FR900482, a close cousin of mitomycin C that exploits mitosene-based DNA cross-linking

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Background: The class of antitumor antibiotics that includes FR900482 has a very close structural analogy to the mitomycins, one of which, mitomycin C, has been in widespread clinical use for more than 20 years. Like mitomycin C, these antitumor antibiotics are reductively activated *in vivo* and covalently cross-link DNA as a result of activity of the mitosene moiety generated on reduction. Owing to differences in structure and the attendant mechanistic differences in bioreductive activation between the mitomycins and FR900482, FR900482 does not produce an adventitious superoxide radical anion during reductive activation and thus does not exhibit oxidative strand scission of DNA. It is postulated that the low clinical toxicity of FR900482 relative to mitomycin C is a direct manifestation of the mechanistic differences of bioreductive activation leading to the highly reactive DNA cross-linking mitosenes.

Results: Using Fe(II)–EDTA footprinting, we showed that the two natural products FR900482 (**1**) and dihydro, FR66979 (**3**), and the semi-synthetically derived triacetate FK973 (**2**), display remarkable selectivity for 5′ deoxy-CG sequences of DNA, and that this selectivity is abolished upon deletion of the exocyclic N2 amine of either participating guanosine residue. In addition, we investigated the mono alkylation abilities of FR66979 with respect to a number of inosine-substituted oligonucleotides and observed that the FR900482 class of compounds were able to give rise to easily separable orientation isomers of their respective cross-links.

Conclusions: The FR900482 class of antitumor antibiotics cross-link DNA in a fashion analogous to the mitomycins. The cross-linking reaction yields two orientation isomers which are of vastly different electrophoretic mobility and which also exhibit radically different DNA–protein recognition properties upon reaction with *Alu*I restriction endonuclease. In addition, mono-alkylation of DNA by FR66979 shows little, if any, dependence upon pre-covalent interactions deemed necessary for the mitomycins. These insights support the proposal that the FR900482 class of compounds represents a compelling clinical replacement for mitomycin C, given its greatly reduced host toxicity and superior DNA interstrand cross-linking efficacy.

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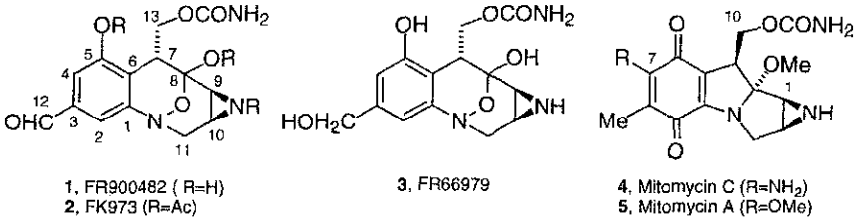
Introduction

The bioreductive alkylating agents are a very important class of clinical agents in that they can become selectively activated upon delivery to hypoxic environments such as those of cancerous tissues [1]. The recently discovered antitumor antibiotics FR900482 (**1**) and FR66979 (**3**) (Fig. 1), a dihydro congener (obtained from the fermentation harvest of *Streptomyces sandaensis* No. 6897 at Fujisawa Pharmaceutical Co. Ltd, Japan), bear close structural similarity to the clinically employed bioreductive alkylating agent mitomycin C (MC, **4**, Fig. 1) [2–5]. Like many antitumor antibiotics, MC exerts multiple biological effects in cells, including selective inhibition of DNA synthesis, chromosome breakage, DNA mutagenesis, stimulation of genetic recombination, as well as mediating non-specific oxidative damage to cellular components. The interaction of MC with cellular nucleic acids has been the subject of intensive investigation for many years. It is now well-established that MC is reductively activated *in vivo* and *in vitro* to provide a highly reactive, bis-electrophilic species called mitosene, which cross-links DNA preferentially at 5′–3′ CG sites [6]. During the single-electron reductive activation pathway that has been implicated, the quinone moiety of MC is initially reduced to a semi-quinone radical anion. This can then either accept a second electron, and proceed to mitosene, or revert back to MC by electron transfer to molecular oxygen, generating a superoxide radical anion [7]. Subsequent Haber–Weiss/Fenton cycling in the presence of trace transition metals produces hydroxyl radical and other related reactive oxidants capable of causing non-selective tissue damage.

Figure 1



The structures of mitomycin C, FR900482 and related molecules (see text for details).

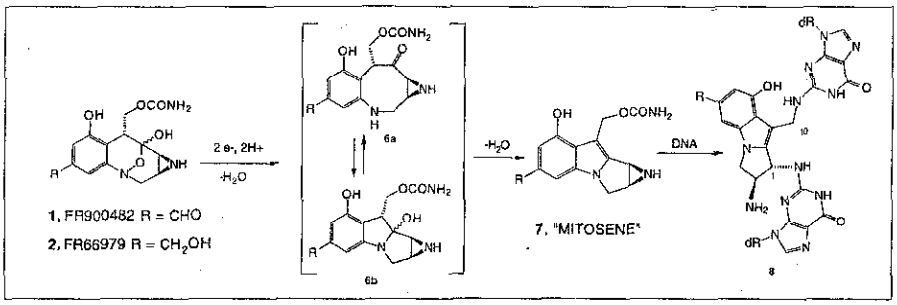
In preliminary studies, it was shown that FR900482 and the derived triacetate, FK973 (**2**), are approximately threefold more potent than MC and display significantly lower toxicity ([8–10] and Fujisawa Pharmaceutical Co. Ltd, personal communication). FK973 has recently been shown to form DNA–DNA interstrand cross-links and DNA–protein cross-links in L1210 cells [4,5]. Like MC, compounds **1–3** also require reductive activation both *in vivo* and *in vitro* [11,12]. Compounds **1–3** do not produce the random single-strand breaks in DNA [4,5,7] that are typically associated with redox cycling of reactive oxygen radical species. The low toxicity of the Fujisawa drugs relative to MC may thus be a manifestation of differences in the chemical means by which the drugs are activated. Both FK973 and MC can cross-link DNA [11–13] through the agency of a bioreductively generated mitosene. The major structural difference between MC and FK973 is the presence of the quinone in MC and the hydroxylamine hemi-ketal of the Fujisawa drugs. In MC, the quinone functionality [4,5] is an essential functional array for the reductive unmasking of the potent, bis-electrophilic mitosene [6]. In the Fujisawa drugs, it appears that the reductively labile functionality resides in the N–O bond of the unique hydroxylamine hemi-ketal. Given the potential clinical promise of the Fujisawa drugs, coupled with their unusual structures and their relationship to MC, a great deal of effort has recently been directed towards the synthesis of the natural products as well as preliminary mechanistic studies concerning the biological mode of action of these secondary metabolites. In this paper, we report on the interaction of FR900482 and FR66979 with DNA and point out the mechanistic similarities and differences between MC and FR900482, which may be useful in designing new classes of more selective and less toxic antitumor agents.

