FR-66979 Covalently Cross-Links the Binding Domain of the High-Mobility Group I/Y Proteins to DNA

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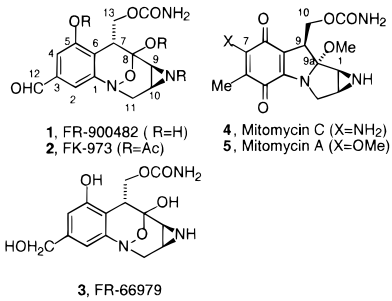
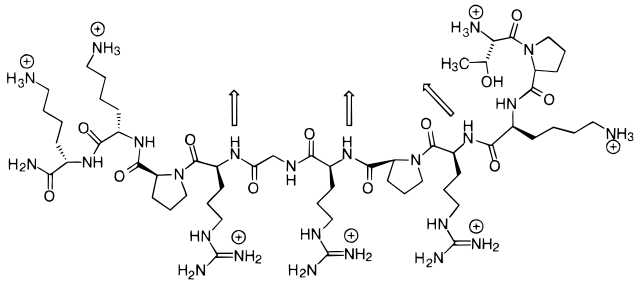
DNA interstrand cross-linking agents have long been regarded as among the most significant chemotherapeutics available.[1,2] The potent antitumor and antibacterial properties of such agents are attributed to their ability to covalently bind the two strands of duplex DNA, thus abrogating events of biological importance such as transcription and DNA replication.[3] The presence of two reactive sites within such molecules hints that other manifolds may exist by which such agents may express biological activity, monoalkylation of biomacromolecules and nucleic acid−protein cross-linking being the most likely. Facile cellular repair mechanisms dictate that DNA monoalkylation is significantly less important in the expression of therapeutic activity.[4] However, DNA−protein cross-linking can be presumed to result in lesions of vastly greater importance than monoalkylation adducts. The importance of DNA−protein cross-links relative to that of DNA−DNA interstrand cross-links is not yet known. Indeed, research aimed at the elucidation of DNA−protein cross-linking mechanisms has been hampered by the generally lower yields of such adducts relative to the interstrand events and the radically more complex and diverse structural possibilities inherent to nucleic acid−protein cross-links. We report herein a demonstration of DNA−protein cross-linking by FR-66979 (**3**), a novel antitumor antibiotic which closely resembles the clinically employed mitomycin C.[5] This represents the first demonstration of mitosene-based cross-linking of DNA to a DNA-binding protein.

We examined the reactions of **3** with duplex DNA in the presence and absence of synthetic peptides corresponding to the highly conserved binding domain (BD) within the high mobility group I/Y (HMG I/Y) proteins. Significantly, the HMG proteins are preferentially expressed in rapidly proliferating undifferentiated cell lines such as those of cancerous origin. The HMG I/Y proteins contain three runs of the BD sequence per protein.[6] The AT-hook motif inherent to TPKRPRGRPKK results from the synergism of the N-terminus TPK hook and the *trans*-proline-induced crescent conformation of the PRGRP palindrome.[6] The N-terminal hook results from an AsnX turn resulting from intramolecular hydrogen bonding between the threonine hydroxyl and the lysine-to-proline amide hydrogen.[6,7] This hook fits tightly into the widened minor groove of AT/GC junctions and has been





implicated as a major factor in DNA binding.[7,8] Notably, the BD peptide binding to the preferred sequence 5′-AAATTT-3′ occurs via minor groove contacts in a fashion similar to that known for netropsin and Hoechst 33258.[6−8]

Early efforts establishing the minor groove specificity of the FR-900482 class of agents[9] suggested that the minor groove HMG I/Y motif might represent a relevant drug target. This was supported by modeling studies in which one of the two arginine moieties (of PRGRP) placed a guanidine nitrogen proximal (within 2−3 Å) to the mitosene C10 position of a FR-66979 monoalkylated deoxyguanosine residue abutting the BD recognition sequence 5′-AAATTT-3′.[10] This suggested that a sequence bearing the FR-66979 alkylation "hot-spot" 5′-CG-3′ to the 5′ side of 5′-AAATTT-3′ would result in a high probability for reaction between an essential arginine and the mitosene C10.[11] This prompted the synthesis of the oligodeoxyribonucleotide (ODN) 5′-CCCACATCACTATACACGCGCGAAATTTCTCA-CTATC-3′ (**ODN 1A**) and its inosine-substituted complement 5′-GATAGTGAGAAATTTCICICITGTATAGTGATGTGGG-3′ (**ODN 1B**) which upon hybridization affords template 1 (**T1**).

