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Monoalkylation of DNA by Reductively Activated FR66979

Manuel M. Paz, Snorri Th. Sigurdsson* and Paul B. Hopkins†

Department of Chemistry, University of Washington, Seattle, WA 98195-1700, USA

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Abstract—The antitumor antibiotic FR66979 has previously been shown to form interstrand cross-links in duplex DNA at the sequence $[5'\text{-d(CG)}]_2$, linking the exocyclic amino groups (N2) of deoxyguanosine (dG) residues. During the reaction of reductively activated FR66979 with DNA, products are formed which have electrophoretic mobility in denaturing polyacrylamide gels which is intermediate between that of unmodified and interstrand cross-linked DNA. We show here that these products are monoadducts between FR66979 and DNA and provide strong evidence for the site of alkylation being N2 of dG. Moreover, the sequence selectivity of monoalkylation reactions between FR66979 and DNA containing either 5′-d(CG)·5′-d(CI) or $[5'\text{-d(CG)}]_2$ was observed to be ca. 5-fold less than for the related antitumor antibiotic mitomycin C (MC). The mechanistic implications of this result are discussed. Furthermore, it was demonstrated that contrary to a previous report, FR66979 requires DNA to be in duplex form for efficient monoadduct formation.

Introduction

The interstrand cross-linking reactions of the antitumor antibiotics FR66979 (**1**), FR900482 (**2**) and FK973 (**3**) with DNA have been extensively studied and shown to be similar to that of mitomycin C (MC) (**4**).[1–5] Reductive activation of **1** in the presence of duplex DNA yields DNA–DNA interstrand cross-links at the sequence $[5'\text{-d(CG)}]_2$, as demonstrated by gel mobility analysis.[1,6] Furthermore, structural characterization of cross-link lesion **6**[2] revealed that the cross-link consists of two dG residues which are linked through their N2 atoms, analogous to lesion **5** found in MC-treated DNA.[7]

More is known about the *inter*strand cross-linking reactions of **1**–**3** than about their abilities to either monoalkylate or form *intra*strand cross-links in DNA. In contrast, MC has been shown to form both intrastrand cross-links (at d(GG))[8] and monoadducts (at dG)[9] in DNA through N2 of dG. Preliminary evidence for the formation of monoadducts between FR66979 and DNA was first obtained in a study of DNA interstrand cross-linking by FR66979.[1] In that study, the analysis of FR66979-treated DNA by denaturing polyacrylamide gel electrophoresis (DPAGE) revealed a band which had electrophoretic mobility between that of single stranded and interstrand cross-linked DNA, consistent with monoadduct formation. More recently, Williams et al. have reported the formation of monoadducts between reductively activated FR66979 and DNA, both in duplexes and in single strands.[6] Furthermore, multiple monoadducts which had discrete electrophoretic mobilities were observed. However, there was no further structural characterization of these products. Here we further characterize the monoadducts formed between reductively activated FR66979 and duplex DNA at dG and provide evidence for the alkylation occurring at N2 of dG. In addition, we show that the sequence selectivity of monoadduct formation of **1** in duplex DNA is less than is found for MC. Furthermore, we demonstrate that contrary to a previous report,[6] FR66979 requires DNA to be in duplex form for efficient monoadduct formation.

Results and Discussion

Monoadducts form at dG nucleotides

In an attempt to isolate and characterize a monoadduct lesion, the DNAs $[5'\text{-d(TATAACCATGGTTATA)}]_2$, $[5'\text{-d(TATAACAGCTGTTATA)}]_2$, and duplex **II** (Table 1), none of which contains the 5′-d(CG) sequence required for interstrand cross-linking, were treated with reductively activated **1**, using previously reported conditions.[4] The reaction mixtures were analyzed by DPAGE, the band corresponding to the putative

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*Corresponding author. Tel.: +1-206-616-8276; fax: +1-206-685-9238; e-mail: sigurdsson@chem.washington.edu

†Second corresponding author. Tel.: +1-206-543-9835; fax: +1-206-685-9238; e-mail: hopkins@chem.washington.edu

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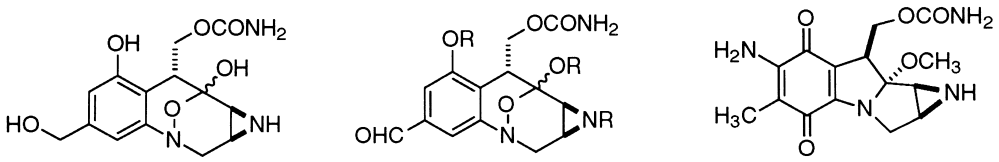
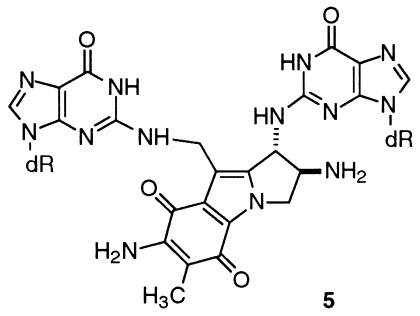


