FR900482 class of anti-tumor drugs cross-links oncoprotein HMG I/Y to DNA in vivo

Lois Beckerbauer [1], Jetze J Tepe [2], Jennifer Cullison [1], Raymond Reeves [1] and Robert M Williams [2]

Background: Overexpression of the high-mobility group, HMG I/Y, family of chromatin oncoproteins has been implicated as a clinical diagnostic marker for both neoplastic cellular transformation and increased metastatic potential of several human cancers. These minor groove DNA-binding oncoproteins are thus an attractive target for anti-tumor chemotherapy. FR900482 represents a new class of anti-tumor agents that bind to the minor groove of DNA and exhibit greatly reduced host toxicity compared to the structurally related mitomycin C class of anti-tumor drugs. We report covalent cross-linking of DNA to HMG I/Y by FR900482 in vivo which represents the first example of a covalent DNA–drug–protein cross-link with a minor groove-binding oncoprotein and a potential novel mechanism through which these compounds exert their anti-tumor activity.

Results: Using a modified chromatin immunoprecipitation procedure, fragments of DNA that have been covalently cross-linked by FR900482 to HMG I/Y proteins in vivo were polymerase chain reaction-amplified, isolated and characterized. The nuclear samples from control cells were devoid of DNA fragments whereas the nuclear samples from cells treated with FR900482 contained DNA fragments which were cross-linked by the drug to the minor groove-binding HMG I/Y proteins in vivo. Additional control experiments established that the drug also cross-linked other non-oncogenic minor groove-binding proteins (HMG-1 and HMG-2) but did not cross-link major groove-binding proteins (Elf-1 and NFκB) in vivo. Our results are the first demonstration that FR900482 cross-links a number of minor groove-binding proteins in vivo and suggests that the cross-linking of the HMG I/Y oncoproteins may participate in the mode of efficacy as a chemotherapeutic agent.

Conclusions: We have illustrated that the FR class of anti-tumor antibiotics, represented in this study by FR900482, is able to produce covalent cross-links between the HMG I/Y oncoproteins and DNA in vivo. The ability of this class of compounds to cross-link the HMG I/Y proteins in the minor groove of DNA represents the first demonstration of drug-induced cross-linking of a specific cancer-related protein to DNA in living cells. We have also demonstrated that FR900482 cross-links other minor groove-binding proteins (HMG-1 and HMG-2 in the present study) in vivo; however, since HMG I/Y is the only minor groove-binding oncoprotein presently known, it is possible that these non-histone chromatin proteins are among the important in vivo targets of this family of drugs. These compounds have already been assessed as representing a compelling clinical replacement for mitomycin C due to their greatly reduced host toxicity and superior DNA interstrand cross-linking efficacy. The capacity of FR900482 to cross-link the HMG I/Y oncoprotein with nuclear DNA in vivo potentially represents a significant elucidation of the anti-tumor efficacy of this family of anticancer agents.

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Introduction

The mammalian high-mobility group, HMG I/Y, non-histone chromosomal proteins are founding members of a new class of gene regulatory proteins called 'architectural transcription factors' that function by recognizing and altering the structure of DNA and chromatin substrates (reviewed in [1–3]). The HMG I/Y protein family consists of three known members: HMG-I and HMG-Y, alternatively spliced protein variants encoded for by a single gene, and HMGI-C, the product of a separate locus [4–8]. The

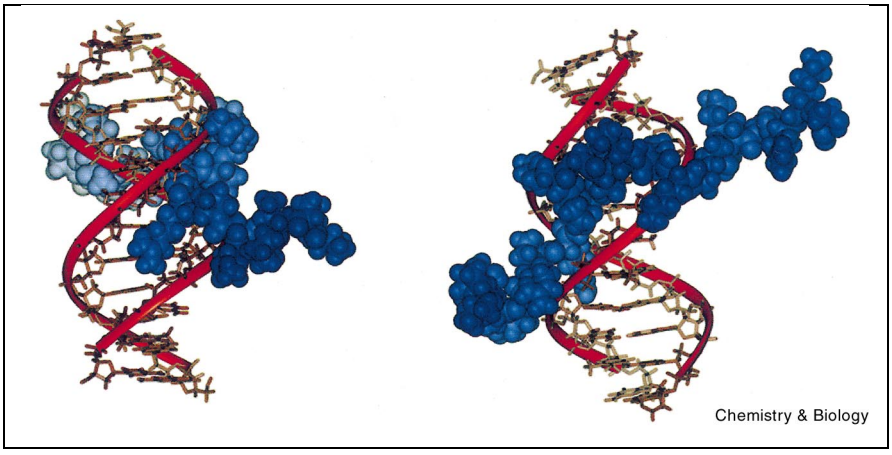

Figure 1. Structure of the DNA-binding domain II of human HMG I/Y bound to DNA showing the association of the conserved A·T hook protruding deeply into the minor groove [10].

HMG I/Y proteins are distinguished from other architectural transcription factors by their ability to preferentially bind to the minor groove of A·T-rich regions via novel DNA-binding motifs called A·T hooks (Figure 1) [9,10]. The HMG I/Y proteins have been implicated in the in vivo transcriptional regulation of numerous mammalian genes [1–3], many of which are associated with the immune system and cell growth [11–16], through participation in the formation of stereo-specific, multi-protein complexes called 'enhanceosomes' on gene promoter/enhancer regions.

Artificial overexpression of HMG I/Y proteins leads to cancerous transformation of cells (Li and Reeves; unpublished data) and elevated levels of these proteins are, therefore, a consistent feature of many different types of tumors. In fact, overexpression of the HMG I/Y proteins has been suggested to be a diagnostic clinical marker for both neoplastic transformation and increased metastatic potential of cancer cells (reviewed in [17–20]). In particular, it has been demonstrated that HMG I/Y proteins are over-expressed in human tumors of the prostate, colon, thyroid and breast, among other tissues. This manifest involvement of HMG I/Y overexpression in neoplasia suggests that drugs that can abrogate the functional integrity of these proteins when bound to DNA may have improved efficacy for tumor cells.

