Interstrand Cross-Linking of DNA by FK317 and Its Deacetylated Metabolites FR70496 and FR157471[†]

Robert M. Williams* and Pascal Ducept

Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523

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ABSTRACT: FR900482 (**1**) and FR66979 (**2**) are structurally novel natural products isolated by Fujisawa in 1987 and have been shown to be highly potent antitumor antibiotics structurally related to the mitomycins. Studies on the mode of action have established that these new agents form covalent DNA interstrand cross-links both *in vitro* and *in vivo* as a result of the reactive mitosene intermediate generated upon bioreductive activation. Semisynthetic analogues such as FK973 (**3**) and FK317 (**4**) were developed in the search for potentially superior clinical candidates. Although FK317 has been shown to be a potent compound, to date no direct evidence of DNA interstrand cross-link sequence specificity has been reported. In this study, DNA interstrand cross-links were generated by treatment of a synthetic duplex DNA substrate with FK317 (**4**) and its deacetylated metabolites FR70496 (**5**) and FR157471 (**6**). Analysis by gel electrophoresis revealed the formation of orientation isomers displaying electrophoretic mobility vastly greater than the mobilities of those generated from FR900482 (**1**). Despite these differences, it was established by Fe(II)−EDTA footprinting that FK317 (**4**) as well as **5** and **6** forms DNA interstrand cross-links within the expected 5′CpG3′ step, clearly demonstrating that the phenolic hydrogen in **1** and **2** is not a prerequisite for efficient DNA interstrand cross-linking by the FR class of compounds.

Compounds which exert their biological activity by covalently binding to nucleic acids, in particular DNA interstrand cross-linking agents, have played a significant role in the discovery of new agents for cancer chemotherapy (*1*−*3*). FR900482 (**1**) (*4*−*8*) and FR66979 (**2**) (*9*) are highly potent natural antitumor antibiotics with a unique dihydrobenzoxazine structure isolated in the late 1980s by the Fujisawa Pharmaceutical Co. from the fermentation harvest of *Streptomyces sandaensis* No. 6897. Initial biological studies have shown highly promising antitumor activity against a variety of cancerous cell lines both *in vitro* and *in vivo* (*4*−*9*). The clinical candidates FK973 (**3**) (*10*−*20*) and more recently FK317 (**4**) (*21*−*26*), both semi-synthetically derived from FR900482, have exhibited highly promising antitumor activity in human clinical trials in Japan and hold significant promise for becoming powerful anticancer drugs alongside the structurally related and widely used antitumor drug mitomycin C (MMC, **7**) (*27*, *28*).

Because of their intriguing structure and biological activity as well as the close structural relationship to the mitomycin family, extensive research efforts have been directed at the study of the mechanism of action of these agents. It has been demonstrated that the natural products FR900482 (**1**) and FR66979 (**2**) as well as FK973 (**3**) exert their cytotoxicity upon activation by a thiol-mediated two-electron reduction of the N−O bond to form a reactive mitosene intermediate. The mitosene generated by reductive activation, in a fashion similar to that for the mitomycins, preferentially cross-links duplex DNA at 5′CpG3′ steps in the minor groove (*29*−*35*). Additionally, early studies on FK973 conducted by Fujisawa scientists revealed that DNA might not be the only biological target and that proteins might also be involved, a property which may contribute to the biological activity of these compounds (*12*). More recently, it was shown in our laboratory that FR66979 covalently cross-links a synthetic oligonucleotide with the peptide-binding domain of the minor groove DNA binding oncoprotein HMG[1] A1 (formerly HMG I/Y) (*36*, *37*). Furthermore, our laboratory, in collaboration with the Reeves laboratory, has very recently shown that FR900482 and FK317 form DNA−protein cross-links with full-length HMG A1 protein (*38*, *39*) in transformed Jurkat cells. Interestingly, these studies have also established that, in contrast to FR900482, FK317 displays at concentrations above 1 μM a necrotic to apoptotic switch in the mode of cell death, which may explain the absence of vascular leak syndrome (VLS) displayed by FK317, a serious side effect that precipitated the early withdrawal of FK973 from clinical development (*17*). FR900482 induces cell death by a necrotic pathway at all concentrations that have been examined.