1 FR66979

2 R = H FR900482
3 R = Ac FK973

4 Mitomycin C (MC)



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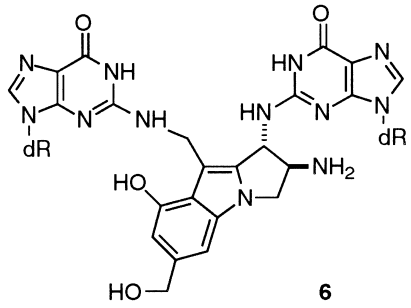

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monoadducts was isolated (for duplex **II**, see Fig. 2, lane 6) and enzymatically digested with snake venom phosphodiesterase and calf intestinal alkaline phosphatase. HPLC analysis of the digest showed in all three cases the disappearance of one dG residue, consistent with monoadduct formation at this nucleotide (see Fig. 1 for HPLC analysis of duplex **II**). However, the HPLC chromatograms did not reveal the presence of a major product corresponding to the dG-FR66979 conjugate. Instead, a series of minor compounds which were more strongly retained than the natural nucleosides were observed, which might be decomposition products of the monoadduct. MS analyses of these new products were inconclusive. The signal to noise ratio was low and none of the major peaks could be shown to correspond to the monoadduct or simple fragments resulting from dissociation of the drug from the nucleoside. This precluded a more extensive structural analysis of the dG-FR669798 conjugate and instead the mass-spectra of intact DNAs containing monoadducts were obtained (see below).

Alkylation occurs at N-2 of dG

The involvement of the exocyclic amino group of dG (i.e. N2) can conveniently be tested by using oligomers in which this functional group has been deleted (Table 1). Duplex **I** contains the 5′-d(CG) sequence required for interstrand cross-link formation, while in duplexes **II** and **III** one of the dG residues at this sequence has been replaced with deoxyinosine (dI), which lacks the exocyclic amino group of dG. These DNA duplexes were incubated with reductively activated **1**. After 24 h incubation, the DNA was precipitated and analyzed by DPAGE (Fig. 2). Duplex **I** yielded the expected interstrand cross-linked material and a small amount of the putative monoadduct (lane 3), while duplex **II**, with dI in the non-radiolabeled strand, gave a significant yield of the monoadduct (lane 6). The use of duplex **III**, where the dI-containing strand **D** is radiolabeled, yields neither monoadducts nor interstrand cross-links. Although this experiment does not constitute a direct

Table 1. DNA oligomers used to study the requirement of dG in monoadduct formation

		Strand descriptor
Duplex **I**	*5′ T T T A A A **C G** T A A T T T	**A**
	A A A T T T **G C** A T T A A A 5′	**B**
Duplex **II**	*5′ T T T A A A **C G** T A A T T T	**A**
	A A A T T T **I C** A T T A A A 5′	**C**
Duplex **III**	*5′ T T T A A A **C I** T A A T T T	**D**
	A A A T T T **G C** A T T A A A 5′	**B**

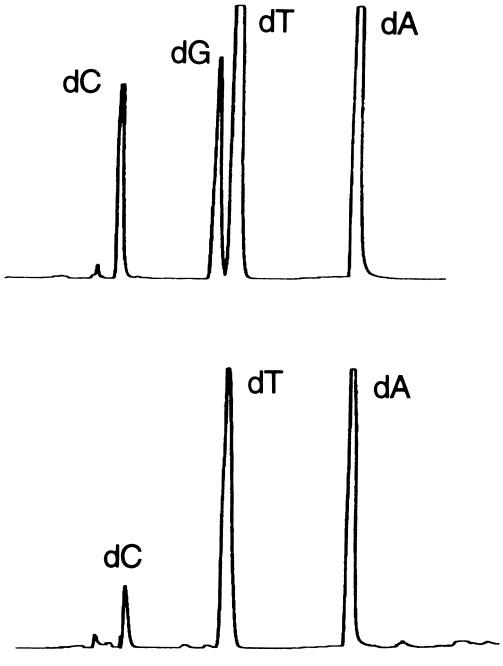


Figure 1. HPLC analysis of enzymatically digested, unmodified (upper) and monoalkylated (lower) DNA strand **A**. The retention times increase to the right.

