Site-specific targeting of aflatoxin addition directed by triple helix formation in the major groove of oligodeoxyribonucleotides

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ABSTRACT

The targeted addition of aflatoxin B1-exo-8,9-epoxide (AFB1-exo-8,9-epoxide) to a specific guanine within an oligodeoxyribonucleotide containing multiple guanines was achieved using a DNA triplex to control sequence selectivity. The oligodeoxyribonucleotide d(AAGAGAGATTTTCTCCTTTTTTTTTCTC), designated ‘3G’, spontaneously formed a triplex in which nucleotides C24*G2+G18 and C29*G4+C16 formed base triplets, and nucleotides G7*C13 formed a Watson–Crick base pair. The oligodeoxyribonucleotide d(AAGAAATTTTTTCTTTTTTTTTTTCCCTCT), designated ‘1G’, also formed a triplex in which nucleotides C24*G3+C24 formed a triplet. Reaction of the two oligodeoxyribonucleotides with AFB1-exo-8,9-epoxide revealed that only the 3G sequence formed an adduct, as determined by UV absorbance and piperidine cleavage of the 5′-labeled adduct, followed by denaturing polyacrylamide gel electrophoresis. This site was identified as G7 by comparison to the guanine-specific cleavage pattern. The chemistry was extended to a series of nicked bimolecular triple helices, constructed from d(AAAAGGGGAA) and d(CnTCTTTTTCTC) (n = 1–5). Each oligomer in the series differed only in the placement of the nick. Reaction of the nicked triplexes with AFB1-exo-8,9-epoxide, piperidine cleavage of the 5′-labeled adduct, followed by denaturing polyacrylamide gel electrophoresis, revealed cleavage corresponding to the guanine closest to the pyrimidine strand nick. By using the appropriate pyrimidine sequence the lesion was positioned within the purine strand.

INTRODUCTION

Aflatoxin B1 (AFB1) is the predominant mutagenic fungal metabolite isolated from several species of Aspergillus. This mycotoxin contaminates many food stuffs and is consequently of world-wide health concern. AFB1 is a mutagen in several strains of bacteria (1); it is a hepatocarcinogen in animals (2,3). Epidemiological studies suggest it may be a carcinogen in humans (2,4). Furthermore, AFB1 may be linked to site-specific transversion in the tumor suppressor gene p53 (5,6) and to proto-oncogene activation (3,7).

AFB1 is metabolized by cytochrome P450 monoxygenases (8,9) to yield the ultimate carcinogen, AFB1-exo-8,9-epoxide (10,11). The exo-epoxide bonds predominantly to guanine N7 to yield trans-8,9-dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin B1 (12); small amounts of the corresponding adenine adduct trans-8,9-dihydro-8-(N7-adenyl)-9-hydroxyaflatoxin B1 also can form (Scheme 1) (13). The synthesis of AFB1-exo-8,9-epoxide allowed sufficient quantities of site-specific aflatoxin B1 adducts to be produced for NMR structural and related biophysical studies (14–16), and for site-directed mutagenesis studies (17,18). Structural studies of the related sterigmatocystin adduct have also been reported (19,20).

One limitation of the synthesis using AFB1-exo-8,9-epoxide is the poor control of sequence selectivity in oligodeoxyribonucleotides containing multiple guanines. Many DNA sequences of biological interest, including iterated repeats and palindromes (21–25), proto-oncogene sequences (26–31) and the p53 tumor suppressor gene sequence (5,6), contain multiple guanines. Uncontrolled reaction with AFB1-exo-8,9-epoxide potentially results in multiple adducts, at low yield, and necessitating complex separation protocols to isolate the desired product. Indeed, the successful post-synthetic separation of multiple adducts may not be possible.