Results and discussion

Fukuyama *et al.* [14] were the first to propose reductive activation of FR900482 *en route* to DNA cross-linking (Fig. 2), in the form of a preliminary synthetic approach [15] to these substances [16,17]. Reductive cleavage of the N–O bond was proposed to generate aniline (**6a** and **6b**) which cyclizes and dehydrates to aziridinomitosene (**7**). Related work [6] on structurally similar mitosenes generated from MC provided a precedent that the interaction of **7** with DNA would yield cross-linked DNA species (**8**). If this proposal proved correct, the 5′C–phosphodiester–G cross-linking sequence specificity for mitosenes derived from MC was also anticipated for the FR900482-generated mitosenes. The 5′CpG cross-linking sequence specificity of mitosenes is based upon a good geometrical fit of the intramolecular distance between the two electrophilic centers of the mitosene (3.4 Å) and the distances between the two cross-linked bases of the duplex substrate (3.1 Å for 5′CpG, Fig. 3).

The dihydro derivative FR66979 was studied because early efforts in our laboratories [18,19] demonstrated that,

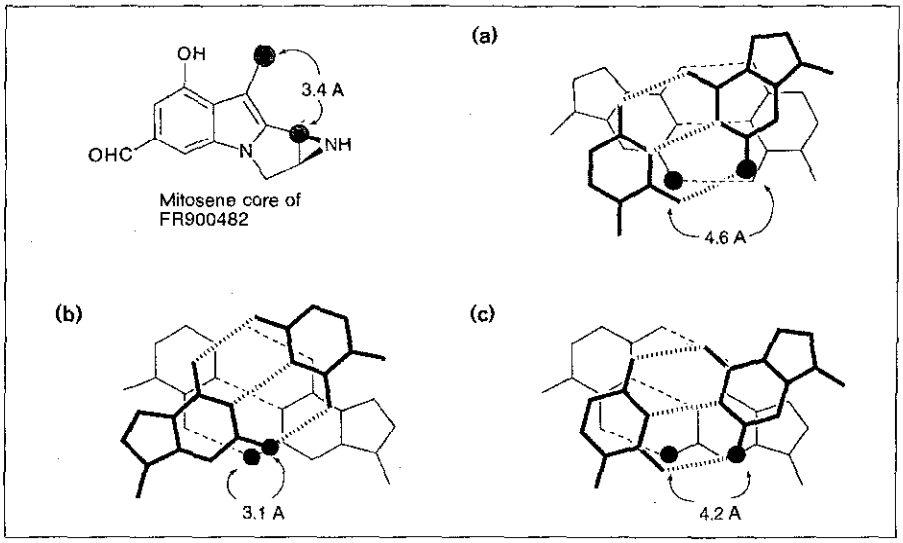
Figure 2



The proposed mechanism of action for FR900482 and FR66979 in DNA cross-linking.

Figure 3

Molecular distances of adjacent guanosine exocyclic amines in sequences of interest: **(a)** 5′GpC, **(b)** 5′CpG and **(c)** 5′GpG.



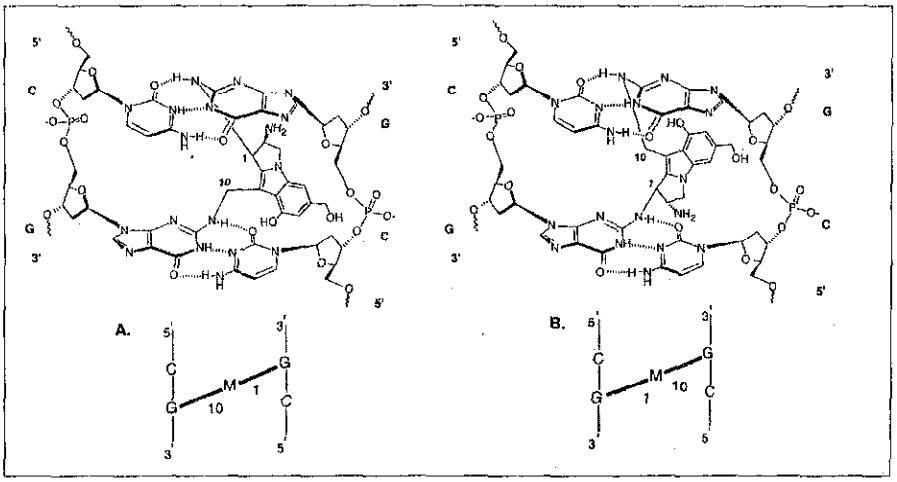
of the two natural products, FR66979 was by far the more efficient DNA interstrand cross-linking agent. At the same time, Hopkins, Woo, Sigurdsson and colleagues [20,21] were investigating the ability of FR900482 and FR66979 to cross-link various self-complementary DNA substrates. Both laboratories concluded that the FR900482 class of compounds displayed a strikingly similar specificity for 5′CpG, as previously shown for MC [6,22]. Collaborative efforts [11] ultimately demonstrated that both FR900482 and FR66979 required reductive activation to cross-link DNA; the DNA-drug lesion from both antibiotics has been fully characterized [20,21]. We have since demonstrated that the natural products FR900482 and FR66979, as well as FK973, display sequence selectivity for 5′CpG sequences, in which a number of guanosine residues preside (unpublished observations). In addition, mono-alkylation of DNA by FR66979 appears to require similar, yet distinct, 'pre-covalent' associations that are thought to be necessary for efficient substrate alkylation (typical yields ≥10%) by MC [22–25], and both FR900482 and FR66979 give rise to separable orientation isomers of the respective interstrand cross-links (Fig. 4).

Fe(ii)-EDTA footprinting of FR900482-, FR66979- and FK973-mediated cross-links

In the absence of exogenous reducing agents and metal ions [12], FR900482 and FR66979 do not cross-link DNA. FR66979 can be synthesized most conveniently by either $NaBH_4$ reduction or catalytic hydrogenation of FR900482. The reactivity of chemically 'over-reduced' FR66979

Figure 4

Orientation isomers of cross-linked DNA. M refers to the mitosene core derived from FR66979. The numbers 1 and 10 refer to carbons 1 and 10 of the DNA-bound mitosene core. A is the 'slow mobility' isomer and B the faster mobility isomer.