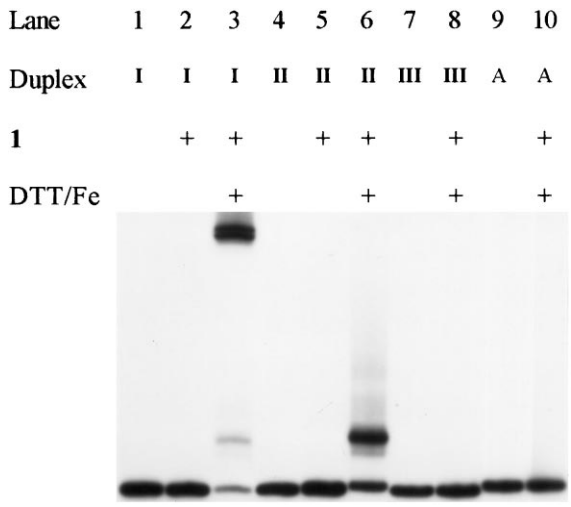


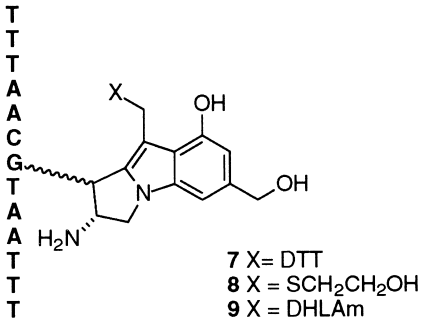
Figure 2. Reactions of reductively activated FR66979 with DNA, analyzed by DPAGE.

proof of the monoadduct structure, it is fully consistent with the alkylation site being N2 of dG.

The possibility that the FR66979-monoadducts had formed at N7 of dG, which is the preferred site of alkylation for a variety of alkylating agents, such as the nitrogen mustards,[10] was also investigated. The putative DNA monoadducts were isolated and treated with piperidine at 90 °C, which results in solvolysis of N7-alkylated purines and subsequent strand cleavage, and the reactions analyzed by DPAGE (data not shown). This included a control containing DNA monoadducts that were not treated with piperidine, showing that there was not considerable decomposition of the monoadducts during purification by gel electrophoresis. The absence of significant DNA fragmentation upon piperidine treatment was fully consistent with monoadduct formation at N2 rather than N7 of dG. The major product of decomposition of the monoadducts had the same electrophoretic mobility as single stranded DNA, presumably resulting from solvolysis of the N2-monoadduct. A less likely interpretation is that N7 is alkylated but that no cleavage is observed because the bond to the alkylating agent solvolyzes in preference to that between N9 and the deoxyribose.

Thiols are incorporated in FR66979 monoadducts

As the monoadduct lesion could not be isolated from an enzymatic digest of DNA-containing monoadducts, the FR66979-modified DNA was further analyzed by mass spectrometry. DNA duplex **II** was incubated with DTT-activated FR66979, the monoadduct isolated by DPAGE and the negative ion electrospray mass spectrum recorded. A series of multiply charged species was observed which allowed the calculation of a molecular weight (*m*/*e* 4635) which was consistent with structure **7** (calcd *m*/*e* 4633), in which the second electrophilic position of FR66979 is connected to one molecule of the thiol. This was also observed in the mass spectra of monoadducts which resulted from activation of FR66979 with other thiols; with mercaptoethanol and dihydrolipoamide molecular weights of *m*/*e* 4558 and *m*/*e* 4689 were observed, respectively, corresponding to structures **8** (calcd *m*/*e* 4557) and **9** (calcd *m*/*e* 4686). Lower abundance peaks in the aforementioned mass spectra of **7**, **8** and **9**, corresponding to Na^+ salts, were also observed. Preliminary evidence suggests that these monoadducts are not on the cross-linking pathway.[11]



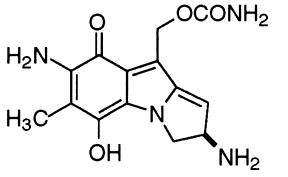
The sequence-selectivity of FR66979 monoadduct formation is lower than that of MC

It has previously been shown that monofunctionally-activated MC preferentially alkylates the sequence [5′-d(CG)]$_2$ and, to a minor extent, the sequence 5′-d(GG).[9] In addition, alkylation of DNA under these conditions at the sequence 5′-d(CG·CI) resulted in a 14-fold lower yield than at the sequence [5′-(CG)]$_2$ (36 versus 2.6%). This selectivity was explained by the formation of a pre-covalent complex between O10 of the carbamoyl group of activated MC and the exocyclic amino group of dG in the non-bonding strand of DNA.