The control of site-specific addition into sequences of biological importance could be achieved by taking advantage of knowledge regarding the mechanism of aflatoxin addition to DNA (32). AFB1 exhibited strong regioselectivity for the N7 position of guanine (12,33), with minor amounts of adduction at adenine N7 (13,34). The regioselectivity of addition at guanine N7 was mechanistically consistent with precovalent intercalation of the epoxide on the 5′ face of the guanine (14,35–38) prior to S2 attack by the epoxide (32). This necessity for precavalent intercalation of the epoxide prior to adduct formation was recently exploited in a clever scheme in which AFB1-exo-8,9-epoxide reaction was blocked by selectively incorporating a covalent 5′-intercalating agent, the cis–syn thymidine benzofuran (39,40), opposite non-targeted guanines in an oligodeoxyribonucleotide containing the p53 mutational hotspot (41). Alternatively, we hypothesized that triple helices could be utilized to control sequence selectivity, since the presence of a third strand of DNA in the major groove should block reactivity at the N7 positions of guanines involved in triplex formation. Using a similar strategy, the third strand of a triplex was shown to block the action of S1 nuclease (42–45).

This work reports the synthesis of site-specific aflatoxin adducts in a DNA sequence with multiple guanines. To develop
**Materials and Methods**

**Adduct synthesis**

Unadducted oligodeoxyribonucleotides were purchased from Midland Certified Reagent Company (Midland, TX), or synthesized in house. AFBl was purchased from Sigma-Aldrich Chemicals, Inc. (Milwaukee, WI). Dimethyldioxirane was reacted with AFBl to give AFBl-exo-8,9-epoxide (10). Caution: crystalline aflatoxins are hazardous due to their electrostatic nature and should be handled using appropriate containment procedures and respiratory mask to prevent inhalation. Aflatoxins can be destroyed by treatment with NaOCl. It should be assumed that aflatoxin epoxides are highly toxic and carcinogenic. Manipulations of AFB1-exo-8,9-epoxide should be carried out in a well-ventilated hood with suitable containment procedures. The epoxide was dissolved at 30 °C in methylene chloride. To form the 3G and 1G adducts, two equal aliquots of 15 μmol of the epoxide in 500 μl methylene chloride were added sequentially to 30 μmol of the appropriate oligonucleotide in 200 μl TH or non-TH buffer to form a two-phase mixture. The mixture was vortexed and allowed to react for 15 min at 5 °C, with a 5 min interval between additions. The reactions for the biomolecular systems utilized 30 μmol of the appropriate triplex in 200 μl buffer. These reactions were carried out at room temperature. The mole ratio of epoxide to oligonucleotide was 0.5:1 for the site-specific cleavage studies of the unimolecular and bimolecular systems, 2:1 for the pH studies and 20:1 for the initial HPLC studies. In all instances, site-selectivity was increased at lower ratios of epoxide:DNA.

**Triple helix formation**

The aqueous buffer used to form triplexes (TH buffer) was 10 mM Na2HPO4, 100 mM NaCl, 10 mM MgCl2, 0.05 mM Na2EDTA, pH 5.8. The phosphate buffer (non-TH buffer) was 10 mM Na2HPO4, 100 mM NaCl, 0.05 mM Na2EDTA, pH 7.0.

**Gel electrophoresis**

The oligodeoxyribonucleotides were 32P-labeled using T4 polynucleotide kinase (Promega Corporation, MD) and [γ-32P]ATP (NEN/Dupont, Newark, DE). Unincorporated nucleotide was removed using spin columns packed with Sephadex G-25 (super fine mesh; BioRad, Richmond, CA). The eluent was reacted with dimethyl sulfate for G specific cleavage, formic acid for GA specific cleavage or with AFBl-exo-8,9-epoxide to generate base labile sites (34,46,47). Beta elimination of the phosphate backbone was accomplished by addition of piperidine, followed by heating. The samples were lyophilized. Loading buffer (Amersharm) was added to the dried aliquots prior to loading the samples onto the denaturing polyacrylamide gel. A 20% (19:1 acryl:bis) (Biorad) 7 M urea, 0.4 mm gel was used for electrophoretic separation. The gel was exposed to X-OMAT film to generate the autoradiogram. The autoradiograms were analyzed densitometrically with a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info).
Spectroscopy (48–50). For the 3G sequence, the magnitude of the triple helix formation was monitored by circular dichroism.