Although it is clear that FK317 yields DNA interstrand cross-links (as well as a DNA−protein cross-link), to our knowledge, no information concerning the DNA sequence specificity of FK317-mediated interstrand cross-links has been reported. This, and the observations mentioned above concerning HMG A1, prompted us to investigate the DNA cross-linking reaction of **4** and its deacetylated metabolites **5** and **6** with several synthetic oligonucleotides.

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* To whom correspondence should be addressed. Phone: (970) 491-6747. Fax: (970) 491-3944. E-mail: rmw@chem.colostate.edu.

[1] Abbreviations: DTT, dithiothreitol; DPAGE, denaturing polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; HMG, high-mobility group; NMR, nuclear magnetic resonance; ODN, oligodeoxynucleotide; TBE, Tris-borate-EDTA buffer; LS, liquid scintillation.

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EXPERIMENTAL PROCEDURES

Materials and General Methods. FK317 was generously supplied by the Fujisawa Pharmaceutical Co., Ltd. All H_2O that was used was obtained from a Barnstead Mega-Pure still. [γ-^{32}P]dATP (6000 Ci/mmol) was obtained from Amersham. T4 polynucleotide kinase was purchased from New England Biolabs and used according to the supplier's instructions in the activity buffer provided. DPAGE gels were run using a model S2 BRL sequencer. $FeSO_4$ (from Mallinckrodt) solutions were made up to 4 mM using 4 mM EDTA 5 min before use. A DTT stock solution was made using ddH_2O immediately prior to use. Centrex MF 0.45 μm cellulose acetate spin filters were obtained from Schleicher & Schuell. Radioactive samples were counted via Cerenkov counting using a Packard Tri-Carb 1500 scintillation counter. Phosphorimaging analysis was carried out using a Molecular Dynamics Storm 840 PhosphorImager equipped with ImageQuant software (version 5.1).

Drugs. FR70496 and FR157471 were both prepared by mono- and dideacetylation upon treatment of FK317 with sodium bicarbonate in methanol at room temperature for 4 and 24 h, respectively. Spectroscopic data (^{1}H and ^{13}C NMR) for both compounds were in agreement with the proposed structure (see the Supporting Information). All drug stock solutions were made up to 50 mM in sterile ddH_2O immediately prior to use (9:1 ddH_2O/DMF mixture for FK317).

Preparation and Radiolabeling of DNA. The oligodeoxynucleotides (ODNs) were synthesized on an Applied Biosystems 380B DNA synthesizer (1 μmol scale) using standard phosphoramidite chemistry (reagents and phosphoramidites from GLEN Research Inc.). All ODNs were purified by 10% preparative (1.6 mm) DPAGE. The DNA concentrations were determined by UV absorption at 260 nm using a Beckman Coulter DU 640 UV spectrometer. ODNs of interest were 5′-end-labeled with [γ-^{32}P]dATP and T4 polynucleotide kinase and then purified once more by 20% DPAGE. Labeled ODNs were then hybridized to their corresponding blunt-ended unlabeled complementary strand (1.2 equiv) in 200 mM Tris buffer (pH 7.5) by heating the mixture of ODNs to 85 °C for 15 min followed by slow cooling to room temperature over the course of 2−3 h and then at 4 °C over the course of an additional 2 h to afford a stock solution (90 μM in duplex DNA) in 200 mM Tris (pH 7.5).

*FK317 (**4**), FR70496 (**5**), and FR157471 (**6**) DNA Cross-Linking Assays and Quantitation by Storage Phosphorimaging*. Cross-linking reactions were carried out at 37 °C with either DNA template A or B, an appropriate amount of DTT (2 equiv), $FeSO_4$ (0.05 equiv), and each drug such that the final drug concentration ranged from 1 to 10 mM. The final reaction volume was 20 μL, and reaction mixtures were incubated at 37 °C for 18 h. The DNA was subsequently ethanol precipitated by cooling the sample in dry ice (20 min) and centrifuging for 10 min at 4 °C (14 000 rpm in an Eppendorf model 5415C instrument). The supernatant was removed, and the resulting pellet was washed with 70% ethanol and water. Following vacuum drying of the precipitate in a Speed-Vac concentrator, the labeled DNAs were counted by liquid scintillation (LS) and resuspended with DPAGE loading dye to afford an equal number of counts for each lane (50 000 cpm/μL). Reaction mixtures (5 μL per lane) were loaded onto a DPAGE gel (20% polyacrylamide; 19:1 acrylamide:bisacrylamide ratio) in TBE-buffered solution [89 mM Tris-borate (pH 8.3) and 1 mM EDTA]. Electrophoresis was performed at 50 W for 4 h. The gels were visualized using PhosphorImager and ImageQuant software (storage screens were exposed to the gels at room temperature for 1 h) to produce the images displayed in Figures 2 and 3. Baseline-corrected scans were analyzed by integrating all the densities between two selected boundaries to determine the optimum concentration for each drug.