We sought to determine the relative reactivity of the duplex sequences [5′-d(CG)]$_2$ and 5′-d(CG·CI) toward activated FR66979. Under suitably forcing conditions, these sequences embedded in DNA duplexes **I** and **II**, respectively, yielded 85% cross-linked DNA and 62% yield of monoalkylated DNA (Fig. 2). However, these saturating conditions do not reflect the relative sequence selectivity. Under sub-saturating circumstances, where a small fraction of the total DNA is consumed, and other nucleophiles in the medium compete favorably for capture of activated FR66979, the ratio of cross-linked duplex **I** to monoadducted duplex **II** was found to be 11%:4%, or ca. 2.8:1. Because cross-links in duplex **I** can arise by initial monoalkylation of either the radiolabeled or non-radiolabeled strand, a statistical correction must be applied, yielding a selectivity of monoadduct formation for [5′-d(CG)]$_2$ versus 5′-d(CG·CI) of 1.4:1. This result was in good agreement with an independent experiment in which the relative rate of monoadduct formation at the sequences [5′-d(CG)]$_2$ and 5′-d(CG·CI) was also measured in a single flask by an internal competition experiment using two different length DNAs. After statistical correction, the yields of 16.3% of cross-linked [5′-d(CG)]$_2$ and 5.3% of monoadduct at 5′-d(CG·CI) yield a 1.5:1 greater reactivity of the former over the latter toward monoadduct formation. These results are similar to that obtained by Williams and co-workers who reported that the

unalkylated dG at the sequence 5′-d(CG) had little effect on the relative amounts of monoalkylation.[6]

Williams et al. proposed that this decreased selectivity was due to a hydrogen bonding interaction between the phenolic proton of FR66979 and N3 of dG in the non-bonding strand, as both dG and dI can participate in this bonding interaction.[6] We suggest an alternative explanation. We propose that the reactive intermediate **11**, derived from reductively activated FR66979, is *more reactive* and thus less selective than the quinone methide of activated MC. The reactivity of the quinone methide **10** derived from activated MC towards nucleophiles is modulated by the strongly electron donating phenolic hydroxy group in position 8. The absence of this attenuation in reactivity for the quinone methide derived from activated FR66979 (**11**) results in a more electrophilic intermediate, and hence in less selective reactions. A similar scenario has been proposed to explain the lack of selectivity of some highly reactive compounds derived from reduced MC.[12]



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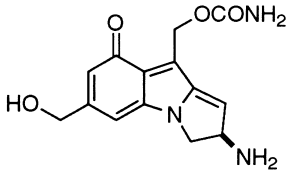

11

Multiple monoadducts are formed in DNA containing many dG nucleotides

Williams and co-workers utilized oligomers which had several dGs in each strand in their studies of monoadduct formation.[6] Incubation with reductively activated FR66979 and analysis of the reactions by DPAGE revealed a ladder of bands with electrophoretic mobility intermediate between that of single strands and interstrand cross-links. The identity of the individual bands was not discussed, but presumably resulted from formation of multiple monoadducts in the same strand. To investigate this further, the oligomers used by Williams and co-workers were prepared (Table 2) and incubated with reductively activated FR66979.

The radiolabeled template **F** was annealed to the different complements (**E**, **G**, **H**, **I**), treated with FR66979 and DTT/Fe(II) for 20 h, and analyzed by DPAGE. The resulting monoadducts were isolated and treated with piperidine at 90 °C, which would induce strand-breaks at N7-alkylated dG, but as before, no cleavage fragments were observed. The reaction was also analyzed using duplexes **IV′**–**VII′**, radiolabeled in strands **E**, **G**, **H**, **I** instead of **F** (data not shown). Duplex **V′**, which contains a single dG in the radiolabeled strand **G**, afforded interstrand cross-linked DNA and a single monoadduct. Duplex **VI′**, which contained only one dI in the radiolabeled strand, at the 5′-d(CG) step, formed multiple monoadducts, but no interstrand cross-links. Duplex **VII**, where all dG residues in the radiolabeled strand were replaced by dI, formed neither cross-links nor monoadducts. The results of these experiments are consistent with FR66979 forming monoadducts at N2 of dG residues. Quantification of the monoadducts revealed that the total yield was only marginally influenced by the substitution pattern in the complement strand, again demonstrating the low selectivity in DNA monoalkylation by FR66979.

We believe that the observed 'ladder' of bands of electrophoretic mobility less than the native single strand for duplexes **IV** and **VI** is the result of formation of multiple adducts in a single strand. We observed that reactions that resulted in a low yield of monoadducts contained mostly the hypothetical $(n+1)$ adduct of mobility just less than the single strand, while an increased yield of total alkylation products increased the relative yield of $(n+2)$, $(n+3)$ and $(n+4)$ adducts. These observations led us to propose a simple scenario for the alkylation reactions of FR66979: the low selectivity of FR66979 leads to competing alkylation reactions at several dG nucleotides. As a consequence, a correlation is observed between the extent of reaction and increased yield of multiple adducts within a given DNA strand.

