type of adduct recognition and repair pathway(s). Possibly relevant to this argument is the observation by Lippard[37] who has recently noted that the nondistortive nature of the *cis*-Pt–DNA adduct apparently leads to a delayed removal of these lesions through DNA repair pathways. In the case of CC-1065, the adduct disturbs the normal electronic character of the adenine nucleus, which might be recognizable by a repair complex by detection of a positive charged imidazole moiety via the major groove. However, the P(1,4)Bs modify the exocyclic N2 of guanine and would be less likely to present an electronically distinct species. Indeed, while excision-deficient human cells derived from *Xeroderma pigmentosum* (XP) patients are defective in removal of anthramycin adducts from DNA,[38] both normal and XP cell lines appear equally adept at recognizing CC-1065 alkylation of DNA,[39] implying two different repair recognition pathways for CC-1065 and the P-(1,4)Bs.

Working upon the assumption that DNA repair might well play an important role in expressing the potency of the minor groove covalent adducts, we have carried out experiments to examine the fate and biological consequences of anthramycin lesions in bacteria, yeast, and human cells. While the P(1,4)Bs are not significantly mutagenic in bacteria or yeast, they are highly recombinogenic.[40] In human cells exposed to anthramycin, excision-dependent single and double strand DNA breaks are produced[38] which may be associated with the recombinogenic characteristics of these drugs. We have speculated that excision repair might not always occur on the damaged DNA strand and may consequently lead to detrimental repair.[38,41] Much less is known concerning the repair of CC-1065 lesions, except that exposure of human cells to the drug leads to a dramatic decrease in NAD levels associated with poly(ADP)ribosylation,[39a] and both normal and XP cells are able to recognize CC-1065–DNA lesions.[39b] The significance of these result in relation to DNA repair and cytotoxicity of CC-1065 remains to be determined.

Concluding Remarks

The mechanism of action work described in this Account has led to considerable insights into the structures of the CC-1065 and P(1,4)B–DNA adducts. Much less is known about the events that lead to the potent cytotoxic effects of these compounds. Our present efforts are designed to understand in more detail the molecular basis for the sequence specificity of these DNA reactive compounds and, through the preparation of site-directed adducts in DNA, begin to unravel the biochemical events that lead to the biological consequences of DNA modification by CC-1065 and the P(1,4)Bs. Perhaps through some of these experiments a more rational approach to antitumor drug design can be discovered.

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Registry No. CC-1065, 69866-21-3.

(37) Ciccarelli, R. B.; Solomon, M. J.; Varshavsky, A.; Lippard, S. J. *Biochemistry* **1985**, *24*, 7533.

(38) Petrusek, R. L. D.; Uhlenhopp, E. L.; Duteau, N.; Hurley, L. H. *J. Biol. Chem.* **1982**, *257*, 6207.

(39) (a) Jacobson, M.; Hurley, L. H. *Biochemistry*, in press. (b) Lambert, M. *Proc. Am. Assoc. Cancer Res.* **1985**, *26*, 1807.

(40) Hannan, M. A.; Hurley, L. H.; Gairola, C. *Cancer Res.* **1978**, *38*, 2795.

(41) Hurley, L. H.; Needham-VanDevanter, D. R. In *Mechanisms of DNA Damage and Repair: Implications for Carcinogenesis a Risk Assessment*; Simic, M. G.; Grossman, L.; and Upton, A. D. Eds.; Plenum: New York, 1986; pp 203–211.