Preparative DNA Cross-Linking. Twenty microliters of a 90 μM duplex stock solution [in 200 mM Tris (pH 7.5)] of 5′-^{32}P end-labeled template A was placed in each of four 1.5 mL Eppendorf tubes. To each tube was added a 50 mM drug stock solution to yield a drug concentration of 10 mM (FK317) or 5 mM (FR70496). To this mixture were added DTT and $FeSO_4$ as described in the previous section. After incubation, each reaction mixture was ethanol precipitated and loaded on a 20% DPAGE gel. Electrophoresis was

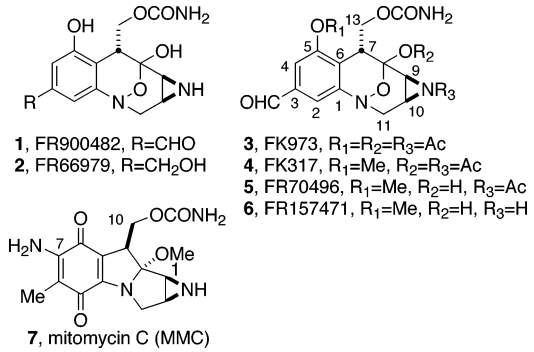


FIGURE 1: Structures of FR900482, mitomycin C, and congeners.

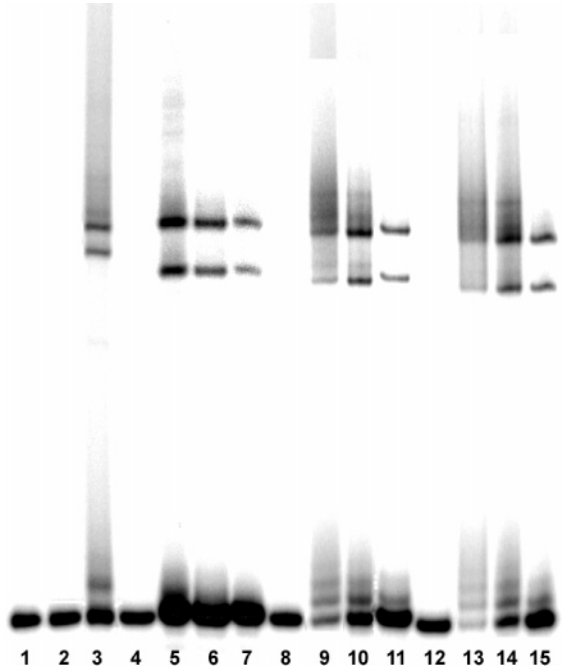


FIGURE 2: Cross-linking of duplex DNA (template A) to **4**−**6** (2 equiv of DTT and 5% $FeSO_4$ related to the drug concentration, 18 h at 37 °C): lanes 1 and 2, standard duplex and duplex with DTT, respectively; lanes 4, 8, and 12, control lanes containing duplex with 10 mM **4**−**6**, respectively; lane 3, DNA with **1** (10 mM); and lanes 5−7, 9−11, and 13−15, DNA with **4**−**6** (10, 5, and 1 mM), respectively.