It has been demonstrated that the FR family of anti-tumor antibiotics (FR900482 (**1**), FR66979 (**2**), FK973 (**3**), FR70496 (**5**) and FK317 (**4**), which is currently in Phase II clinical trials in Japan; Figure 2) undergo reductive activation in vitro to form the reactive mitosene derivative **9** (Figure 3), which preferentially cross-links duplex DNA at $^{5'}$CpG$^{3'}$ steps [21–25]. The mechanism of reductive activation involves the thiol-mediated two-electron reduction of the N–O bond [25] in the presence of trace Fe(II) [26,27] generating the transient ketone **7** that rapidly cyclizes to the carbinolamine **8**. Expulsion of water has been inferred as the rate-determining step en route to the electrophilic mitosene **9** [26,27]. Therefore, FR900482 and the structurally related mitomycin C [28] are naturally occurring clever 'pro-drugs' that must be reductively activated in vivo to expose the highly reactive electrophilic mitosene derivatives that are responsible for the biological activity displayed by these substances [24,26,27].

Results and discussion

Early efforts establishing the minor groove specificity of the FR900482 class of agents suggest that the minor groove-binding HMG I/Y motif might represent a relevant peptide target for these drugs. We have recently reported the capacity of FR66979 to covalently cross-link the binding domain peptide of the non-histone chromosomal DNA-

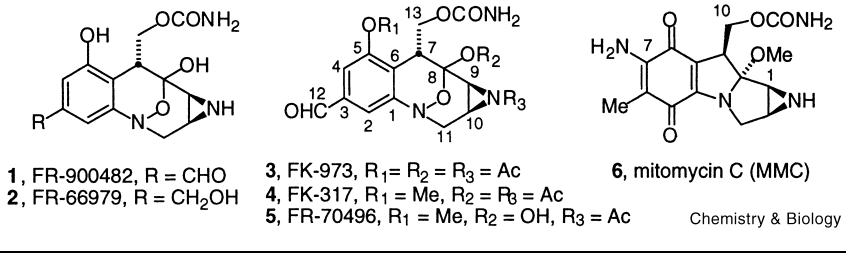

Figure 2. Structures of the clinically significant FR class of compounds (**1**–**5**) and mitomycin C (**6**).

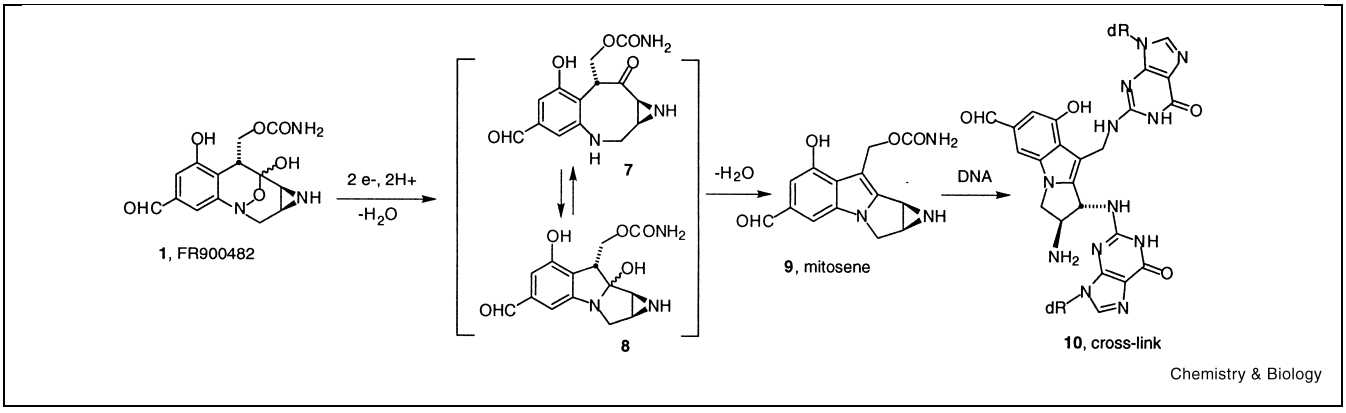

Figure 3. Mechanism of reductive activation and DNA interstrand cross-link formation of FR900482.

binding proteins of the HMG I/Y class in vitro [29]. This was further supported by modeling studies in which one of the two palindromic arginine moieties (of PRGRP run) place a guanidine nitrogen proximal (within 2–3 Å) to the mitosene C10 position of a FR66979 monoalkylated deoxyguanosine residue abutting the binding domain recognition sequence 5′ AAATTT 3′. This suggested that a sequence bearing the FR66979 alkylation 'hot-spot' $^{5'}$CpG$^{3'}$ to the 5′ side of 5′ AATT 3′ would result in a high probability for the reaction between an essential arginine and the C10 center of the mitosene. As a significant extension, we now report the *first* example of a small organic anti-tumor drug that forms an in vivo covalent cross-link to an entire DNA-binding protein of relevance to cancer cells. The ability of these anti-tumor antibiotics to cross-link the HMG I/Y protein with nuclear DNA in living cells suggests a mechanism for anti-tumor efficacy by abrogating the functional integrity of these oncoproteins.

Determination of in vivo cytotoxicity of FR900482

Human Jurkat T-leukemia cells were chosen for drug toxicity and cross-linking studies because they contain high endogenous levels of the HMG I/Y proteins and also because they can be induced to express specific cytokine genes, such as interleukin-2 (IL-2) and the α subunit of the IL-2 receptor (IL-2Rα), whose transcriptional expression in vivo is regulated by these proteins [11–16,30]. Jurkat cells (3×10^6) were exposed for 24, 48 and 72 h to varying concentrations (1.0, 1.5 and 2.0 μM) of FR900482 and the remaining viable cells were counted in each culture using a hemocytometer. Twenty-four hours after the addition of FR900482, the cells showed significant inhibition of cell proliferation at the lowest concentration of the drug, with death occurring at high concentrations (Figure 4).