Support for this model is found in the time course of the alkylation reaction with duplex **VII** (Fig. 3A). It is observed that the putative monoadduct appears with the highest initial rate. The diadduct, triadduct, and

Table 2. DNA oiligomers used to study sequence selectivity of monoadduct formation

		Strand descriptor
Duplex **IV**	5′ C C C T C A **G** C A A **G** A **G** A C **G** T A T **G** A **G** T A T T C A A C A	**E**
	G G G A **G** T C **G** T T C T C T **G** C A T A C T C A T A A **G** T T **G** T 5′*	**F**
Duplex **V**	5′ C C C T C A **I** C A A **I** A **I** A C **G** T A T **I** A **I** T A T T C A A C A	**G**
	G G G A **G** T C **G** T T C T C T **G** C A T A C T C A T A A **G** T T **G** T 5′*	**F**
Duplex **VI**	5′ C C C T C A **G** C A A **G** A **G** A C I T A T **G** A **G** T A T T C A A C A	**H**
	G G G A **G** T C **G** T T C T C T **G** C A T A C T C A T A A **G** T T **G** T 5′*	**F**
Duplex **VII**	5′ C C C T C A **I** C A A **I** A **I** A C **I** T A T **I** A **I** T A T T C A A C A	**I**
	G G G A **G** T C **G** T T C T C T **G** C A T A C T C A T A A **G** T T **G** T 5′*	**F**

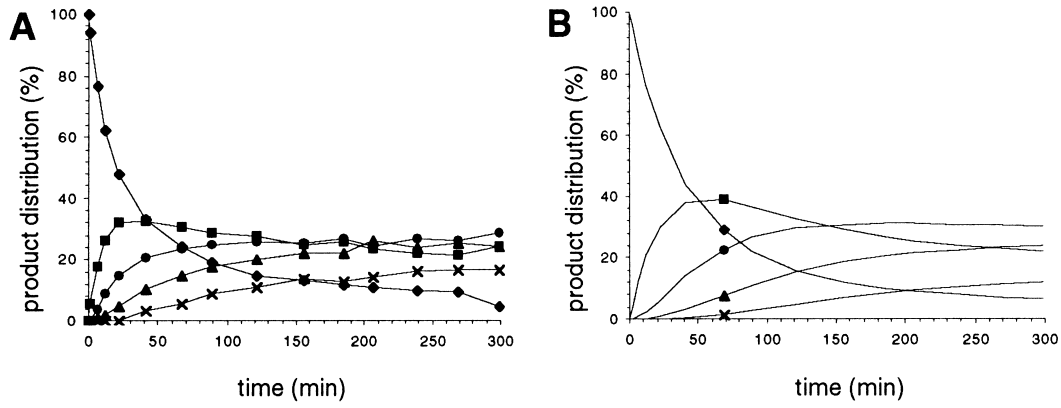

Figure 3. Product distribution as a function of time for the reaction of reductively activated FR66979 with duplex **VII** from (**A**) experimental data and (**B**) simulation (described in Experimental). (◆) unmodified **F**; (■) monoalkylated **F**; (●) dialkylated **F**; (▲) trialkylated **F**; (**X**) tetraalkylated **F**.

tetraadduct appear with decreasing initial rates. Consistent with this model, the yield of monoadduct falls at longer times, as it is consumed to form diadducts, etc. For rough comparison, the time-dependence of the product distribution was calculated for an oligomer containing eight dG sites of identical reactivity. The model assumed an initial molar ratio of 2.4 FR66979 per DNA duplex, a rate limiting appearance of alkylating agent in a unimolecular process with a half life of ca. 75 min, and finally rapid bimolecular reaction with DNA with a rate constant directly proportional to the number of dG nucleotides available for alkylation (Fig. 3B).[5] The experimental (Fig. 3A) and simulated (Fig. 3B) distributions are strikingly similar. These data are supportive of the hypothesis that the ladder of bands observed in denaturing gels arises from sequential alkylation events in a given DNA strand by reductively activated FR66979.

Monoadducts are not formed in single stranded DNA

It has been reported that incubation of single strand **F** (Table 2) with reductively activated FR66979 yielded a significant amount of monoadducts.[6] In contrast, we observed that the use of single strand **A** resulted in a yield of monoadduct lower than 2% (lane 11, Fig. 2). Therefore, we repeated the experiment with strand **F** and indeed, a 25% yield of alkylated DNA was observed. We postulated that this result might be the result of the propensity of strand **F** to form secondary structures of duplex-like regions. To further test this hypothesis, alkylation of oligonucleotides with a low propensity to form such secondary structures was studied (Table 3).