RESULTS AND DISCUSSION

1G oligonucleotide reaction mixture showed absorbance at 255 nm simultaneously at 362 and 255 nm. The major peak eluting from the oligodeoxyribonucleotide reaction mixtures were monitored 3G oligomers. HPLC chromatograms of the 1G and the 3G deoxyribonucleotide mole ratio was reacted with each of the 1G and the 3G purine tract spontaneously formed a bimolecular triple helix when combined with any one of the five pyrimidine tracts under appropriate buffer conditions. The buffer was 100 mM NaCl, 1 mM spermine HCl, 50 mM Tris–acetate, pH 5.5. Each of the guanines in the purine strand formed protonated base triplets. There was a nick in the pyrimidine tract where the 5′-end and the 3′-end phobicities of the two molecules. At 255 nm, two peaks were seen for the 3G oligodeoxyribonucleotide reaction mixture. The first peak showed absorbance at 255 nm only, indicating that it was unmodified oligodeoxyribonucleotide, while the second peak also contained absorbance at 362 nm, characteristic of the aflatoxin chromophore. The magnitude of the 362 nm absorbance indicated that one guanine was adducted.

Identification of the site of adduction

Following adduction at a mole ratio of 0.5:1.0 epoxide:DNA at pH 5.8, treatment of the adducted 3G oligodeoxyribonucleotide with piperidine, followed by heating, caused cleavage of the phosphate backbone at the lesion (34). This was compared to cleavage patterns generated by G- and GA-specific reactions (46). Figure 1A shows the 32P autoradiogram. In the aflatoxin-adducted lane, a single cleavage product was observed, which corresponded to the lone guanine not protected from adduction by the DNA triplex. Figure 1B shows the 32P autoradiogram following treatment of the adducted 1G oligodeoxyribonucleotide. In the 1G experiment, AFB1-exo-8,9-epoxide was reacted with the DNA under two different buffer conditions. The first buffer (TH buffer) promoted triplex formation in the 1G sequence, while the second (non-TH) buffer shifted the intrasstrand equilibrium toward what was presumed to be a Watson–Crick duplex with an overhanging end. Figure 1B reveals that under the non-TH buffer conditions, a cleavage product corresponding to the single guanine in the 1G sequence was observed, while under the TH buffer conditions, this guanine was protected from adduction, resulting in the absence of a cleavage product.

pH dependence of the reaction between AFB1-exo-8,9-epoxide and the 3G oligodeoxyribonucleotide

The reaction of AFB1-exo-8,9-epoxide with the 3G sequence was examined as a function of pH. Figure 2 shows densitometric analysis of electrophoretic gels from a series of reactions between the epoxide and the 3G sequence, over the range from pH 4.2 to 7.0. The plot shows the percentage of cleavage products located at the targeted G7 in comparison to the adducts at the non-targeted guanines, G2 and G4. This increased from 38 to 62% as pH was lowered, consistent with the expectation that C-G+C-G triple helix formation was favored at lower pH, making G7 the preferred target.

Control of temperature and pH was crucial in triplex-mediated site selection. The data obtained in Figure 2, using an epoxide:DNA ratio of 2:1, exhibited residual non-targeted adduct formation, illustrating that excess epoxide in the reaction compromised site-selectivity. Additionally, at pH values ~5, reaction yield decreased despite favorable triple helix formation, a phenomenon
Figure 1. (A) $^{32}$P autoradiogram of the cleavage products following reaction of 3G with AFB1-exo-8,9-epoxide. Lane 1, unreacted 3G; lane 2, G-specific cleavage; lane 3, GA-specific cleavage; lane 4, AFB1-specific cleavage. (B) $^{32}$P autoradiogram of the cleavage products following reaction of 1G with AFB1-exo-8,9-epoxide. Lane 1, G-specific cleavage; lane 2, GA-specific cleavage; lane 3, AFB1-specific cleavage in TH buffer; lane 4, AFB1-specific cleavage in non-TH buffer.