The viability of all cells in each culture treated with FR900482 was assessed by exclusion of the dye Trypan blue (2%). Staining of cells by the blue dye was indicative of cell death. Throughout the 72 h period, most cells treated with 1.0 μM drug remained viable ($>90\%$) but were non-proliferating. In contrast, after 48 h cells that had been treated with either 1.5 or 2.0 μM concentrations of the drug showed significant decreases in viability ($\ll90\%$). These results indicate that at low concentrations of FR900482, cells remain alive but quiescent while at higher drug concentrations overt toxicity and death occurs.

Determination of the mode of cell death: necrosis versus apoptosis

We determined that cell death mediated by FR900482 was due to necrosis rather than apoptosis. Nuclear DNA was isolated from cells after 24 and 48 h of drug exposure and analyzed on a 2% agarose gel for DNA fragmentation into a nucleosome ladder that is characteristic of apoptosis. DNA fragmentation was also assessed using the commercial TUNEL assay. Neither of these assay procedures detected

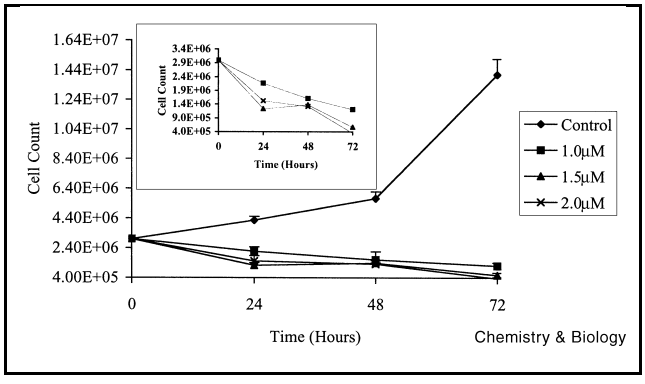

Figure 4. Cell growth inhibition caused by various concentrations of FR900482 over 0, 24, 48 and 72 h time periods, respectively. The inset shows a magnified view of the results from the drug-treated cells. Error bars are ±S.D.

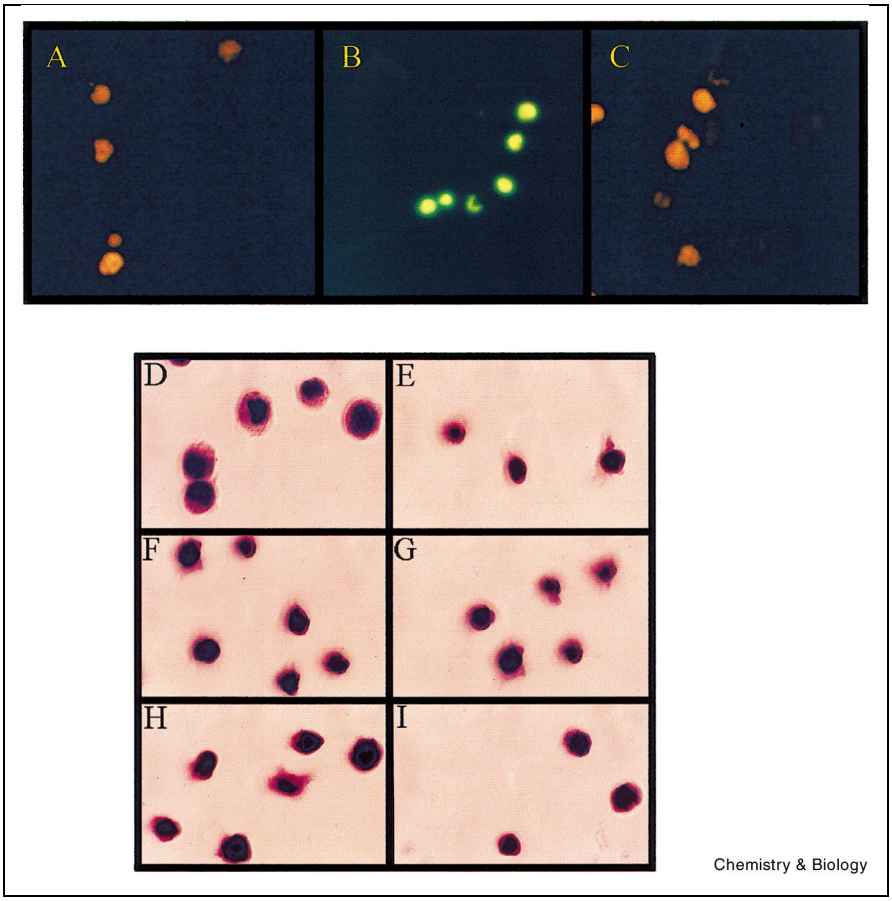

Figure 5. Histochemical staining of Jurkat cells analyzed by a fluorescence microscope. **A**: Control cells (20×); **B**: cells treated with anisomycin to induce DNA fragmentation as seen in apoptosis (20×); **C**: cells treated with 1.0 μM FR900482 for 24 h (20×). Histochemical staining of Jurkat cells using hematoxylin and eosin B. Cells were treated with 1 μM FR900482 for 0 (**D**), 6 (**E**), 12 (**F**), 24 (**G**), 36 (**H**) and 48 (**I**) h. The nucleus stained by hematoxylin is shown in dark red while the cytoplasm stained by eosin B is pink.

significant DNA fragmentation in cells exposed to FR900482 although treatment of the cells with the drug anisomycin, which is known to induce apoptosis, did result in significant DNA fragmentation (Figure 5). In addition, histochemical staining failed to detect gross nuclear fragmentation indicative of apoptosis but did show extensive cytoplasm loss and considerable nuclear condensation, characteristics of necrosis, in the drug-treated cells (Figure 5). We therefore conclude that the toxicity of FR900482 is likely the result of induction of necrotic cellular death.