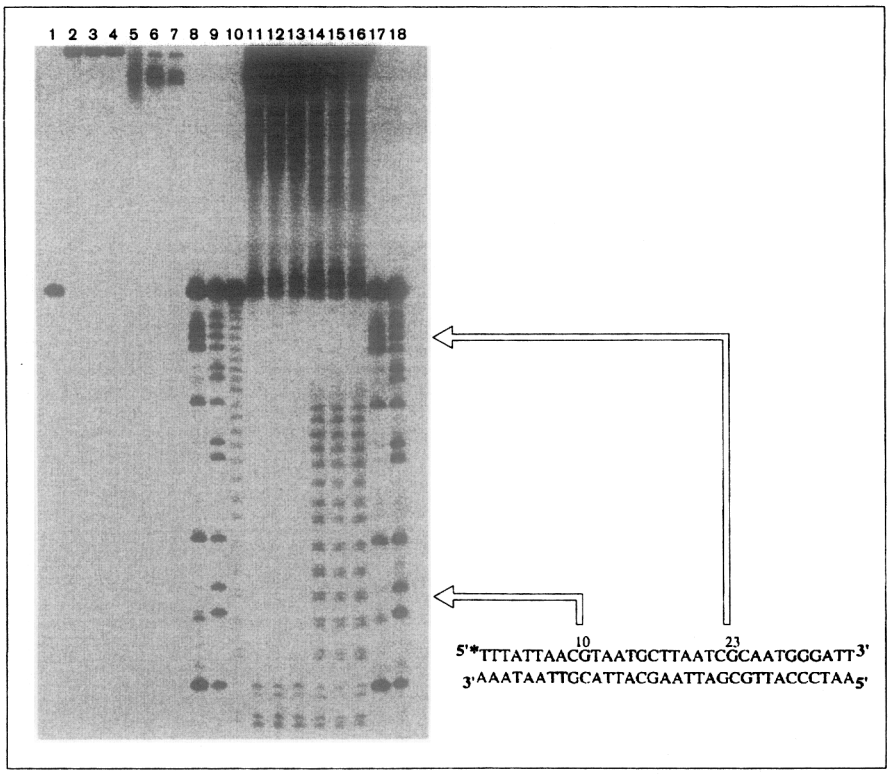
proved to be highly specific for 5′CpG, providing provocative evidence for the production of trace amounts of a reactive mitosene. Although the isolation of such an intermediate (**7**) or the carbinolamine (**6**) has thus far been elusive, it was deemed necessary to investigate the specificity of purified compounds under reducing conditions. Using various synthetic DNA substrates, cross-linking and Fe(II)–EDTA footprinting were used to secure both the sequence specificity and the regio-specificity of the DNA-alkylation processes.

The substrate, 5′TTTATTAACGTAATGCTTAATCGCAATGGGATT3′ **(template 1)**, and a modified version bearing inosine (guanosine lacking the N2 exocyclic amine) at the G10 position **(template 2)**, and their mutual complement, were synthesized. Cross-linking of both substrates followed by isolation and Fe(II)–EDTA digestion rendered the cleavage patterns in lanes 11–17 of Figure 5. Native DNA subjected to Fe(II)–EDTA cleavage yields an equimolar assortment of all fragment sizes up to and including the full-length strand (lane 10, Fig. 5), whereas analogous treatment of cross-linked DNA yields short fragments corresponding to cleavage at or to the radiolabeled side of the alkylated residue. As such, the observed cleavage patterns show that, for cross-linking of the unsubstituted duplex, G10 is the principal site of alkylation (lanes 11–13, Fig. 5), whereas cross-linking and digestion of the duplex bearing inosine at the G10 position reveals G23 as the primary alkylation site (lanes 14–16, Fig. 5). This 'cleavage shift' is the result of deleting the exocyclic amine (N2) at the G10 residue. Substituting with inosine at this position halts cross-link formation, and thus implicates the N2 residues of adjacent guanosines (within only the 5′CpG box) exposed in the minor groove as the sole reactive groups responsible for interstrand cross-linking by compounds **1–3**. This conclusion is in agreement with the reasonable expectation that the interaction of **1–3** with DNA should occur in the minor groove of DNA, and it supports the hypothesis that FR900482, FR66979 and FK973 cross-link DNA via the same mechanism.

Orientation isomerism of the FR66979-mediated 5′ deoxy CG cross-link

The 5′ ^{32}P end-labeled substrate 5′TGTTGAATACTCATACGTCTCTTGCTGAGGG3′ **(template A)** was synthesized and annealed to its complement. Cross-linking of this substrate by FR66979 revealed the presence of two bands corresponding to cross-linked product with only one 5′CpG site. One possibility was that over-mono-alkylation gave rise to the slower band. Of particular concern was the GAGGG run at the 3′ terminus. By analogy to MC, this sequence posed problems entailing not only possible mono-alkylation, but also intrastrand bis-alkylation [22–25]. The presence of another interstrand site was considered

Figure 5



Autoradiogram of Fe(II)–EDTA footprinting of cross-linked 5′ labeled 5′TTTATTAA**CG**TAATGCTTAAT**CG**CAATGGGATT 3′ and 5′TTTATTAA**CI**TAATGCTTAAT**CG**CAATGGGATT 3′ to their corresponding complement (resulting duplexes are **templates 1** and **2**, respectively). Lane 1 is standard 5′-labeled **template 1**. Lanes 2, 3 and 4 are **template 1** cross-linked with FR900482 (**1**), FR66979 (**3**) and FK973 (**2**), respectively. Lanes 5, 6 and 7 are **template 2** cross-linked with **1**, **3** and **2**, respectively. Lanes 8, 9 and 10 are Maxam–Gilbert G, G+A, and 1 mM Fe(II)– EDTA lanes, respectively. Lanes 11, 12 and 13 are **template 1** cross-linked by **1**, **3** and **2**, respectively, followed by Fe(II)–EDTA digestion. Lanes 14, 15 and 16 are **template 2** cross-linked by **1**, **3** and **2**, respectively, followed by Fe(II)–EDTA digestion. Lanes 17 and 18 are Maxam–Gilbert G and G+A lanes, respectively.

highly unlikely, but, on the basis of available data, could not be ruled out. To probe these issues, two additional oligonucleotides were synthesized. One substrate replaced the GAGGG3′ run with the sequence TTAAT3′ (**template B**), while the other substrate had the cross-linkable 5′CpG site replaced with 5′TpT (**template C**). As a control, the self-complementary oligonucleotide 5′TATTAAAATTA-ATACGTATTAATTTTAATA3′ was synthesized. Treatment of the duplexes made up of **templates A** and **B** with FR66979 and DTT revealed the two band pattern not only for the known **template A**, but also for the substrate lacking the deoxy-G (dG) rich 3′ terminus (**template B**).