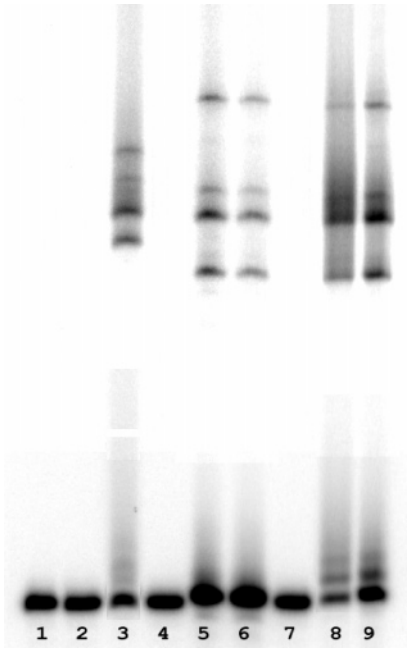


FIGURE 3: Cross-linking of duplex DNA (template B) to **4** and **5** (2 equiv of DTT and 5% $FeSO_4$ related to the drug concentration, 18 h at 37 °C): lanes 1 and 2, standard duplex and duplex with DTT, respectively; lanes 4 and 7, control lanes containing duplex with 10 mM **4** and **5**, respectively; lane 3, DNA with **1** (10 mM); lanes 5, 6, 8, and 9, DNA with **4** and **5** (10 and 5 mM), respectively.

carried out at 40 W for 5 h and the gel analyzed by phosphorimaging. The cross-link material was recovered by the crush and soak method as described below.

Recovery of Radiolabeled DNAs from DPAGE (crush and soak method). All DNAs (native DNA and cross-linked DNA) were isolated as follows. Following drug reactions, samples were ethanol precipitated and then redissolved in DPAGE loading dye (50 μL). Using analytical thickness (0.4 mm) 20% DPAGE, the samples were loaded into an appropriate number of wells (10 μL per well), and electrophoresis was carried out at 50 W for 4 h. Electrophoresis was typically carried out until the xylene cyanol dye (the dye with the slower mobility) was within 15 cm of the gel bottom. The gel was removed from the apparatus, and the plates were separated to render the gel on only one plate. A solution of the radiolabeled DNA standard in DPAGE dye was placed at various positions on the gel ($\sim$0.3 μL for each drop), and the gel was wrapped with plastic wrap. Storage screen was exposed for 30 min and analyzed by phosphorimaging. A picture of the area of interest was printed out at 100% scale and a transparency made. The bands of interest were cut out of the transparency; the bands that could be seen through the transparency and the gel were aligned, and the band of interest was excised from the gel. DNAs were finely crushed using a glass rod and then eluted into 3−5 mL of 500 mM NH_4OAc and 1 mM EDTA at 37 °C overnight. After elution, the solution was filtered through a Centrex MF 0.45 μm filter and the eluant was butanol extracted to a volume of 200−300 μL, and then the DNA was ethanol precipitated, dried, and finally resuspended in ddH_2O.

Maxam−Gilbert G and G+A Reactions. The 5′-end-labeled template A (typically 1 μg) was dissolved in 10 μL of ddH_2O and reacted according to the standard protocol (*40*, *41*).

*Fe(II)−EDTA Footprinting of FK317 (**4**)- and FR70496 (**5**)-Mediated Cross-Links.* After cross-linking reactions, cross-linked material was isolated as described above. Dried pellets were dissolved in 20 μL of ddH_2O, and a 5 μL aliquot was kept as a standard. To the remaining 15 μL were added phosphate buffer (pH 8, 7.5 μL) and 4 mM Fe(II)−EDTA (7.5 μL) to give a final concentration of 1 mM. Reaction mixtures were incubated at 37 °C for 8 h and ethanol precipitated. The dried pellets were counted by LS and were loaded on a 20% DPAGE gel such that standard duplex and cross-linked species contained 1000 cpm, and Fe(II)−EDTA lanes contained 10 000 cpm (standard DNA) and 15 000 cpm (cross-linked material). To G and G+A lanes were added 8000 and 16 000 cpm, respectively. Electrophoresis was carried out at 1100 V for $\sim$5 h (until the bromophenol blue dye migrated $\sim$26 cm from the bottom of the gel plates). The resolved DNA fragments were analyzed by exposing a phosphor screen for 24 h to yield the image displayed in Figure 4. After phosphorimaging, the alkylation site was assigned through reference to Maxam−Gilbert G and G+A lanes.