The purine-rich single strand **J** did not yield a significant amount of monoadducts when treated with reductively activated FR66979 (data not shown). However, when duplexes **VIII** or **IX**, both of which contain a short oligomer complementary to strand **J**, were incubated with FR66979, a high yield of monoadducts was observed. Furthermore, when single strand **M** was annealed to strand **J** (duplex **X**), the yield of monoadducts further increased. This preference was quantified using strand **A**; only 2% of **A** was alkylated as a single strand whereas 62% of **A** formed monoadducts when annealed to strand **C** (ca. 30:1 preference). These results demonstrate conclusively a strong correlation between duplex formation and the extent of alkylation with FR66979, analogous to that observed with MC, suggesting that monoalkylation reactions are strongly favored in duplex DNA structures.[9]

Conclusions

We have presented evidence which supports the hypothesis that products from the reaction of reductively activated FR66979 with duplex DNA, which have electrophoretic mobility between that of unmodified and interstrand cross-linked DNA, are monoadducts formed at N2 of dG. Specifically, enzymatic digestion of

Table 3. DNA oligomers used to study monoadduct formation in single stranded DNA

		Strand descriptor
Duplex **VIII**	*5′ T C G C A T G A G A A G A G A A G A G A C C A G A A G G A G A G G A G A C T G T C G	**J**
	C T C T T C T C T T C T C T G 5′	**K**
Duplex **IX**	*5′ T C G C A T G A G A A G A G A A G A G A C C A G A A G G A G A G G A G A C T G T C G	**J**
	G T C T T C C T C T C C T C T 5′	**L**
Duplex **X**	*5′ T C G C A T G A G A A G A G A A G A G A C C A G A A G G A G A G G A G A C T G T C G	**J**
	C T C T T C T C T T C T C T G G T C T T C C T C T C C T C T 5′	**M**

DNA containing monoadducts and subsequent HPLC analysis revealed disappearance of dG. Furthermore, substitution of dI for dG results in abolishment of monoadduct formation. The sequence selectivity of monoadduct formation at CG·CG versus CG·CI sites was found to be less than for MC, which we explain by the increased reactivity of the reactive intermediate. In contrast to previous reports,[6] we find that monoadducts form with much lower efficiency in single stranded DNA than in duplex DNA.

Experimental

Materials and methods

Materials and their sources were as follows: DNA synthesis reagents were from Applied Biosystems. Oligonucleotides were synthesized on an Applied Biosystems Model 392 synthesizer and purified by preparative DPAGE. FR900482 was a gift from Fujisawa Pharmaceutical Co., Ltd. (Japan). FR66979 was prepared by reduction of FR900482 with $NaBH_4$ as reported.[13] Water was purified on a Millipore Milli-Q deionizer. All other reagents were commercial and used as received. Unless otherwise specified, solutions were aqueous. Samples were concentrated on a Savant Speed Vac concentrator. The gel loading buffer was 90% aqueous deionized formamide containing 10 mM Tris/Tris·HCl (pH 7.5) and 1 mM Na_2EDTA. Gels were prepared as follows: Urea (48 g) was added to 19/1 acrylamide/bisacrylamide (40% in H_2O, 50 mL) and stirred until the urea was completely dissolved. Ammonium persulfate (10% w/v in H_2O, 350 μL) was added and the mixture filtered through Whatman #5 filter paper. To the resulting solution TEMED (17 μL) was added and the gel was poured and allowed to polymerize for 1 h. Gels were subjected to electrophoresis conditions for at least 45 min before loading the samples. DNA was ^{32}P-radiolabeled at the 5′ terminus as described by Sigurdsson and Hopkins.[14] Electrospray ionization mass spectra (ESIMS) were measured on a FISONS VG Quattro II, triple quadrupole model with a megaflow electrospray source, with infusion of sample. The program MaxEnt was used to calculate the true mass from each spectrum, which contained a series of multiply charged ions. HPLC was performed on an Alltech, 5 mm, C18, 250×4.6 mm column, using an SSI 200B/220B dual pump system with an SSI controller and sequential SSI 500 UV–vis (output to a Linear 255/M recorder and an HP 3390A electronic integrator) detector. The solvent was run at 1 mL/min. Gradient: solvent A: 10 mM ammonium acetate (pH 7.0); solvent B: CH_3CN; isocratic 92% A for 4 min, 11 min linear gradient to 25% A, 10 min linear gradient to 40% A, then 10 min linear gradient to initial conditions. Kinetic simulations were performed with Chemical Kinetics Simulator 1.01, IBM's Almaden Research Center, IBM Corp. (1996).