HMG I/Y–drug–DNA cross-link formation in vivo

For cross-linking studies, Jurkat cells were treated for 6–24 h with 1.0 μM of FR900482 or with 1% formaldehyde for 60 min. The chromatin was subsequently isolated, sheared by sonication and the cross-linked HMG I/Y protein–DNA adducts reacted with a specific anti-HMG I/Y antibody. The resulting antibody–protein–DNA complexes were isolated on protein A Sepharose beads as previously described [31–33]. The protein A Sepharose beads non-covalently bind to the specific anti-HMG I/Y antibody, thus enabling the isolation of any potential cross-linked HMG I/Y protein–DNA adducts in the cells. In this modified chromatin immunoprecipitation (CHIP) procedure, fragments of DNA that have been covalently cross-linked by FR900482 to HMG I/Y proteins in vivo are thus tightly bound to the Sepharose beads whereas fragments of DNA not cross-linked to these proteins are eluted from the beads. This immunofractionation procedure efficiently differentiates the chromatin isolated from control cells (not treated with FR900482) from the chromatin isolated from the experimental drug-treated cells. Thus, the nuclear samples from control cells attached to the beads should be devoid of DNA fragments whereas the nuclear samples attached to the beads from the experimental cells should contain DNA fragments specifically cross-linked by the drug to the HMG I/Y proteins in vivo. The DNA fragments bound to the Sepharose beads from control and experimental drug-treated cells were isolated and screened for specific DNA sequences postulated to be bound in vivo by the HMG I/Y protein and were amplified by polymerase chain reaction (PCR).

The DNA sequences chosen for investigation were the highly repetitive, A·T-rich Alu sequences which are present in approximately 500 000 copies in the human genome [34,35] and the unique sequence promoter regions of the human IL-2 and IL-2Rα genes which bind, and are

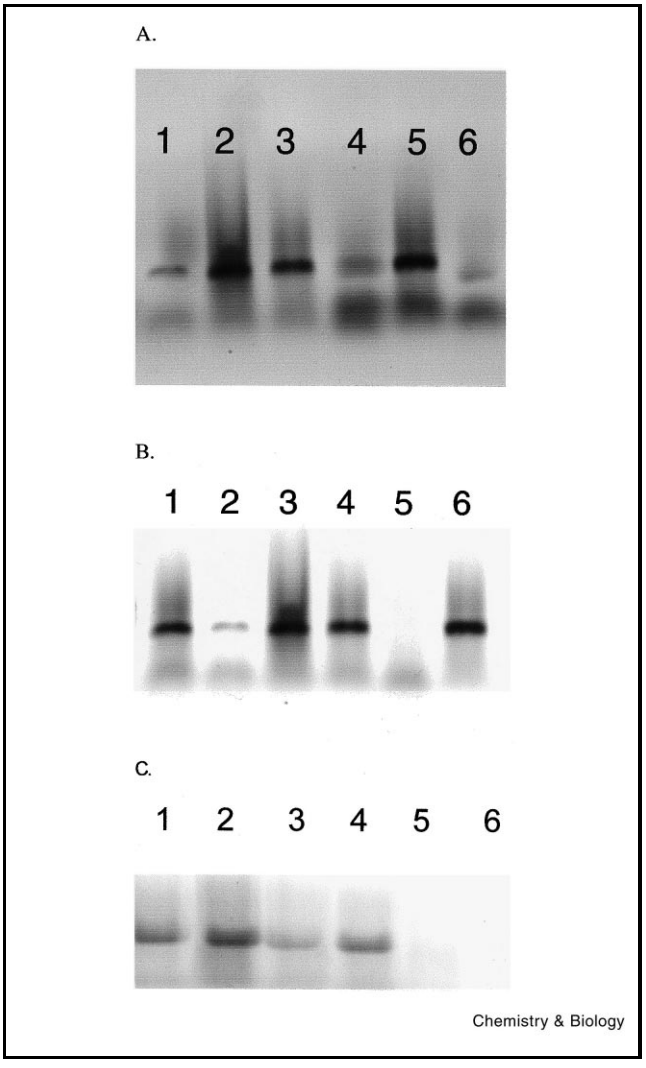

Figure 6. PCR of pellet and supernatant of immunoprecipitated DNA-protein crosslinks. In all panels, the PCR product is the upper band, while the lower band contains primer dimers. **A**: CHIP assay using HMG I/Y serum. PCR performed using primers designed for the IL-2 gene promoter [15]. Lanes **1–3** are PCR results from the supernatants and lanes **4–6** are PCR results from the immunoprecipitated pellets. Lanes **1** and **4** are cells treated with 1% formaldehyde; lanes **2** and **5** are cells treated with 1 μM FR900482; and lanes **3** and **6**, are the cells left untreated. **B**: CHIP assay using HMG I/Y serum. PCR was done using primers designed for a IL-2Rα gene promoter. Lanes **1–3** are PCR results from the supernatants and lanes **4–6** PCR results from the immunoprecipitated pellets. Lanes **1** and **4** are cells treated with 1 μM FR900482; lanes **2** and **5** are cells left untreated; and lanes **3** and **6** are cells treated with 1% formaldehyde. **C**: CHIP assay using Elf-1 antibodies. PCR performed using primers designed for the IL-2R α gene promoter. Lanes **1–3** are PCR results from the supernatants and lanes **4–6** PCR results from the immunoprecipitated pellets. Lanes **1** and **4** are cells treated with 1% formaldehyde; lanes **2** and **5** are cells treated with 1 μM FR900482; and lanes **3** and **6**, cells left untreated.

regulated by, the HMG I/Y in T lymphocytes in vivo [11–16,30].