Figure 2. Densitometric analysis of the cleavage patterns seen in the 3G reaction as a function of pH. The % adduct formation at guanine N7 versus pH.

probably the consequence of acid-catalyzed hydrolysis of the epoxide (51,52). At ratios of epoxide:DNA <1:1, and at pH 5.8–6.0, site-selective targeting within the 3G oligomer was optimal.

Bimolecular triplexes

The oligomers shown in Scheme 3 were designed to spontaneously form bimolecular triple helices having a nick opposite the targeted guanine. The bimolecular system provided the possibility of facile removal of the blocking triple strand after the reaction. Initially, the strategy had been to leave a 1 nt gap in the blocking triplex strand, opposite the guanine at which reaction was desired, allowing a specific guanine within an iterated guanine repeat to be adducted. However, this approach failed to achieve the desired sequence selectivity. Inspection of model structures suggested that this probably occurred because the 1 nt gap allowed adduction to occur either at the targeted unprotected guanine, or at its 3‘-neighbor guanine, which potentially also had a free intercalation site. An improved protocol was developed, in which the 1 nt gap was replaced by a nick opposite the targeted guanine; in the nicked system only a single intercalation site was readily available to the epoxide (Scheme 4).

Figure 3 shows an autoradiogram demonstrating the site-specific placement of the aflatoxin lesion at each guanine within the iterated guanine tract. In the triplex system, reactivity was dependent not only upon sequence, but upon the relative stabilities of the blocking strand triplexes, i.e., the relative amounts of triplex fraying differed at each of the guanines, leading to a source of differential reactivity. Densitometric analysis revealed that the yield of AFB1-adducted oligodeoxyribonucleotide at sites G⁴ and G⁸ was 40% using the blocking strands Y⁴ and Y⁸, respectively; this decreased to 25% yield at sites G⁵ and G⁶, using the blocking strands Y⁵ and Y⁶, respectively. In the present bimolecular reaction system, the Y⁵ and Y⁶ blocking strands were expected to form the most stable triplexes since the termini of the blocking strands were farthest from the hairpin loops. This probably explained the lower reactivity when the reaction was targeted at G⁵ or G⁶ (Fig. 3). The differences in yields at the different sites might additionally reflect intrinsic differences in reactivity at the various sites. At higher ratios of AFB1 epoxide:DNA, secondary products were seen in subsequent HPLC purification, which probably resulted from a transient melting of the triple helix beyond the 5′ terminal pyrimidine tract base.

The DNA cleavage reactions (Fig. 3) did not eliminate the possibility that diadducts could have formed, as only the cleavage site nearest the 5′-end label would be visible. Figure 4 shows the isolation by gel electrophoresis of the intact site-specifically modified oligodeoxyribonucleotide d(AAAGGGAFBGGGA) prepared using this triplex methodology, in which the aflatoxin adduct was embedded within a run of five guanines. Under our reaction conditions, there was no evidence for formation of diadducts, which would have appeared as additional bands in this gel. We have not investigated the fate of the monoadducted product with respect to further reaction with AFB1 epoxide. The possibility exists that formation of the monoadduct may disrupt the DNA triplex and facilitate undesired reactions at non-targeted guanines. Thus, control of the [epoxide]:[DNA] concentration ratio to minimize multiple ‘hits’ should be considered important.
A number of lines of evidence support this mechanism (54). Reaction mixture (Fig. 4, lane 1) suggested that FAPY formation by electrophoresis of the cationic adduct as a single band from the fact that gels were run at slightly alkaline pH. The successful occurrence of the cationic adduct was consistent with the cationic species; had quantitative conversion to the neutral FAPY species occurred, the band would have been expected to have had an electrophoretic mobility comparable to the unadducted oligomer. This result also correlated with our experience in synthesizing the FAPY adduct of AFB1. We have found FAPY formation to be slow at slightly alkaline pH 7–8. At pH 9 and 25°C, FAPY formation remained slow (on the order of many hours for complete conversion to FAPY). On the other hand, depurination of the cationic adduct proved to be a greater problem at even slightly acidic conditions. At pH 7–8 and low temperatures, the cationic adduct was reasonably stable. Manipulation of the cationic adduct between pH 7.5 and 8.0, minimized both depurination and FAPY formation. Nevertheless, small amounts of FAPY or depurinated adducts might pose a problem in purifying material for mutagenesis or other studies where absolute purity is crucial.