The BD peptide **6** was constructed which was identical to that examined by Wemmer for which the DNA−peptide contacts were identified by elegant ^{1}H NMR studies.[6a] Simultaneous addition of dithiothreitol (DTT) (to reductively activate **3**), **3**, and **6** failed to afford gel-shifted materials. This was not unexpected, since binding of BD peptides to DNA is highly salt-dependent the result of electrostatic guanidinium-to-phosphate interactions.[6−8] An alternative reaction series involved initial alkylation of the DNA followed by rigorous desalting of the alkylated substrate via size exclusion chromatography (Sephadex G-50) and repetitive pre-

(1) Lawley, P. D. *Bioessays* **1995**, *17*, 561.
(2) Paustenbach, D. J.; Finley, B. L.; Kacew, S. *Proc. Soc. Exp. Biol. Med.* **1996**, *211*, 211.
(3) Bridgewater, L. C.; Manning, F. C. R.; Patierno, S. R. *Carcinogenesis* **1994**, *15*, 2421.
(4) (a) Sancar, A. *Annu. Rev. Biochem.* **1996**, *65*, 43. (b) Lawley, P. D.; Lethbridge, J. H.; Edwards, P. A.; Shooter, K. V. *J. Mol. Biol.* **1969**, *39*, 181.
(5) (a) Uchida. I.; Takase, S.; Kayakiri, H.; Kiyoto, S.; Hashimoto, M.; Tada, T.; Koda, S.; Morimoto, Y. *J. Am. Chem. Soc.* **1987**, *109*, 4108. (b) Fugita, T.; Takase, S.; Otsuka, T.; Terano, H.; Kohsaka, M. *J. Antibiotics* **1988**, *41*, 392. (c) Masuda, K.; Makamura, T.; Shimomura, K.; Shibata, T.; Terano, H.; Kohsaka, M. *J. Antibiot.* **1988**, *41*, 1497.
(6) (a) Geierstanger, B. H.; Volkman, B. F.; Kremer, W.; Wemmer, D. E. *Biochemistry* **1994**, *33*, 5347. (b) Reeves, R.; Nissen, M. S. *J. Biol. Chem.* **1990**, *265*, 8573. (c) Reeves, R.; Langan, T. A.; Nissen, M. S. *Proc. Nat. Acad. Sci. U.S.A.* **1991**, *88*, 1671.
(7) Siino, J. S.; Nissen, M. S.; Reeves, R. *Biochem. Biophys. Res. Commun.* **1995**, *207*, 497.
(8) Zajicek, J.; Nissen, M. S.; Munske, G.; Smith, V.; Reeves, R. *Int. J. Pept. Protein Res.* **1995**, *45*, 554.
(9) (a) Williams, R. M.; Rajski, S. R. *Tetrahedron Lett.* **1993**, *34*, 7023. (b) Huang, H.; Pratum, T. K.; Hopkins, P. B. *J. Am. Chem. Soc.* **1994**, *116*, 2703.
(10) Coordinates for the PRGRP fragment of HMG I/Y bound to the recognition sequence 5′-AATT-3′ were courtesy of Prof. D. Wemmer (UC Berkeley).
(11) For thorough discussions of the mitosene pathway to DNA−DNA interstrand cross-linking by the FR-900482 class compounds, see: (a) Williams, R. M.; Rollins, S. B.; Rajski, S. R. *Chem. Biol.* **1997**, *4*, 127. (b) Huang, H.; Rajski, S. R.; Williams, R. M.; Hopkins, P. B. *Tetrahedron Lett.* **1994**, *35*, 9669. (c) Paz, M. M.; Hopkins, P. B. *Tetrahedron Lett.* **1997**, 38, 343.