PCR primers were designed to amplify DNA fragments of both the repetitive Alu repeat sequences and the unique IL-2 and IL-2Rα promoter sequences, all of which contain numerous A·T-rich, potential HMG I/Y binding sites. In the case of the IL-2 [30] and the IL-2Rα [15] promoters, the binding sites for HMG I/Y have been footprinted in vitro and are indicated by the bold, underlined letters in Figure 7. All three of these substrates (i.e. Alu, IL-2 and IL-2Rα) have several deoxyguanosine residues, which are well known potential drug monoalkylation sites [26–27,29], adjacent to one or more of the A·T-rich stretches, therefore providing potential protein–DNA cross-linking sites. It should be noted, however, that the 'exact' in vivo drug alkylation sites have not yet been characterized for any of these substrates.

Figure 6 shows the results of PCR amplifications of DNA fragments present in immunoprecipitates after 24 h of drug treatment using primers for the IL-2 promoter (Figure 6A) and the IL-2Rα promoter (Figure 6B). This figure illustrates the presence of amplified DNA products that resulted from the formation of the drug-induced HMG I/Y–DNA cross-links in the experimental, but not the control, cells. Similar results were obtained with the Alu primers (data not shown). The presence of these DNA fragments in the experimental cells is indicative of the formation of HMG I/Y–drug–DNA covalent cross-links in vivo during the period of drug exposure. As seen in Figure 6A,B, none of the control (i.e. non-drug treated) cells produced PCR products with anti-HMG I/Y, as expected. In

A.

TAATGTAACA AAGAGGGATT TCACCTACAT CCATTCAGTC AGTCTTTGGG
GGTTTAAAG**A** **AATT**CCAAAG AGTCATCAGA AGAGG**AAAAA** **T**GAAGGTAAT
GTTTTTTCAG ACAGGTAAAG TCTTCG**AAAA** **TATGT**GTAAT ATGTAAA**ACA**
TTTTGACACC CCC**ATAATAT** **TTTT**CCAG**AA** **TTAA**CAG**TAT** **AAATT**GCATC
TCTTGTTCAA GAGTTCCCTA TCACTCTCTT TAATCACTAC TCACAGTAAC
CTCAACTCCA

B.

CCAGCCCACA CCTCCAGC**AA** **TT**GAACTTG**A** **AAAAAAAA**CC TGGTTTG**AAA**
AATTACCGCA AAC**TATATTT** CATC**AAAAAA** **AAAAAAAAAA** **AA**CACTTCC**T**
ATATTTGAGA TGAGAGAAGA GAGTGCTACG CAGTTTCCTG GCTGAACACG
CCAGCCC**AAT** **ACTTAAA**GAG AGCAACTCCT GACTCCGATA GAGACTGGAT
GGACCCACAA GGGTGACAGC CCAGGCGGAC CGATCTTCCC ATCCCACATC
CTCCGGCGCG ATGCC**AAAAA** GAGG

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Figure 7. A: Sequence of the cross-linked product PCR amplified with primers designed for the IL-2 promoter (GenBank accession No. J006884). **B**: Sequence of the cross-linked product PCR amplified with primers designed for the IL-2Rα promoter (GenBank accession No. M15864). The bold, underlined letters indicate A·T-rich sequences footprinted in vitro by the HMG I/Y proteins on the IL-2 promoter [30] and the IL-2Rα promoter [15].

addition, control cells did not produce PCR products with preimmune sera (data not shown). Figure 6 also shows that all of the samples contained DNA, as PCR on the supernatants collected after the protein A Sepharose step did indeed, as expected, show the same size bands in all lanes. On the other hand, DNA fragments of the sizes predicted (~300 bp) for the promoter regions of the IL-2 and the IL-2Rα genes were obtained in the specific PCR amplification reactions employing anti-HMG I/Y (but not preimmune) immunoprecipitates obtained from the drug-treated experimental cells. These bands could be detected as soon as 6 h after addition of drug to the cells (data not shown). As a positive control, drug-free cells that had been fixed with the non-specific cross-linking agent formaldehyde also rendered similar PCR products (Figure 6). These results are clearly consistent with the in vivo covalent cross-linking of HMG I/Y proteins to the nuclear DNA of Jurkat cells by FR900482.

FR900482 cross-links other minor groove-binding proteins to DNA in vivo

Since it is known that FR900482 binds in the minor groove, it is then important to determine if FR900482 is specific for HMG I/Y or if it cross-links other minor groove-binding proteins as well. Immunoprecipitation experiments were therefore performed with two other minor groove-binding proteins, HMG-1 and HMG-2. Although HMG-1 and HMG-2 are also members of the high-mobility class of chromatin proteins they are unrelated structurally to the HMG I/Y proteins and exhibit no DNA sequence binding specificity (reviewed in [2]). HMG-1 monoclonal antibodies or HMG-2 serum were used in the CHIP assay and PCR was done using primers designed for the Alu repeat sequence. HMG-1 and HMG-2 were cross-linked by both formaldehyde and FR900482 to DNA (data not shown). These results show that FR900482 is not specific for HMG I/Y, but cross-links other minor groove-binding proteins of which there are only a few known members, such as HMG I/Y, HMG-1, HMG-2 and others. However, HMG I/Y is the only known minor groove-binding oncoprotein so FR900482's ability to cross-link this nuclear protein may represent a new mechanism of action for anti-tumor drugs.

FR900482 does not non-specifically cross-link proteins to the DNA major groove in vivo

Since both FR900482 and HMG I/Y are known to bind specifically in the minor groove of DNA, an important control for the modified CHIP assays described above is to demonstrate that the drug does not non-specifically cross-link proteins in the major groove. Immunoprecipitation experiments were therefore, repeated using antibodies against the T lymphocyte-specific protein Elf-1 which is present in Jurkat cells and binds to the major groove of A·T-rich sequences in the promoter of the IL-2Rα gene overlapping a site bound and regulated by the HMG I/Y protein in vivo [11–16]. Two independent immunoprecipitation experiments show that Elf-1 is not cross-linked to DNA with FR900482 (e.g. lane 5, Figure 6C) while, in control experiments, Elf-1 is cross-linked to the major groove of the IL-2Rα gene promoter by the non-specific reagent formaldehyde (lane 4, Figure 6C). In addition, antibodies against NF-κB (p50), a non-T lymphocyte restricted major groove-binding transcription factor that binds the promoter region of IL-2Rα, were used in the immunoprecipitation experiment. These experiments show that NF-κB is not cross-linked to the DNA by FR900482, but is cross-linked by formaldehyde (data not shown). These results demonstrate that FR900482 cross-linking of proteins to DNA has specificity for the minor groove.