Targeting adduction

The short half-life of the epoxide (53) aids in limiting the reaction to the formation of monoadducts. Another concern was the potential for base-catalyzed hydrolysis at C8 (imidazole ring opening leading to FAPY formation) due to the fact that gels were run at slightly alkaline pH. The successful electrophoresis of the cationic adduct as a single band from the reaction mixture (Fig. 4, lane 1) suggested that FAPY formation did not occur under these electrophoretic conditions. The fact that the single band observed in lane 1 migrated more slowly than the unadducted oligomer (lane 2) was consistent with the cationic species; had quantitative conversion to the neutral FAPY species occurred, the band would have been expected to have had an electrophoretic mobility comparable to the unadducted oligomer. This result also correlated with our experience in synthesizing the FAPY adduct of AFB1. We have found FAPY formation to be slow at slightly alkaline pH 7–8. At pH 9 and 25°C, FAPY formation remained slow (on the order of many hours for complete conversion to FAPY). On the other hand, depurination of the cationic adduct proved to be a greater problem at even slightly acidic conditions. At pH 7–8 and low temperatures, the cationic adduct was reasonably stable. Manipulation of the cationic adduct between pH 7.5 and 8.0, minimized both depurination and FAPY formation. Nevertheless, small amounts of FAPY or depurinated adducts might pose a problem in purifying material for mutagenesis or other studies where absolute purity is crucial.

Afatoxin G1, which substitutes a δ-lactone ring for the cyclopentenone ring of AFB1, exhibits lower binding affinity for B-DNA, and forms lower levels of DNA adducts (37). AFB1-exo-8,9-epoxide reacts readily with B-form DNA, but not with A or Z form DNA, or single-stranded DNA (38). Reaction with the sequence isomeric oligodeoxyribonucleotides d(AATGCT)2 and d(AATGGCT)2 proceeds with differing stoichiometries: d(AATGCT)2 will react with only one equivalent of epoxide, while d(AATGGCT)2 will react with two equivalents (15). AFB1-endoxo-8,9-epoxide (55) does not form DNA adducts and is not mutagenic (32). The epoxide is highly reactive in aqueous solution, and hydrolyzes to the dihydrodiol with a rate constant of 0.6 s⁻¹ (51) which probably accounts for the low yield of adducts in single-stranded DNA and mononucleotides.

The present results showing that the C·G·C·C·G base triplet prevents adduction at guanine N7 suggest that AFB1 adduction is prevented because the epoxide does not intercalate into the DNA triplex, or alternatively, that it does intercalate but due to the presence of the Hoogsteen base, fails to achieve the proper geometry to allow for attack on the epoxide. The present data do not distinguish between these two possibilities. While triplex formation itself should not prevent intercalation, the process of intercalation requires formation of a suitable intercalation site, which might be more difficult in the triplex. The pH dependence of the reaction with the 3G oligodeoxyribonucleotide confirmed that sequence selectivity was achieved by the DNA triplex, modulated by reaction pH.

A recent report documented the utilization of the cis–syn thymidine benzofuran adduct to site-specifically target aflatoxin adduction in a p53 mutational hotspot (41). That approach also exploited the intercalative mechanism of aflatoxin adduction, but through formation of a covalently attached intercalating agent opposite non-targeted guanines. The use of triplexes does not require non-standard phosphoramidites in the blocking strand, which will be important in large scale preparations required for structural biology. Also, with the triplex approach the aflatoxin adduction was targeted within iterated guanine sequences, which would be problematic using the covalently intercalated blocking group due to the tendency toward nearest neighbor exclusion at intercalation sites. On the other hand, the selectivity of the triplex methodology is dependent upon the equilibrium binding between the triplex-forming blocking strand and the targeted duplex. As
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