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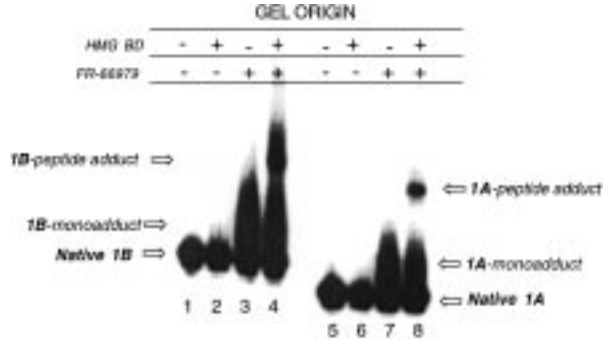

Figure 1. DNA−protein cross-linking by reductively activated **3**. All reactions were incubated 37 °C in 5 mM Tris (pH = 8.0) for 12 h. All DNA substrates were desalted by Sephadex G-50 chromatography and subsequently EtOH-precipitated prior to peptide addition. Lanes 1−4: reactions of template 1 (5′-end-labeled **1B**). Lanes 5−8: reactions of template 1 (5′-end-labeled **1A**). Lanes 1 and 5: template **1** standards. Lanes 2 and 6: 50 μM **6** + DNA controls. Lanes 3 and 7: DNA + FR-66979/DTT controls. Lanes 4 and 8: FR-66979-alkylated template **1** + 50 μM **6**.

cipitation from ethanol. Titration of FR-66979-monoalkylated DNA with 50 μM BD peptide (a 5-fold molar excess) resulted in the image depicted by Figure 1. As shown in this same experiment, binding was not only drug-dependent but also unexpectedly occurred with both strands of **T1**, indicative of peptide−drug interactions not restricted to the anticipated dG N2 of the DNA-bound FR-66979-derived mitosene. Inosine incorporation across from the 5′-CG-3′ run proximal to 5′-AAATTT-3′ of **ODN 1A** was anticipated to facilitate identification of the HMG−DNA cross-link by abrogating interstrand cross-linking and accentuating the formation of the peptide−DNA cross-link.

To confirm the covalency of this adduct, ligand/DNA exchange reactions were performed. Purified radiolabeled complexes of **6** with each strand of **T1** were incubated with unlabeled competitor **T1**. Incubation of each drug-mediated complex with increasing amounts of competitor DNA at 37 °C for 24 h failed to exchange peptide **6** from either radiolabeled strand. This is consistent with the expectation that the observed gel-shifted materials in Figure 1 are the result of covalent attachment of the peptide to DNA. Complete retention of each labeled complex was observed even in the presence of a >1000-fold molar excess of unlabeled duplex.

This result was further supported by negative-ion electrospray mass spectral analysis in which a mixture of high molecular weight materials were obtained for the DNA−peptide conjugate. Isolation of the drug-dependent complex that formed between **1A** and **6** was achieved by 10% denaturing polyacrylamide electrophoresis (DPAGE). The slow-mobility adduct was then eluted into 500 mM NH_4OAc/1 mM EDTA (pH 8.0) and repetitively precipitated from 1 M NH_4OAc as previously described for the interstrand cross-link.[11c] Electrospray mass spectral analysis revealed the presence of unmodified **1A** and a collection of signals just higher in mass than the anticipated 12 721.3 Da for the **1A**−drug−**6** adduct and significantly lower than the 13 094.8 Da expected for any **1B**−drug−**6** complex. The highest series of masses (12 899.1, 12 903.1, 12 906.0, and 12 910.0 Da) is consistent with protonation of the pendant guanidines of the three arginine residues leading to nonvolatile ion-pairing with buffer-derived acetate ion.[12] Significantly, the series of highest mass signals not only is consistent with protonation/ion-pairing of the guanidines with acetate but also supports the anticipated alkylation of both C1 and C10 positions of the FR-66979-derived mitosene. It is likely that the drug-mediated cross-link underwent fragmentation under the conditions of the mass spectral analysis, but nonetheless, these data are consistent with the adduct's covalent nature.