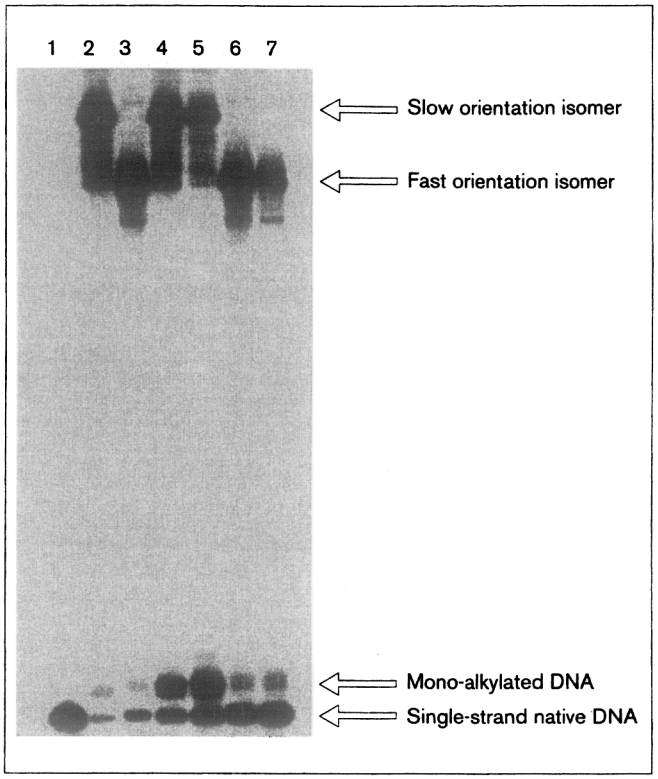
As expected, the substrate lacking the 5′CpG site (**template C**) gave no reaction, thus ruling out the possibility of another interstrand cross-linkable site, and the self-complementary substrate yielded only one band corresponding to a cross-linked species. Retention of the two-band pattern between substrates with and without the GAGGG3′ terminus suggested that the observed two-band pattern was not due to over-alkylation, but rather to the separation of orientation isomers. Each cross-linked species was isolated and subjected to electrospray mass spectral analysis. The slow orientation isomer had an observed molecular weight of 19261.39±10.78 Da; the faster orientation isomer had an observed molecular weight of 19259.73±9.36 Da (calculated molecular weight = 19259.75 Da). Following isolation, each orientation isomer was subjected to Fe(II)–EDTA digestion, which revealed that each band corresponded to the singly cross-linked species. The presence of the cross-link in the minor groove was verified by digesting each orientation isomer with piperidine; major base-labile (major groove) lesions were not revealed to be involved on either strand of the cross-linked duplex.

Assignment of connectivity for FR66979 orientation isomers

Efforts to determine whether one orientation isomer of cross-linked **template A** could revert to the other orientation isomer, or to a mixture of both isomers, were carried out by separating the two products and subsequently exposing each to denaturing conditions. Analysis by 20% denaturing polyacrylamide gel electrophoresis (DPAGE) revealed that heat denaturation did not generate the other orientation isomer but instead yielded hydrolysis products. Cross-linked adducts in which **template A** was 5′ labeled and its complement was unlabeled were purified, then subjected to heating at 80°C in 200 mM Tris buffer (pH = 7.5) for 20 min and then cooled to room temperature.

As depicted in lanes 4–7 of Figure 6, it is clear that upon heating the slow orientation isomer, low molecular weight DNA was produced corresponding to native single stranded DNA (ssDNA), along with a large proportion corresponding to mono-alkylated DNA. In contrast, upon heating the faster orientation isomer, only a tiny fraction of mono-alkylated DNA was produced, the majority being ssDNA (5′ labeled **template A**).