Characterization of DNA cross-linked to HMG I/Y by FR900482 in vivo

The PCR amplified products from the anti-HMG I/Y immunoprecipitates isolated from drug-treated cells were excized from gels and subcloned into the pGEM T-Easy vector and sequenced. Figure 7 shows the sequences of the DNA obtained from these CHIP assays of drug-treated cells. A Blast search revealed that they were from the expected human IL-2 and IL-2Rα gene promoter regions.

Significance

In conclusion, we have illustrated that the FR class of antitumor antibiotics, represented in this study by FR900482, are able to produce covalent cross-links between the HMG I/Y proteins and DNA in vivo. The ability of these compounds to cross-link the HMG I/Y oncoproteins in the minor groove of DNA represents, to our knowledge, the first demonstration of drug-induced cross-linking of a specific cancer-related protein to DNA in living cells. These results do not exclude the potential in vivo cross-link formation with other minor groove-binding proteins that are not directly related to neoplastically transformed cells. Indeed, we have demonstrated that other minor groove-binding proteins such as HMG-1 and HMG-2 are also cross-linked in cells by this agent. However, the capacity of FR900482 to cross-link the HMG I/Y proteins, the only known minor groove-binding oncoproteins, with nuclear DNA in vivo represents a potentially novel mechanism that might contribute to the anti-tumor efficacy of these compounds by abrogating the functional integrity of these oncoproteins. These compounds have already been assessed as representing a compelling clinical replacement for mitomycin C due to their greatly reduced host toxicity and superior DNA interstrand cross-linking efficacy [23,24,36–38]. Based on these observations, it is reasonable to anticipate that other drugs that covalently cross-link DNA in the minor groove might also form covalent adducts with the HMG I/Y proteins. Efforts to investigate the generality of these lesions are under study in these laboratories and will be reported in due course.

Materials and methods

Human Jurkat T-leukemia cells (clone E6-1; American Type Culture Collection, Rockville, MD, USA) were grown in RPMI-1640 Media (Gibco-BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum, penicillin (614 μg/ml, Sigma, St. Louis, MO, USA), streptomycin (10 μg/ml, Sigma, St. Louis, MO, USA) and HEPES buffer, pH 7.2 at 37°C, 5% CO_2.

Determination of the mode of cell death

Jurkat cells (1×10^7 cells) were treated with anisomycin (2 μg/ml; Sigma, St. Louis, MO, USA) for 5 h, and FR900482 for 24 and 48 h at 37°C, 5% CO_2. To test for nucleosome fragmentation, the DNA was isolated and analyzed on a 2% agarose gel at 100 V for 90 min. For TUNEL assays, the kit Apoptosis Detection System, Fluorescein (Promega, Madison, WI, USA) was used. Briefly, Jurkat cells (3×10^7 cells) were spread on a poly-L-lysine slide (Sigma, St. Louis, MO, USA), the cells were washed in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. The cells were incubated with 50 μM fluorescein-12-dUTP, equilibration buffer (supplied with kit) and terminal deoxynucleotidyl transferase (TdT) for 1 h at 37°C. The reaction was stopped by washing the cells in 2× SSC followed by washes in PBS. The cells were counterstained using 1 mg/ml propidium iodine and washed in dH_2O. The cells were analyzed using a fluorescence microscope. Cells that displayed green fluorescence were indicative of DNA fragmentation due to labeling by the fluorescein-12-dUTP by TdT. Histochemical staining of cells was done using Gill's hematoxylin and counterstained in eosin B.