Reactions of FR66979 with DNA

The DNA (0.3 OD units of each strand in 10 μL of H_2O) were admixed with 25 μL Tris buffer (200 mM, pH 7.5) and spiked with approximately 20000 cpm of the complementary 5′-^{32}P-labeled strand. The solutions were heated for 5 min at 90 °C and allowed to cool slowly to 25 °C. FR66979 (4 μL of a 50 mM solution in H_2O, 0.2 μmol) and DTT (4 μL of a 200 mM solution in Ar-purged H_2O, 0.8 μmol) were added, the mixture was deairated by bubbling Ar through the solution for 10 min, and $Fe(SO_4)_2(NH_4)_2$ (1 μL of a 2.5 mM solution in Ar-purged H_2O, 2.5 nmol) was then added. The mixture was vortexed and incubated at 25 °C for 18 h. The DNA was precipitated by addition of 100 μL of 0.3 M NH_4OAc (pH 5.2) and 800 μL of absolute EtOH. The sample was centrifuged, the supernatant decanted and the resulting pellet was dried. The DNA was dissolved in 20 μL of a 1/1 mixture of water and loading buffer and analyzed by DPAGE as described by Kirchner et al.[15] Quantification of the products was performed by phosphorimagery.

Large-scale preparation of oligonucleotides containing FR66979 monoadducts

The procedure reported here is representative for all the sequences used in our studies, except for self-complementary strands, where the annealing step was omitted. The DNA strands **A** (5′-TTT AAA CGT AAT TT) and **C** (5′-AAA TTA CIT TTA AA) (7.5 OD units of each, 0.053 μmol duplex) in 100 μL of H_2O were admixed with 200 μL Tris buffer (200 mM, pH 7.5), heated for 5 min at 90 °C and allowed to cool to 25 °C over 3 h. FR66979 (100 μL of a 50 mM solution in H_2O, 5.0 μmol) and DTT (100 μL of a 200 mM solution in Ar-purged H_2O, 20.0 μmol) were added, Ar bubbled through the solution 10 min, followed by addition of $Fe(SO_4)_2(NH_4)_2$ (20 μL of a 2.5 mM solution in Ar-purged H_2O, 50 nmol). The mixture was vortexed and incubated at 25 °C for 18 h. The DNA was precipitated by addition of 100 μL of 0.3 M NH_4OAc (pH 5.2) and 800 μL of absolute EtOH. The sample was centrifuged, the supernatant decanted and discarded, and the resulting pellet was dried. The DNA was dissolved in 100 μL of a 1/1 mixture of water and loading buffer and purified by 20% DPAGE (1 mm thick, 20×18 cm). Electrophoresis was performed at 400 V, until bromophenol blue had traveled ca. 17 cm. The bands were visualized by UV light, and the band with slightly less mobility than the native strand was cut out from the gel and the modified oligonucleotide eluted from the gel matrix[2] to give 2.2 OD units of modified DNA. The dihydrolipoamide and mercaptoethanol derivatives were prepared in a similar fashion, substituting the corresponding thiols for DTT. ESMS **7**: *m/e* 4635 (M), 4657 ($M-H^+ + Na^+$). **8**: *m/e* 4558 (M), 4582 ($M-H^+ + Na^+$) 4603 ($M-2H^+ + 2Na^+$). **9**: *m/e* 4689 (M) and 4711 ($M-H^+ + Na^+$).

Enzymatic digestion of oligonucleotides containing FR66979 monoadducts

The modified oligonucleotides (0.2 OD units) were dissolved in water (16 μL), admixed with 10×AP buffer (2 μL), alkaline phosphatase (1 μL, 10 units) and snake venom phosphodiesterase (1 μL, 0.5 units), and

incubated for 1 h at 25 °C. A 5-μL aliquot was diluted with 25 μL of 20% DMSO in water, briefly sonicated, and analyzed by HPLC. For purposes of comparison, a control reaction and HPLC analysis was performed using the unmodified native strand.

Kinetic analysis of the formation of multiple adducts in 30-mer F

Oligonucleotides **F** and **I** (0.4 OD units of each strand, 0.12 nmol duplex, in 40 μL H_2O) were admixed with 50 000 cpm of 5′-^{32}P-labeled **F** in 20 μL H_2O and 100 μL of Tris buffer (200 mM, pH 7.5). The solution was heated for 5 min at 90 °C and allowed to cool slowly to 25 °C. FR66979 (40 μL of a 50 mM solution in H_2O, 2.5 μmol) and DTT (20 μL of a 1.0 M solution in Ar-purged H_2O, 16 μmol) were added. The mixture was vortexed and incubated at 25 °C for 18 h. Aliquots of this mixture (10 μL) were periodically removed and admixed with non-radiolabeled oligonucleotide (1 O.D. unit) and stored at −20 °C. The DNA was precipitated by addition of 100 μL of 0.3 M NH_4OAc (pH 5.2) and 800 μL of absolute EtOH. The samples were centrifuged, the supernatants decanted and the resulting pellet was dried. The DNA pellets were dissolved in 20 μL of a 1/1 mixture of water and loading buffer and analyzed by DPAGE as described by Kirchner et al.[15] Quantification was performed by phosphorimagery.