RESULTS AND DISCUSSION

The semisynthetic drug FK317 (**4**) is chemically prepared from FR900482 (**1**) by methylation of the C5 phenolic oxygen atom and acetylation of both the free aziridine and the hydroxylamine hemiketal (*24*). The acetyl groups facilitate entry of the drug into cells. However, Fujisawa workers have revealed that once FK317 has crossed the cell membrane barrier, deacetylation occurs (likely through the action of esterases). The first deacetylation takes place rapidly on the hydroxylamine hemiketal position to give FR70496 (**5**), followed by the second deacetylation on the aziridine which occurs more slowly to provide FR157471 (**6**) (*22*) (Scheme 1). Although it is believed that the acetyl groups are not necessary for the cytotoxic activity of FK317, without them, FK317 cannot be transported out of the cell by P-glycoprotein, thus leading to increased intracellular concentrations of the drug (*23*). It is thus clear that FK317, like FR900482, is a prodrug that is metabolized to both FR70496 (**5**) and FR157471 (**6**), which are believed to be the active metabolites responsible for the chemotherapeutic activity of FK317. Consequently, it is essential from a biochemical point of view as well as from a drug design perspective to understand why two drugs of almost identical structure display such different biological profiles.

*Reaction between **4**−**6** and DNA Templates A and B.* The 5′-^{32}P end-labeled substrate 5′d(TTTATTAACGTAATGCTTAATGCCAATGGGATT)3′ (template A) possessing one cross-linkable 5′CpG3′ site was synthesized and annealed to its complementary strand. Cross-linking of this substrate by compounds **4**−**6** at different concentrations (1, 5, and 10 mM) was performed. After incubation, reaction mixtures were ethanol precipitated, and gel electrophoresis was used to separate the cross-linked DNA from unmodified DNA and monoalkylated DNA. Thus, analysis by 20% DPAGE of the crude reaction products followed by phosphorimaging of the resulting gel produced the image shown in Figure 2, which revealed the presence of two bands corresponding to the cross-linked product.

Densitometric reading allowed us to determine the optimum concentration for each drug (10 mM for FK317 and