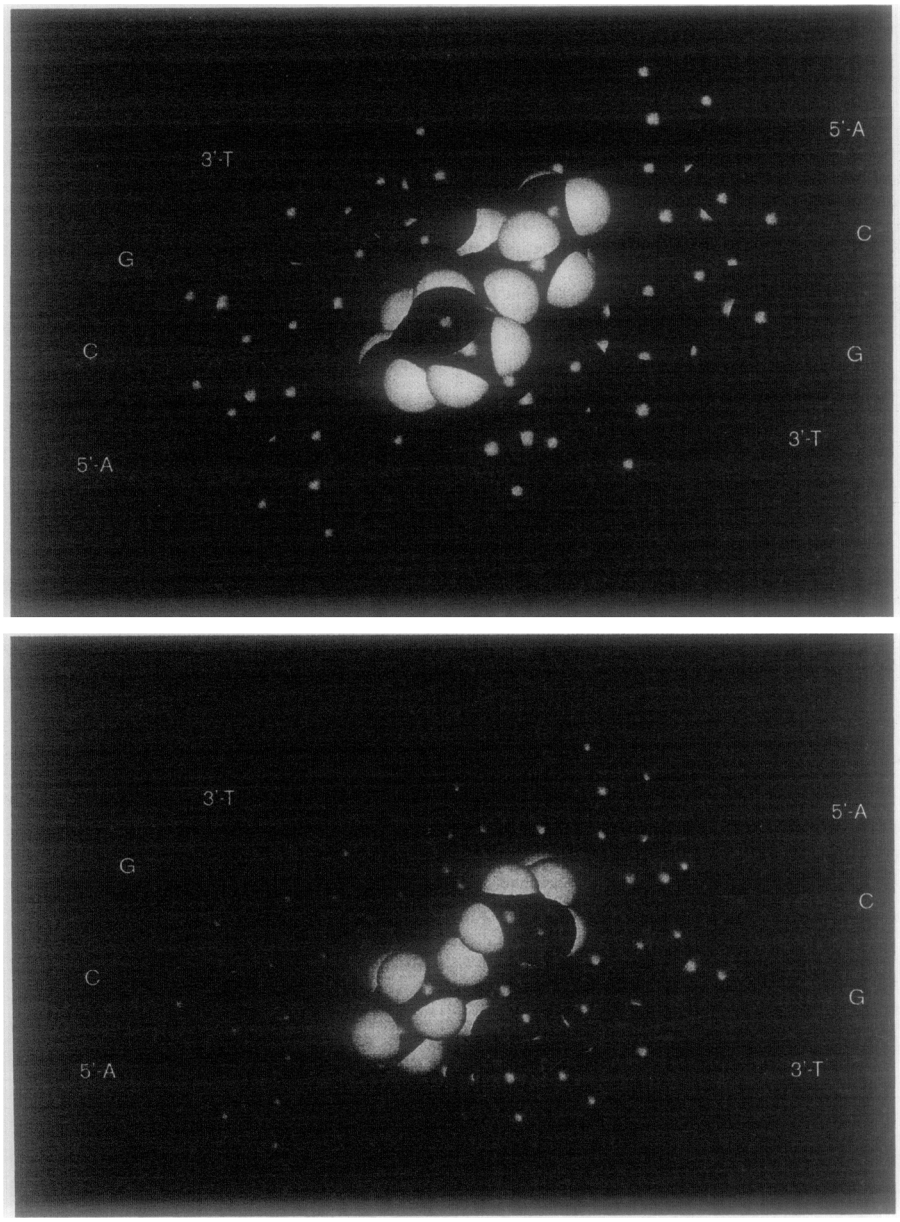
Figure 6



Analysis by 20% DPAGE of sequential denaturing/renaturation reactions using orientation isomers of 5′ ^{32}P labeled 5′ TGTTGAAT-ACTCATACGTCTCTTGCTGAGGG 3′ cross-linked with FR-66979. Lane 1, standard duplex; lane 2, slow orientation isomer of cross-linked substrate standard; lane 3, fast orientation isomer standard; lane 4, the product of heat denaturation 80°C for 20 min followed by cooling to 25°C for 2 h for the slow orientation isomer and lane 5, the same sample as lane 4 following two iterations of the same heat treatment. Lanes 6 and 7 are analogous to lanes 4 and 5 as applied to the fast orientation isomer of the FR66979 cross-linked material.

The reversibility of alkylation of mitosenes derived from MC is more easily achieved (under reductive conditions) at C1 than at C10 [26,27]; this suggested that the slow orientation isomer of the FR66979 cross-link is that corresponding to alkylation in which radiolabeled 5′TGT-TGAATACTCATACGTCTCTTGCTGAGGG3′ is connected to the C10 of the mitosene core of FR66979. Likewise, the faster of the two orientation isomers results from the connection of this strand at C1, which is more labile towards hydrolytic DNA release [27]. To provide confirmation and corroboration of this result, 5′-radiolabeling of the complementary strand to **template A** was performed and the assay repeated. This verified that the faster of the two FR66979 derived orientation isomers results from connection of **template A** to the mitosene C1 (see

Figure 7

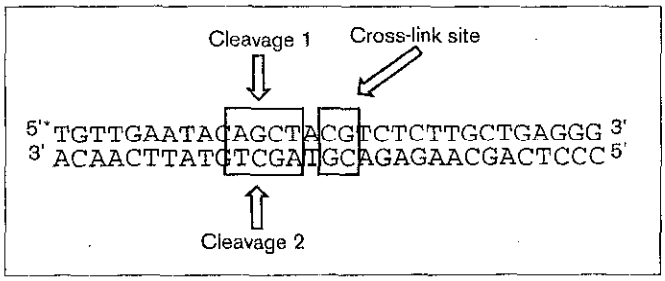


Orientation isomers of FR66979 cross-linked 5′ d(GACGTA)$_2$ (only the 5′d(ACGT)$_2$ is shown). The strands on the right for each duplex are those which were 5′ radiolabeled and had the 5′ AGCT *AluI* recognition site to the 5′ side of the bis-alkylated 5′ d(CG) box. **(a)** Space-filling model of 'slow' orientation isomer. **(b)** Space-filling model of 'fast' orientation isomer. Molecular mechanics were carried out using the cvff force-field in the molecular modeling package INSIGHT II v. 2.3.5 (BIOSYM/Molecular Simulations, Inc.). The double-stranded hexamer was constructed using the Biopolymer program. Once the mitosene was bonded to the duplex, 500 steps of energy minimization using the conjugate gradient algorithm were carried out by fixing the DNA strands and allowing the cross-linked drug to reach an energy minimum.

Supplementary material). These experiments allow the relative connectivity of the mitosene core to be assigned in each orientation isomer. It is likely that this behavior can be correlated with the orientation isomer's melting temperature (T_m) for the cross-linked substrate. Orientation isomers of 4,5,8-trimethylpsoralen cross-linked oligonucleotides have been shown to be easily separable, due to base-pair disruptions in the vicinity of the cross-link [28]. The fact that we have observed an analogous difference in migration between the two orientation isomers derived from compound **3**, as well as from compounds **1** and **2** (data not shown for **1** and **2**), suggests that the cross-link formed results in one orientation giving rise to the fully base-paired cross-link while the other orientation isomer compromises the Watson–Crick base-pairing network, thus resulting in the mobility difference observed in Figure 6.

Cross-linking of a self-complementary substrate with FR66979 resulted in only one band by DPAGE. This is due to the presence of a C_2 axis of symmetry throughout the cross-linked adduct. In cases giving rise to separable cross-linked products, however, the DNA substrates are asymmetric with respect to the bases flanking 5′dCpG. Thus, the two orientation isomers are not chemically equivalent. Also noteworthy is the finding that cross-linking of **template A** with reductively activated MC gave

Figure 8



Template A modified with 5′AGCT *Alu*I cleavage site 1 base pair upstream of the cross-linkable 5′ACGT.

rise to separable orientation isomers of similar electrophoretic mobilities to those seen with the FR-derived cross-links (data not shown). It is possible that perturbation of the cross-linked DNA derives from a functionality common to both the mitomycins and the FR900482 class antibiotics; this is likely to be the C2 primary amine derived from the aziridine. Molecular modeling of both orientation isomers indicates that, in each isomer, an N2 hydrogen of the FR66979-derived mitosene core is within hydrogen-bonding distance of the C2 carbonyl oxygen of the thymine that is on the 3′ side of the alkylated guanosine residue (Fig. 7).

Reaction of orientation isomers of the FR66979 cross-link with *Alu*I restriction endonuclease

The ability of the Fujisawa drugs to yield different cross-linked species at the same site with the same connectivity raises numerous questions regarding the physical basis for the observed electrophoretic differences. An issue of particular significance is the possibility that the observed orientation isomers may exert unique effects on processes of biological importance. The well-defined structural constraints of numerous protein–DNA recognition motifs suggested that such interactions might easily be affected by the DNA distortions induced by one or both orientation isomers. An initial probe of this was performed using the restriction endonuclease *Alu*I, which recognizes the palindrome 5′AGCT3′ and cleaves both strands of the duplex through the central G–C phosphodiester linkage (Fig. 8).

Given the ease with which FR66979-derived cross-link orientation isomers of 5′TGTTGAATACTCATACGTCTC-TTGCTGAGGG3′ could be separated, we chose to follow the protocol described by Tomasz and coworkers [29] in which an *Alu*I site was incorporated one base upstream (to the 5′-side) of the cross-linkable 5′ACGT3′ run [28]. Substrate alkylation and purification of the resulting orientation isomers, followed by incubations of each isomer and of a standard duplex control, with 4 units of *Alu*I for various times gave rise to the autoradiogram shown in Figure 9. Each deoxyoligonucleotide duplex was 5′ labeled on the sequence detailed in the legend of Figure 9.