HMG I/Y–drug–DNA cross-link formation in vivo

Jurkat cells (3×10^6) were treated with either 1 μM FR900482 for 6, 12, 24, 36 and 48 h at 37°C, 5% CO_2 or 1% formaldehyde for 60 min at 4°C. Glycine (0.125 M) was added to the formaldehyde cross-linked cells to stop the cross-linking reaction. The nuclei from the treated and untreated Jurkat cells were isolated using a buffer containing 0.34 M sucrose, 0.2 M KCl, 100 mM EGTA, 500 mM EDTA, 494 mM spermine, 689 mM spermidine, 10 mM HEPES and 5% NP-40. The nuclei were washed in the same buffer without NP-40 and redissolved in TE buffer. The nuclei were fragmented by sonication for 10 min on ice. Following sonication, SDS (1%) was added, the DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitated. The DNA was subsequently redissolved in PBS and sonicated for a further 2 min on ice. Protease inhibitors (PMSF, 200 μg/μl; aprotinin, 60 μg/μl) were added along with HMG I/Y serum at a ratio of 50:1 or 100:1, HMG-1 monoclonal antibody (10:1), HMG-2 serum (50:1), Elf-1 (50:1 or 100:1, Santa Cruz Biotechnology) or NF-κB p50 (50:1 or 100:1 ratio, Santa Cruz Biotechnology). The protease inhibitors and either HMG I/Y (50:1) serum, HMG-1 monoclonal antibody, HMG-2 serum, Elf-1 antibody, NF-κB antibody or preimmune serum (50:1) were also added to untreated Jurkat cells. The reaction was allowed to proceed overnight at 4°C, after which protein A Sepharose beads were added (1:10 ratio) and the mixture was incubated for 90 min at 4°C. The DNA–protein cross-link was subsequently isolated by centrifugation and collection of the protein A Sepharose beads. The beads were resuspended in PBS and SDS (0.1%) and proteinase K (5 μg) was added. The samples were allowed to incubate for 30 min at 37°C and the samples were extracted with a phenol:chloroform:isoamyl alcohol (25:24:1) mixture. PCR primer sequences for human Alu sequences (accession number: U73024) are Alu1 (sense): 5′ GCCCATGCCCTGTTGAGCTT 3′ (bp 3982–4002) and Alu2 (anti-sense): 5′ GGCCTGTGAATTCAGGCCTA 3′ (bp 4423–4463). PCR primer sequences for the human IL-2 gene promoter (accession number: J006884) are IL-2-1 (sense): 5′ TAATGTAACAAAGAGGGATTTCACC 3′ (bp 193–217) and IL-2-2 (anti-sense): 5′ GGAGTTGAGGTTACTGTGAGTAGTC 3′ (bp 450–427). PCR primer sequences for the human IL-2Rα promoter (accession number: M15864) are IL-2Rα-1 5′ CCAGCCCACACCTCCAGCAA 3′ (bp 1052–1071) and IL-2Rα-2 5′ CCTCTTTTTGGCATCGCGCCG 3′ (bp 1306–1327). PCR conditions were 0.5 mM dNTPs, 2 μM primers, 4 mM $MgCl_2$ (for IL-2 and IL-2Rα primers) or 6 mM $MgCl_2$ (Alu primers), 100 mM Tris, pH 9.0, 50 mM KCl, 1% Triton X-100, 1 mM DTT. Taq polymerase was used with 30 cycles of 96°C for 30 s, 52°C for 30 s (Alu and IL-2 primers) or 60°C for 30 s (IL-2Rα) and 72°C for 45 s. The PCR samples were then run on a 1% agarose gel. For the major groove cross-link experiments involving Elf-1 and NF-κB, Jurkat cells (1×10^7) were treated with PMA (10 μg/ml) and ConA (10 ng/ml) for 6 h followed by the addition of FR900482 for 12 h or 1% formaldehyde for 1 h. The samples were then processed as for the reactions with HMG I/Y with the exception of using Elf-1 antibodies or NF-κB instead of the HMG I/Y serum.

Sequencing of PCR products

Gel slices were excized using GenElute columns (Sigma, St. Louis, MO, USA) and cloned using the T/A cloning kit and pGem-T-Easy vector (Promega, Madison, WI, USA). The resulting plasmid was transformed into Dh5α strain of *Escherichia coli* and selected on Luria broth (LB)–ampicillin–IPTG–X-Gal plates. Positive clones were grown overnight in LB–ampicillin and the plasmids were isolated. The sequencing of plasmids was done using a PE Applied Biosystems ABI Prism 377 DNA sequencer utilizing the Big Dye Terminator Cycle sequencing Kit (Perkin-Elmer, Norwalk, CT, USA).

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References

1. Grosschedl, R., Giese, K. & Pagel, J. (1994). HMG domain proteins: architectural elements in the assembly of nucleoprotein complexes. *Trends Genet.* **10**, 94–100.
2. Bustin, M. & Reeves, R. (1996). High-mobility-group chromosomal proteins: architectural components that facilitate chromatin function. *Prog. Nucleic Acid Res. Mol. Biol.* **54**, 35–100.
3. Bustin, M. (1999). Regulation of DNA-dependent activities by the functional motifs of the high-mobility-group chromosomal proteins. *Mol. Cell. Biol.* **19**, 5237–5246.
4. Johnson, K., Lehn, D., Elton, T., Barr, P. & Reeves, R. (1988). Complete murine cDNA sequence, genomic structure, and tissue expression of high mobility group protein HMG-I(Y). *J. Biol. Chem.* **263**, 18338–18342.
5. Johnson, K., Lehn, D. & Reeves, R. (1989). Alternative processing of mRNAs encoding mammalian 'high mobility group' proteins HMG-I and HMG-Y. *Mol. Cell. Biol.* **9**, 2114–2123.
6. Friedmann, M., Holth, L.T., Zoghbi, H. & Reeves, R. (1993). Organization, inducible-expression and chromosome localization of the human HMG-I(Y) nonhistone protein gene. *Nucleic Acids Res.* **21**, 4259–4267.
7. Chau, K.Y., Patel, U.A., Lee, K.L., Lam, H.Y. & Crane-Robinson, C. (1995). The gene for the human architectural transcription factor HMGI-C consists of five exons each coding for a distinct functional element. *Nucleic Acids Res.* **23**, 4262–4266.
8. Manfioletti, G., Rustighi, A., Mantovani, F., Goodwin, G.H. & Giancotti, V. (1995). Isolation and characterization of the gene coding for murine high-mobility-group protein HMGI-C. *Gene* **167**, 249–253.
9. Reeves, R. & Nissen, M.S. (1990). The A-T-DNA-binding domain of mammalian high mobility group-I chromosomal proteins: a novel peptide motif for recognizing DNA structure. *J. Biol. Chem.* **265**, 8573–8582.
10. Huth, J.R., Bewley, C.A., Nissen, M.S., Evans, J.N.S., Reeves, R., Gronenborn, A.M. & Clore, G.M. (1997). The solution structure of an HMG-I(Y)–DNA complex defines a new architectural minor groove binding motif. *Nat. Struct. Biol.* **4**, 657–665.
11. Fashena, S.J., Reeves, R. & Ruddle, N.H. (1992). A poly(dA-dT)