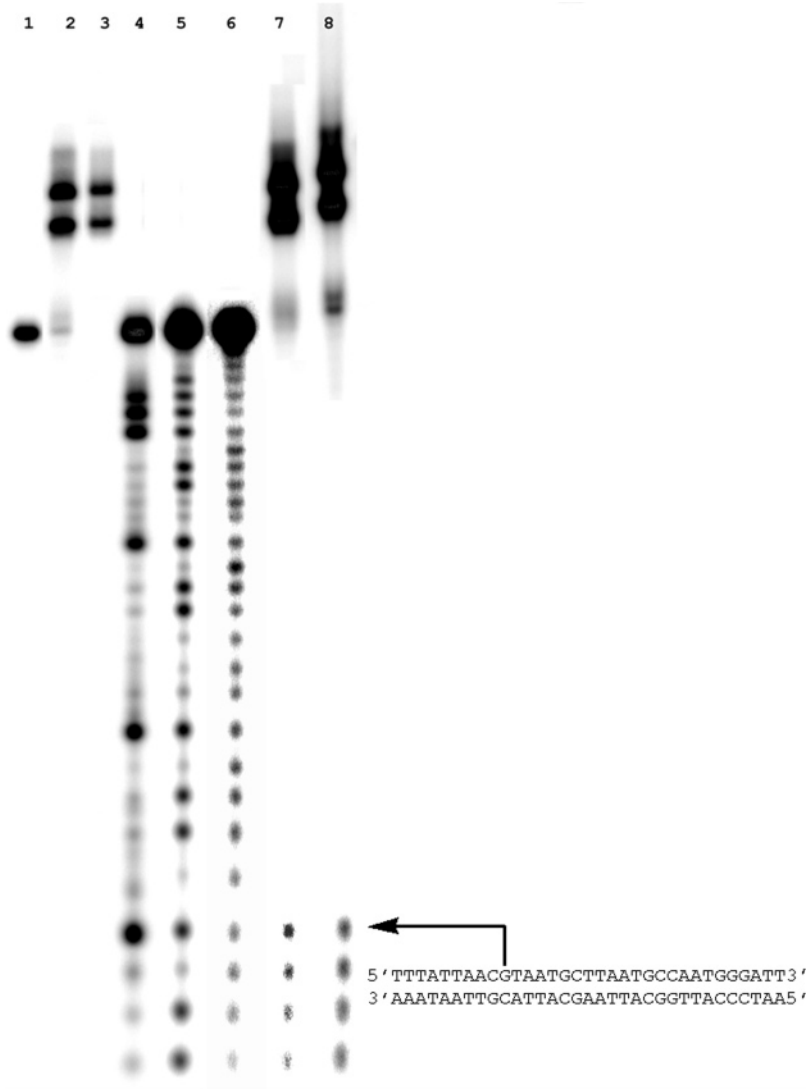


FIGURE 4: Fe(II)−EDTA footprinting of cross-linked template A with **4** and **5**. Lane 1 contained the standard duplex. Lanes 2 and 3 contain template A cross-linked with **4** and **5**, respectively. Lanes 4−6 show Maxam−Gilbert G, G+A, and 1 mM Fe(II)−EDTA template A digestion, respectively. Lanes 7 and 8 contain template A cross-linked by **4** and **5**, respectively, followed by 1 mM Fe(II)−EDTA digestion.

FR157471 and 5 mM for FR70496). Interestingly, analysis by gel electrophoresis revealed the formation of orientation isomers displaying differences in electrophoretic mobility between each orientation isomer much greater than those generated upon cross-linking with FR900482 (**1**). This clearly is due to the presence of the methoxy group since it is the only structural difference between **1** and **6**.

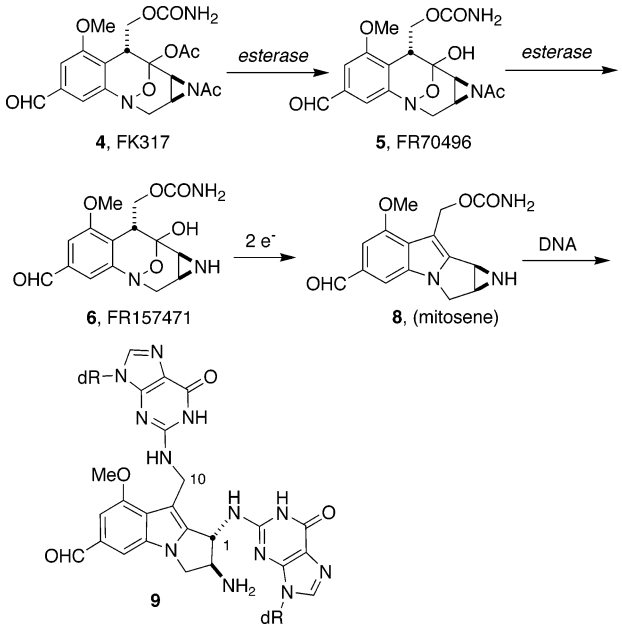
A possible rationalization for this observation is likely an increase in the extent of perturbation of the Watson−Crick base paring of adjacent base pairs compared to the free phenol in compound **1**, thus resulting in the cross-linked duplex possessing a greater difference in T_m values. Good fidelity cross-linking activity by FK317 also suggests that the formation of hydrogen bonds between the phenolic proton of **1** and N3 of the guanosine opposite that responsible for connectivity to C1 of the activated mitosene is not necessary for efficient monoalkylation by compounds **4**−**6**. The methoxy group of **4**−**6** apparently donates sufficient electrons to activate the aziridine for ring opening to an electrophilic species that is first monoalkylated and subsequently leads to cross-link formation. Additionally, recent results in our laboratory (*42*) using a synthetic phototriggered FR900482 mitosene progenitor lacking the carbamate functionality also suggest that the carbamoyl moiety, essential for MMC (**7**) for efficient alkylation of the 5′CpG3′ site (*43*), may not be obligate for the FR family of compounds.

Next, we were interested in reacting the 5′-^{32}P end-labeled substrate 5′TTTATTAACGTAATGCTTAATCGCAATGGGATT3′ (template B) that possesses two cross-linkable 5′CpG3′ sites. Upon reaction with compounds **4** and **5** and analysis by 20% DPAGE, the data in Figure 3 were obtained. Analysis by one-dimensional (1D) densitometry (see the Supporting Information) revealed that FK317 and FR70496 are sensitive to the flanking bases adjacent to the 5′CpG3′ step, which is similar to **1** and MMC (**7**) (*45*).