The faster mobility orientation isomer inhibits *Alu*I cleavage of the radiolabeled strand, while the other orientation isomer shows very little inhibition when compared to the standard duplex. This observation is consistent with related findings by Tomasz (pertaining to MC) which invoked one orientation isomer in orienting the bulky aromatic ring toward the *Alu*I cleavage site, thus inhibiting strand cleavage by the protein, while the other orientation isomer has the aromatic ring oriented away from the *Alu*I site, thus exerting minimal influence on the degree of *Alu*I cleavage.

These observations, taken in conjunction with the reversibility studies, indicate that the faster of the two orientation isomers is that in which C1 of the FR66979-derived mitosene core is covalently attached to the radiolabeled strand 5′TGTTGAATACAGCTACGTCT-CTTGCTGAGGG3′. Conversely, the slower orientation isomer requires C10 connection of the mitosene to the radiolabeled strand. Interestingly, we found that cleavage 1 (Fig. 8) is selectively inhibited while cleavage 2 proceeds at about the same rate for each orientation isomer. This is in contrast to the findings of Tomasz and

Figure 9

Analysis by 20% DPAGE of restriction fragments obtained from treatment of cross-link orientation isomers of double-stranded 5′ TGT-TGAATACAGCTACGTCTCTTGCTGAGG-G3′ with *Alu*I restriction endonuclease (4 units). Lane 1 standard duplex; lane 2, slow orientation isomer; and lane 3, fast orientation isomer. Lanes 4–8 standard duplex treated with 4 units of *Alu*I at 1, 2, 5, 10 and 30 min, respectively. Lanes 9–13 contain the slower FR66979 orientation isomer treated with 4 units of *Alu*I at 1, 2, 5, 10 and 30 min and lanes 14–18 contain the faster orientation isomer treated analogously to the slower cross-link.

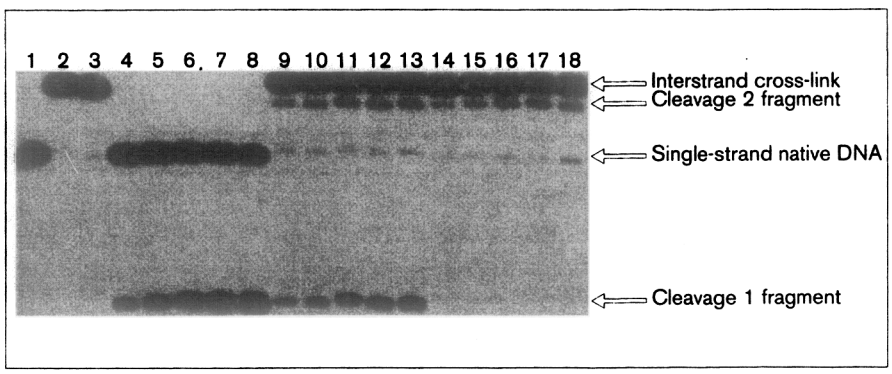
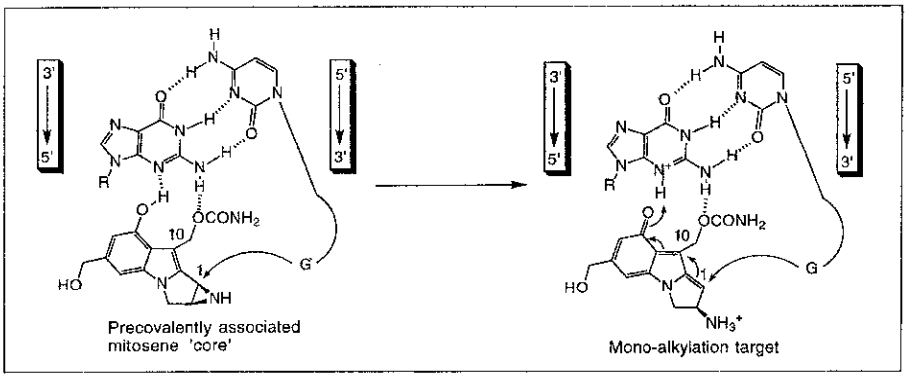


Figure 10



Potential hydrogen-bonding network leading to mono-alkylation of DNA by activated FR66979. Note that the guanosine N3 phenol contact not only represents a means of precovalent association but also may facilitate collapse to the aziridinyl ring-opened mono-alkylation target depicted above.

colleagues [29], which demonstrated inhibition of cleavage 2 for the MC orientation isomer in which C1 was connected to the cleavage 1 target strand. Verification of the Tomasz results with MC, using the electrophoretic assay described herein, was performed and found to be consistent with the HPLC results originally reported. As such, FR66979 cross-link orientation isomers do indeed demonstrate an extremely interesting departure from the known mechanism of *Alu*I inhibition inherent to MC.

Melting temperature determinations and molecular modeling of FR66979 cross-link orientation isomers

The ability to assign the structures and connectivity to the orientation isomers of the 5′ACGT cross-linked sequence of **template A** to the mitosene core derived from FR66979 prompted us to probe additional structural differences between these species by molecular modeling. All the experimental data generated suggested that the differing electrophoretic mobilities observed stemmed not from significant conformational differences in the cross-linked substrates (confirmed by T4 DNA ligase experiments and diethylpyrocarbonate hyper-reactivity studies), but rather from slightly differing T_m values for each cross-linked duplex. This was confirmed when the T_m values for the two cross-links were determined. It was found that the faster isomer had $T_m = 79.4°C$ while for the slower isomer $T_m = 77.1°C$. This order was consistent with the observed electrophoretic properties, in that, under denaturing conditions, the faster isomer would be expected to have the more compact structure inherent to double-stranded DNA for a longer period of time than would the lower melting (slower mobility) adduct. It is noteworthy that this same concept has been applied to the comparison of cross-linked substrates bearing a single inosine for guanosine substitution with cross-linked duplexes bearing only the native deoxynucleotides (data not shown).

Molecular modeling (Fig. 7) of the 5′d(GACGTA)$_2$ sequence cross-linked in both orientations at the 5′d(CG) box revealed no discernible difference between the two isomers, such as distances between the 'mitosene' nitrogens and various hydrogen-bond acceptors and donors of neighboring bases. The palindromic (and thereby C_2 symmetric) nature of the 5′d(ACGT)$_2$ box supports this finding. Importantly, the overall asymmetry of the oligonucleotide duplex is likely to facilitate the slight perturbation manifested preferentially by one isomer over the other. That this perturbation equates to the experimentally measured T_m difference of 2.3°C suggests that a single hydrogen bond difference may exist. The full-length sequence- dependent origin of such a perturbation and its relative complexity limits its structural elucidation within our model system. Potential repercussions of such drug-induced DNA distortions are clearly evident, however, as discussed earlier.