upstream activating sequence binds high- mobility group I protein and contributes to lymphotoxin (tumor necrosis factor-β) gene regulation. *Mol. Cell. Biol.* **2**, 894–903.
12. Thanos, D. & Maniatis, T. (1992). The high mobility group protein HMG I(Y) is required for NF-κB-dependent virus induction of the human IFN-βm gene. *Cell* **71**, 777–789.
13. Du, W., Thanos, D. & Maniatis, T. (1993). Mechanism of transcriptional synergism between distinct virus-inducible enhancer elements. *Cell* **74**, 887–898.
14. Thanos, D. & Maniatis, T. (1995). Virus induction of human IFN-β expression requires the assembly of an enhanceosome. *Cell* **83**, 1091–1100.
15. John, S., Reeves, R., Lin, J-X., Child, R., Leiden, J.M., Thompson, C.B. & Leonard, W.J. (1995). Regulation of cell type specific interleukin-2 receptor a chain gene expression: potential role of physical interactions between Elf-1, HMG-I(Y) and NF-κB family proteins. *Mol. Cell. Biol.* **15**, 1786–1796.
16. Himes, S.R., Coles, L.S. & Reeves, R. (1996). High-mobility-group protein I(Y) is required for function and for c-Rel but not Rel A binding to CD28 response elements within the GM-CSF and IL-2 promoters. *Immunity* **5**, 479–489.
17. Reeves, R. & Nissen, M.S. (1995). Cell cycle regulation and functions of HMG-I(Y). *Prog. Cell Cycle Res.* **1**, 339–349.
18. Wunderlich, V. & Bottger, M. (1997). High-mobility-group proteins and cancer-an emerging link. *J. Cancer Res. Clin. Oncol.* **123**, 133–140.
19. Hess, J.L. (1998). Chromosomal translocations in benign tumors: the HMG I proteins. *Am. J. Clin. Pathol.* **109**, 251–261.
20. Tallini, G. & Dal Cin, P. (1999). HMG I(Y) and HMG I-C dysregulation: a common occurrence in human tumors. *Adv. Anat. Pathol.* **6**, 237–246.
21. Williams, R.M. & Rajski, S.R. (1993). Determination of DNA cross-linking sequence specificity of FR66979: observations on the mode of action of the FR900482 class of anti-tumor compounds. *Tetrahedron Lett.* **34**, 7023–7026.
22. Huang, H., Pratum, T.K. & Hopkins, P.B. (1995). Covalent structure of the DNA–DNA interstrand cross-link formed by reductively activated FR66979 in synthetic DNA duplexes. *J. Am. Chem. Soc.* **116**, 2703–2709.
23. Williams, R.M., Rajski, S.R. & Rollins, S.B. (1997). FR900482, a close cousin of mitomycin C that exploits mitosene-based cross-linking. *Chem. Biol.* **4**, 127–137.
24. Denny, W.A. (1995). Hypoxia-selective cytotoxins. In *Cancer Chemotherapeutic Agents* (Foye, W.O., ed.), Chap. 14, pp. 483–500, ACS Press, Washington, DC.
25. Fukuyama, T. & Goto, S. (1989). Synthetic approaches toward FR900482. I. Stereoselective synthesis of a pentacyclic model compound. *Tetrahedron Lett.* **30**, 6491–6494.
26. Paz, M.M. & Hopkins, P.B. (1997). DNA–DNA interstrand cross-linking by FR66979 and FR900482: requirement of metal ions during reductive activation. *Tetrahedron Lett.* **38**, 343–346.
27. Paz, M.M. & Hopkins, P.B. (1997). DNA–DNA interstrand cross-linking by FR66979: intermediates in the activation cascade. *J. Am. Chem. Soc.* **119**, 5999–6005.
28. Tomasz, M. (1995). Mitomycin C: small, fast and deadly (but very selective). *Chem. Biol.* **2**, 575–579.
29. Williams, R.M. & Rajski, S.R. (1998). FR66979 covalently cross-links the binding domain (BD) of the high mobility group I/Y (HMG I/Y) proteins to DNA. *J. Am. Chem. Soc.* **120**, 2192–2193.
30. Himes, S.R., Reeves, R., Attema, J., Nissen, M., Li, Y. & Shannon, M.F. (2000). The role of high-mobility group I(Y) proteins in expression of IL-2 and T cell proliferation. *J. Immunol.* **164**, 3157–3168.
31. Hecht, A. & Grustein, M. (1999). Mapping DNA interaction sites of chromosomal proteins using immunoprecipitation and polymerase chain reaction. *Methods Enzymol.* **304**, 399–414.
32. Orlando, V., Strutt, H. & Paro, R. (1997). Analysis of chromatin structure by in vivo formaldehyde cross-linking. *Methods: Companion Methods Enzymol.* **11**, 205–214.
33. Reeves, R. & Nissen, M. (1999). Purification and assays for high mobility group HMG-I(Y) protein function. *Methods Enzymol.* **304**, 155–188.
34. Deininger, P.L. & Batzer, M.A. (1999). Alu repeats and human disease. *Mol. Gene. Met.* **67**, 183–193.
35. Batzer, M.A., Stoneking, M., Alegria-Hartman, M., Bazan, H., Kass, D.H., Shaikh, T.H., Novick, G.E., Ioannou, P.A., Scheer, W.D., Herrera, R.J. & Deininger, P.L. (1994). African origin of human specific polymorphic Alu insertions. *Proc. Natl. Acad. Sci. USA* **91**, 12288–12292.
36. Nakamura, T., Masuda, K., Matsumoto, S., Oku, T., Manda, T., Mori, J. & Shimomura, K. (1989). Effect of FK973, a new anti-tumor antibiotic, on the cell cycle of L1210 cells in vitro. *Jpn. J. Pharmacol.* **49**, 317–324.
37. Hirai, O., Shimomura, K., Mizota, T., Matsumoto, S. & Mori, J. (1987). A new anti-tumor antibiotic, FR900482. IV. Hematological toxicity in mice. *J. Antibiot.* **40**, 607–611.
38. Hismomura, K., Hirai, O., Mizota, T., Matsumoto, S., Mori, J., Shibayama, F. & Kikuchi, H. (1987). A new anti-tumor antibiotic, FR900482 III. Anti-tumor activity in transplantable experimental tumors. *J. Antibiot.* **40**, 600–606.