*Fe(II)−EDTA Footprinting of FK317 (**4**) and FR70496 (**5**).* Treatment of template A with **4** and **5** yielded cross-linked species (see Figure 2), which were easily isolated following ethanol precipitation and purification by 20% DPAGE. Following isolation of the cross-linked material from the gel, both native and cross-linked DNAs were subjected to Fe(II)−EDTA cleavage (*31*, *44*, *45*).

Following Fe(II)−EDTA digestion and Maxam−Gilbert reactions, all samples were ethanol precipitated and subjected

Scheme 1: Deacetylation and DNA Cross-Linking of FK317



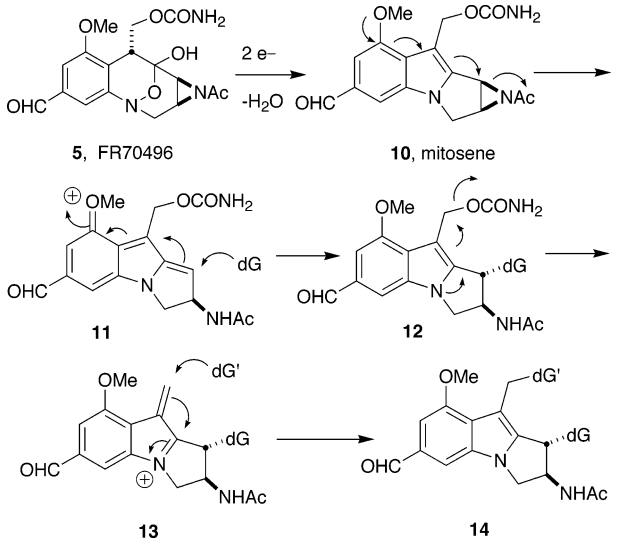
to 20% DPAGE at 1100 V for 4.5 h. Phosphorimaging of the gel produced the image shown in Figure 4. As expected for a mitosene, cross-link formation appears to occur at the exocyclic amine of the G10 residue of the 5′CpG3′ step. Corroboration of the footprinting data was secured by substitution of the 2′-deoxyguanosine base in template A with 2′-deoxyinosine at the 5′CpG3′ step (see the Supporting Information). Reactions with the inosine-modified duplex did not result in any observable cross-link, while the use of the 2′-deoxy-7-deazaguanosine-modified duplex resulted in cross-link formation in the same way as the unmodified template, thereby confirming that alkylation occurred, as expected for a compound based on the FR900482 framework, into the minor groove.

The similar biochemical reactivity of **4**−**6**, with respect to interstrand cross-linking, demonstrates that the aryl *O*-methyl ether in these structures is sufficiently electron-rich to facilitate the ring opening activation of the aziridine ring to form electrophilic species **11** (illustrated in Scheme 2 for **5**). This is thought to be the initial point of formation of a covalent bond with the DNA substrate (*1*, *20*).

CONCLUSIONS

In summary, we have demonstrated, using Fe(II)−EDTA footprinting, that the cross-linking of DNA by FK317 (**4**) and related deacetylated metabolites FR70496 (**5**) and FR157471 (**6**) follows the same general pattern seen with other cross-linking agents in this family of compounds by forming DNA interstrand cross-links within the expected 5′CpG3′ step. In addition, these results clearly demonstrate that the phenolic proton present in natural products **1** and **2** is not obligate for efficient cross-linking, although the replacement of the phenolic hydrogen (FR900482) with a methyl group (FK317) plays a significant, and as yet poorly understood, chemical role in the observed differences in the cell biological and clinical properties between these drugs. This is most notable in the manner in which the two drugs differ with respect to the mode of cell death.

Scheme 2: Mechanism of Interstrand Cross-Linking by **5**



Efforts to further probe the structural and mechanistic underpinnings of these important and interesting phenomena are the subject of current study in these laboratories.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Synthesis and ^{1}H NMR for FR70496 and FR157471, 1D densitometric scan for slow and fast mobility cross-linking isomers (Figure 2), 20% DPAGE for cross-linking of inosine and 7-deaza-modified template A with FR70496, and cross-linking assay with modified template A with **1** and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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