Mono-alkylation of DNA by FR66979

Tomasz and colleagues [29] showed that, upon bioreductive activation, loss of the carbamate functionality is the major obstacle to cross-link formation by MC; the presence of this moiety, however, is obligate for MC to efficiently alkylate the 5′CpG site [28]. Hydrogen bonding between the C10′–O of the activated form of MC and one of the exocyclic amines within the 5′CpG site aligns the drug properly for ring opening of the aziridine by the 'non-associated' guanosine of the opposing strand, directing the initial mono-alkylation within the 5′CpG site — an event that ultimately results in interstrand cross-linking.

DNA mono-alkylation by reductively activated MC is often accompanied by adventitious re-oxidation back to the non-electrophilic quinone, thus abrogating formation of the interstrand cross-link. In this regard, FR900482 and FR66979 display a distinct advantage over the redox-labile mitomycins, in that competitive oxidation of the mono-alkylated species is precluded.

Our results suggest, however, that the carbamoyl hydrogen-bonding interactions deemed necessary for MC to efficiently alkylate or cross-link DNA are not obligatory for target modification by FR66979. We propose that one

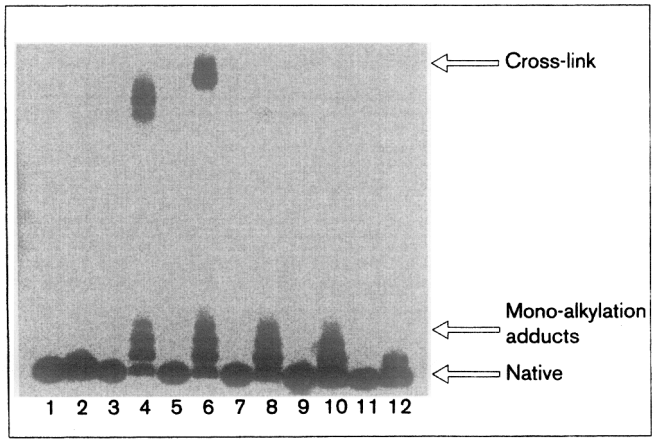
Figure 11

1	5′CCCTCAGCAAGAGACGTATGAGTATTCAACA3′
2	5′CCCTCAICAAIAIACGTATIAITATTCAACA3′
3	5′CCCTCAGCAAGAGACITATGAGTATTCAACA3′
4	5′CCCTCAICAAIAIACITATIAITATTCAACA3′

DNA strands in which guanosines were replaced by inosine.

possible explanation for this is the ability of the phenolic proton of **3** to hydrogen bond with N3 of the guanosine opposite that responsible for connectivity to C1 of the activated mitosene. Li, Kohn and coworkers [23–25] have carried out modeling studies on MC in which they proposed hydrogen bond contacts for the reduced hydroquinone form of **5** *en route* to DNA mono-alkylation. The importance of such a contact was later questioned by Tomasz given that when N2 was deleted from the 'pre-covalently' associated guanosine, the yield of mono-adduct at 5′CpG was decreased more than ten-fold under both reductive and acidic activation conditions (in which MC is reported to be in the quinone form thus lacking the C8 OH group). Such an interaction might be considerably more important for the FR compounds, however, because the phenolic proton is available to form a hydrogen bond to the dG N3 as well as to undergo deprotonation, resulting in the formation of the quinone methide species depicted in Figure 10.

Figure 12



Reaction of 5′ labeled 5′ TGTTGAATACTCATACGTCTCTTGCT-GAGGG 3′ annealed to complementary deoxyoligonucleotides 1–4 with reductively activated FR66979 (**2**). Lanes 1 and 2 are radiolabeled oligo and oligo + DTT standards, respectively. Lanes 3, 5, 7, 9 and 11 are control lanes containing substrate + 10 mM FR66979. Lanes 4, 6, 8, 10 and 12 are reaction lanes containing substrate after reaction with 10 mM FR66979 + 100 mM DTT. Lanes and corresponding complements to 5′ labeled strand are as follows: Lanes 1–4, complement 1; lanes 5 and 6 complement 2; lanes 7 and 8 complement 3; lanes 9 and 10 single-strand 5′ labeled oligo control; and lanes 11 and 12 complement 4.

To determine the importance of such interactions with FR66979, reactions were carried out with 5′ ^{32}P end-labeled 5′TGTTGAATACTCATACGTCTCTTGCT-GAGGG3′ **(template A)** annealed to complementary strands in which various guanosines were replaced by inosine (Fig. 11).

The data depicted in Figure 12 show clearly that use of strands 1, 2 or 3 as the complement resulted in nearly identical patterns of mono-alkylation adduct formation. Reaction of duplex 4 resulted in greatly diminished yields of mono-alkylation adduct and, surprisingly, significant mono-alkylation was observed in the single-strand control reaction. The ability to form the interstrand cross-link with substrate 2 in comparable yields to those using substrate 1 demonstrates that denaturing of substrate 3 was not a factor in these reactions. Substitution of dI for dG at the 5′CpG site had no effect on the relative amounts of mono-alkylation; this represents an interesting departure from the MC pathway with respect to non-covalent pre-association of the drug with the DNA. These results suggest that in the case of FR66979, alternative non-covalent pre-association phenomena might be operative; these would bear greater influence on sequestering the drug to the DNA target than that exerted by the carbamoyl–N2 interactions strongly implicated in MC–DNA interactions.

Significance

Structure elucidation and Fe(II)-EDTA footprinting of cross-linked and alkylated DNA products have demonstrated that members of the FR900482 class of antitumor antibiotics interact with DNA in a manner very similar to that of mitomycin C (MC). Like the mitomycins, FR900482 and congeners are 'pro-drugs' that must be activated reductively both *in vitro* and *in vivo* to produce the potent, electrophilic mitosene species that alkylate and cross-link DNA. Of particular interest is the role played by the phenolic portion of the FR compounds versus the quinone/semi-quinone of reductively activated MC. We have presented evidence that the phenol functionality may be crucial in enabling these compounds to mono-alkylate DNA very efficiently, thus facilitating DNA–DNA cross-links and potentially also DNA–protein cross-links. It is clear from the results presented that the guanosine N2 to C10′–O contact, involving the activated mitosene core of FR66979, plays a much smaller role than does the analogous (and necessary) contact with activated MC. It is likely that the presence of the phenol OH is infinitely more important for directing the FR pharmacophore to

its initial mono-alkylation target than the C7 carbamate. Also, it is interesting to note the relative ease of formation of separable orientation isomers of DNA–DNA cross-links mediated by FR900482 and MC. Structural differences aside, the lesions isolated from FR900482, FR66979, FK973 and MC cross-linked oligonucleotides all give rise to orientation isomers of considerably different electrophoretic mobilities. In addition, these isomers exert vastly different influences on the cleavage of 5′AGCT by *Alu*I restriction endonuclease, thus demonstrating an additional means by which both classes of compounds may exert biological activity. The ability of the FR compounds not only to shut down (via cross-linking) the strand separation events necessary for transcription, but also to inhibit DNA–protein recognition motifs, clearly provides additional insights into the mechanism by which these agents inflict cellular damage. Such information should prove invaluable in the design of second generation FR900482 compounds and will no doubt shed further light on the mechanism of action of the clinically employed mitomycins.