Kinetic simulation of alkylation

For the kinetic simulation, it was assumed that FR66979 formed activated FR66979 (FR*) in a unimolecular reaction with rate constant k'. It was further assumed that FR* reacted with DNA (**F**) in eight sequential alkylation steps: $\mathbf{F}(\mathrm{FR66979})_n + \mathrm{FR}^* \rightarrow \mathbf{F}(\mathrm{FR66979})_{n+1}$, where $n = 0$–7 and the rate constant $k_{n+1} = (8-n)\,\mathrm{M}^{-1}\ \mathrm{s}^{-1}$. The initial concentration of **F** was 1 M. The values for the rate constant of the activation step (k') and initial concentration of FR were floated to fit the experimental data, and found to be $1.5\times10^{-4}\ \mathrm{s}^{-1}$ and 2.4 M, respectively.

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References and Notes

1. Woo, J.; Sigurdsson, S. T.; Hopkins, P. B. *J. Am. Chem. Soc.* **1993**, *115*, 1199.
2. Huang, H.; Pratum, T. K.; Hopkins, P. B. *J. Am. Chem. Soc.* **1994**, *116*, 2703.
3. Huang, H. K.; Rajski, S. R.; Williams, R. M.; Hopkins, P. B. *Tetrahedron Lett.* **1994**, *52*, 9669.
4. Paz, M. M.; Hopkins, P. B. *Tetrahedron Lett.* **1997**, *35*, 343.
5. Paz, M. M.; Hopkins, P. B. *J. Am. Chem. Soc.* **1997**, *119*, 5999.
6. Williams, R. M.; Rajski, S. R.; Rollins, S. B. *Chemistry & Biology* **1997**, *4*, 127.
7. Tomasz, M.; Lipman, R.; Chowdary, D.; Pawlak, J.; Verdine, G. L.; Nakanishi, K. *Science* **1987**, *235*, 1204.
8. Bizanek, R.; McGuinnes, B. F.; Nakanishi, K.; Tomasz, M. *Biochemistry* **1992**, *31*, 3084.
9. Kumar, S.; Lipman, R.; Tomasz, M. *Biochemistry* **1992**, *31*, 1399.
10. Rink, S. M.; Solomon, M. S.; Taylor, M. J.; Rajur, S. B.; McLaughlin, L. W.; Hopkins, P. B. *J. Am. Chem. Soc.* **1993**, *115*, 2551.
11. To explore the possibility that the isolated monoadducts were intermediates on the interstrand cross-linking pathway, the FR66979-modified DNA monoadduct **7** was annealed to its dG-containing complementary strand and incubated for 20 h at 25 °C. The analysis of the mixtures by DPAGE showed the formation of cross-linked DNA (10% yield) with the same mobility as the one obtained from treatment of duplex **I** with FR66979 (data not shown). The relatively low yield of cross-linking indicated that the monoadducts were not efficiently cross-linked. Further indication that these monoadducts were not on the major cross-linking pathway was provided by the observation that increased amounts of DTT, in a DTT-activated FR66979 cross-linking of duplex **I**, decreased the amount of interstrand cross-links, relative to that of the monoadducts (data not shown). The interstrand cross-linked DNA, obtained by incubation of monoadduct **7** with its complementary strand, was enzymatically digested and analyzed by HPLC (data not shown). The HPLC chromatogram revealed that dG was absent and instead, a strongly retained peak was observed which coeluted with an authentic sample of the bisadduct **6**.[2] This provided further evidence for conjugation of the monoadduct to N2 of dG. However, it cannot be conclusively ruled out that this cross-linked material originated from a minor component of the monoadduct mixture (only 10% yield of cross-linking) and that the majority of the monoadducts were connected to a site different than N2 of dG.
12. Li, V.-S.; Choi, D.; Tang, M.-S.; Kohn, H. *J. Am. Chem. Soc.* **1996**, *118*, 3765.
13. Williams, R. M.; Rajski, S. R. *Tetrahedron Lett.* **1992**, *33*, 1992.
14. Sigurdsson, S. T.; Hopkins, P. B. *Tetrahedron* **1994**, *50*, 12065.
15. Kirchner, J. J.; Sigurdsson, S. T.; Hopkins, P. B. *J. Am. Chem. Soc.* **1992**, *114*, 4021.