Depurination has been shown to very efficiently induce DNA−histone cross-links via lysine addition to the resulting aldehyde of the abasic site.[13] The generation of abasic sites within the peptide-bound DNAs was viewed as a possible mode of interaction. Mitomycin C has previously been shown to induce abasic sites within DNA, and this prompted examination of the FR-66979 mediated cross-links from this perspective.[14] To probe this potential "depurinative" means of cross-linking and to determine the minor versus major groove nature of the observed complex, piperidine digestion of the complexes formed between **6** and both strands of duplex **1** was performed.[15]

Isolation of both **1A**−**6** and **1B**−**6** complexes was followed by 3′-end-labeling of each single-stranded species. Digestion of each complex in 1 M piperidine at 80 °C (peptide-bound as well as unbound) and subsequent electrophoretic analysis revealed that the peptide-bound ODNs did not undergo significant strand scission over that seen in the piperidine control reactions. This suggests that the peptide−DNA lesion formed does not go through a depurination pathway since significant DNA scission would have been observed at such a site.[15] More importantly, this substantiates the anticipated minor groove nature of the drug-induced DNA-peptide cross-link (C1- and C10-dependent).[11]

DNA interstrand cross-linking agents figure prominently in current chemotherapeutic approaches to the treatment of cancer due to their ability to inhibit strand separation processes. Their ability to induce DNA−protein cross-links is beginning to be recognized, and the biochemistry of such lesions merits additional scrutiny. Several DNA cross-linking agents have been reported to induce such cross-links, including the psoralens and mono- and dimeric cisplatin-based metalloagents.[16] Mitosene/pyrrole-based agents have also been reported to form DNA−protein cross-links, but no defined DNA-binding protein system has been reported in which this occurs.[17,18] It is thus highly significant that FR-66979, whose mechanism of DNA modification chemistry closely parallels that of the mitomycins, pyrrolizidine alkaloids, and simple pyrroles,[11,18] forms DNA−protein cross-links between the BD of HMG I/Y proteins and a synthetic DNA bearing the protein recognition sequence 5′-AAATTT-3′. The exact mechanism of this reaction has not yet been elucidated, and this, along with issues of regiochemistry, stereochemistry, and sequence specificities, is currently being examined.

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Supporting Information Available: Electrospray mass spectral data for FR66979-mediated ODN 1A-6 cross-link and autoradiogram of piperidine treatment of the peptide-drug−DNA complex (2 pages). See any current masthead page for ordering information and Web access instructions.

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(12) This suggests that the previously anticipated guanidine−mitosene connectivity involved in cross-linking (as predicted on the basis of molecular modeling) may not be operative in our system.

(13) Levina, E. S.; Bavykin, S. G.; Shick, V. V.; Mirzabekov, A. D. *Anal. Biochem.* **1981**, *110*, 93

(14) Tomasz, M.; Lipman, R. *J. Am. Chem. Soc.* **1979**, *101*, 6063.

(15) The susceptibility towards β-elimination of the peptidic Schiff base formed with apurinic sites upon digestion with piperidine is supported by: (a) Singh, M. P.; Hill, C. G.; Peoc'h, D.; Rayner, B.; Imbach, J. L.; Lown, W. J. *Biochemistry* **1994**, *33*, 10271. (b) Bailly, V.; Verly, W. G. *Biochem. J.* **1988**, *253*, 553.

(16) Of particular interest, is the recent report by Lippard on the cisplatin-HMG1-DNA cross-link: (a) Kane, S. A.; Lippard, S. J. *Biochemistry* **1996**, *35*, 2180. (b) Sancar, A.; Hearst, J. H.; Sastry, S. S.; Spielmann, P. H.; Hoang, Q. S. *Biochemistry* **1993**, *32*, 5526. (c) Farrell, N.; Appleton, T. G.; Qu, Y.; Roberts, J. D.; Soares-Fontes, A. P.; Skov, K. A.; Wu, P.; Zou, Y. *Biochemistry* **1995**, *34*, 15480.

(17) (a) Kim, H. Y.; Stermitz, F. R.; Coulombe, R. A., Jr. *Carcinogenesis* **1995**, *16*, 2691.

(18) (a) Weidner, M. F.; Sigurdsson, S. T.; Hopkins, P. B. *Biochemistry* **1990**, *29*, 9225. (b) Woo, J.; Sigurdsson, S. T.; Hopkins, P. B. *J. Am. Chem. Soc.* **1993**, *115*, 3407.