Materials and methods

FR900482 was generously supplied by the Fujisawa Pharmaceutical Co. Ltd, Japan. FR66979 and FK973 were synthesized according to the Fujisawa patent Kokai 61–10590. All drug stock solutions were made up to 50 mM in 33% methanolic water immediately prior to use. Deoxyoligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer using standard phosphoramidite chemistry (reagents and phosphoramidites from GLEN Research). Deoxyoligonucleotides were deprotected by heating 15 h at 55°C in NH_4OH, followed by filtering of the CPG resin and concentration of supernatant *in vacuo*. All oligos were purified by 20% denaturing polyacrylamide gel electrophoresis (DPAGE). Oligos of interest were 5′ end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs) and then purified once more by 20% DPAGE. Labeled deoxyoligonucleotides were then hybridized to their corresponding blunt-ended complements in 200 mM Tris (pH 7.5) by heating the equimolar mixture of oligos to 75°C for 15 min, and cooling to room temperature over 2 h then to 4°C over another 2 h. $FeSO_4$ (from Mallinkrodt) solutions were made up to 4 mM using 4 mM EDTA 5 min before use. Mercaptoethanol (from Kodak) and dithiothreitol (from Gibco BRL) stock solutions were made using distilled deionized water immediately prior to use. Sodium acetate, Tris, EDTA and boric acid were also obtained from Gibco. Gel-loading buffer contained 0.03% bromophenol blue, and 0.03% xylene cyanol in formamide. Dimethyl sulfate and formic acid (88%) for Maxam–Gilbert sequence reactions were obtained from Mallinkrodt. Centrex MF 0.45 μm cellulose acetate spin filters were obtained from Schleicher & Schuell. Samples were counted on a Packard 1500 Tri-Carb liquid scintillation analyzer.

Cross-link formation and subsequent footprinting reactions

To 15 μl of a 90 μM (in duplex) stock solution (in 200 mM Tris pH 7.5) of 5′ ^{32}P end-labeled DNA was added 50 mM drug stock solution to yield a drug concentration of 10 mM. To this mixture was added 1 μl β-mercaptoethanol to afford a reaction concentration of 500 mM in thiol. Reactions were incubated at 4°C for 4 h and were then allowed to warm to room temperature overnight. Modified deoxyoligonucleotides were then ethanol-precipitated, dried *in vacuo* and resuspended in 80 μl DPAGE loading dye. Reactions were then loaded onto a 20% denaturing gel, electrophoresis was carried out for 5 h at 2000 V, and the bands were visualized by autoradiography. Cross-linked product bands were excised from the gel and the DNA recovered by electroelution (0.1×TAE, 500 V, 2 h) using a Schleicher & Schuell Elutrap electroseparation system. The DNA isolated was once again ethanol- precipitated and then dried *in vacuo*. To each alkylation adduct was added 20 μl distilled de-ionized water. From each deoxyoligonucleotide solution, a 5 μl aliquot was removed for use as a standard. To the remaining 15 μl of each product was added 7.5 μl 80 mM phosphate buffer (pH 8) and 7.5 μl 4 mM $FeSO_4$–EDTA solution to afford reactions 1 mM in Fe(II)–EDTA. Footprinting reactions were then incubated at 37°C for 8 h, after which time samples were ethanol-precipitated and the pellets rinsed with cold 70% ethanol and dried *in vacuo*. Dried pellets were resuspended in 10 μl DPAGE dye, and the samples counted by liquid scintillation (LSC). Samples were loaded such that standard duplex and standard cross-linked product lanes contained 500 counts, Fe(II)–EDTA lanes contained 5000 counts (for Fe(II)–EDTA standard and native duplex after cross-linking and Fe(II)–EDTA digestion) and 10 000 counts for inosine-substituted duplex after cross-linking and Fe(II)–EDTA digestion. To G and G+A Maxim–Gilbert sequencing lanes were added 4000 and 8000 counts, respectively. Electrophoresis was carried out at 1500 V for 5 h followed by autoradiography at −80°C for 3 days. The results are depicted in Figure 5.

Analytical scale formation of FR66979–DNA mono-adducts

Mono-alkylation studies involved reactions 6.5 μM in duplex (a stock DNA solution 9 μM in duplex was used instead of the 90 μM stock used previously). Reactions were set up as outlined for orientation isomer studies, with the exception that reactions were 100 mM in DTT. Reaction conditions and workup were the same as discussed earlier except that reaction contents were dissolved in DPAGE dye to afford solutions of 2000 counts per μl activity. Reactions were analyzed by 20% DPAGE (8000 counts per well) followed by autoradiography.

Preparation of FR66979 cross-linked oligos for mass spectrometry

To 275 μl of a 90 μM (in duplex) solution of duplex (dissolved in 200 mM Tris, pH 7.5) was added 75.9 μl FR66979 and 27.5 μl of 277 mM DTT at 0°C. The reaction was allowed to warm to room temperature overnight (10 h) and the contents ethanol-precipitated. Resuspension in 150 μl DPAGE dye followed by 20% preparative DPAGE at 800 V for 10 h afforded clean separation of the two cross-linked species. Each band was excised from the gel, crushed and eluted for 2 h at 37°C in 500 mM NH_4OAc, 1 mM EDTA buffer. The supernatant was filtered through a Centrex spin frit, butanol extracted to a volume of 200 μl and ethanol-precipitated. Each resulting pellet was resuspended in 150 μl double-distilled H_2O and to each solution was added 50 μl 5 M NH_4OAc (pH 5.2). Each sample was kept at room temperature for 10 min, followed by the addition of 600 μl ethanol and placed in a dry ice bath for 15 min. The products were re-pelleted by centrifugation at 14 000 rpm at 4°C followed by removal of supernatant. The series of steps starting with resuspension in 150 μl double-distilled H_2O was performed once more followed by a 70% ethanol rinse and drying *in vacuo*

Supplementary material available

Supplementary material on the internet includes: gel electrophoretic analysis of FR66979 reactions with **templates A–C** (first paragraph on orientation isomers of cross-linked 5′dCG), details for **template A–C** reactions with FR66979, T_m determinations, T4 DNA ligase experiments, diethylpyrocarbonate hyper-reactivity studies, *Alu*I digestions of FR66979 orientation isomers, and cross-link reversibility studies.

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