The aflatoxin B_1-induced imidazole ring-opened guanine adduct: high mutagenic potential that is minimally affected by sequence context

Irina G. Minko[1], Andrew Kellum, Jr[2], Michael Stone[2], and R. Stephen Lloyd[1]

[1]Oregon Health and Science University
[2]Vanderbilt University Medical Center

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Abstract

Consumption of foods contaminated with aflatoxin B_1 (AFB_1) is a recognized risk factor for developing hepatocellular carcinomas (HCCs). The mutational signature of AFB_1 is characterized by high frequency G > T transversions in a limited subset of trinucleotide sequences. The 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl-formamido)-9-hydroxyaflatoxin B_1 (AFB_1-FapyGua) has been implicated as the primary DNA lesion responsible for AFB_1-induced mutations. This study evaluated the mutagenic potential of AFB_1-FapyGua in four contexts, including hot- and cold-spot sequences as apparent in the mutational signature. Vectors containing AFB_1-FapyGua were replicated in primate cells and the products of replication were isolated and sequenced. Regardless of the sequence context, AFB_1-FapyGua caused base substitutions at frequencies of ~ 80-90%, with G > T transversions being most common. Spectra of mutations were only slightly modulated by the sequence context. These data suggest that mechanism(s) defining sequence context-dependent distribution of AFB_1-induced mutations likely operates prior to replication.

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Irina G. Minko[1], Andrew H. Kellum, Jr[2]., Michael P. Stone[2+], R. Stephen Lloyd[1,3+]

[1] Oregon Institute of Occupational Health Sciences, Oregon Health & Science University, 3181 SW Sam Jackson Park Rd., Portland, OR 97239

[2] Department of Chemistry, Vanderbilt University, Nashville, TN

[3] Department of Molecular and Medical Genetics, Oregon Health & Science University, 3181 SW Sam Jackson Park Rd., Portland, OR 97239

[+] Correspondence to: R. Stephen Lloyd, Oregon Institute of Occupational Health Sciences, Oregon Health & Science University, L606, 3181 SW Sam Jackson Park Rd, Portland, OR 97239, USA; Tel: + 1 503 494 9957; Fax: + 1 503 494 4278; Email: lloydst@ohsu.edu

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Abstract

Consumption of foods contaminated with aflatoxin B_1(AFB_1) is a recognized risk factor for developing hepatocellular carcinomas (HCCs). The mutational signature of AFB_1 is characterized by high frequency G

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INTRODUCTION

Several environmental mutagenic agents, including ultraviolet light, components of tobacco smoke, aristolochic acid, and aflatoxin B_1 (AFB_1), are established human carcinogens (Report on Carcinogens, National Toxicology Program, U.S. Department of Health and Human Services, Public Health Service, https://doi.org/10.22427/NTP-OTHER-1003). Following exposures of cellular DNA, these agents or their metabolic byproducts induce distinct mutational patterns that differ by the types of mutations, local sequence contexts in which base substitutions preferentially occur, and distribution of mutations throughout genome (Kucab et al. 2019; Alexandrov et al. 2020; Volkova et al. 2020). Such agent-specific mutational signatures are evident in deconstructed profiles of somatic mutations in human cancers (Catalogue Of Somatic Mutations In Cancer (COSMIC), https://cancer.sanger.ac.uk/signatures/) (Alexandrov et al. 2020).

AFB_1 is implicated as a significant risk factor for developing hepatocellular carcinoma (HCC) (reviewed in (Kobets et al. 2022)). The most common route of human exposure to $AFB_1$is through consumption of food products contaminated with*Aspergillus flavus* and other related fungi producing this toxin (reviewed in (Kensler et al. 2011)). Although the carcinogenic effect of AFB_1 has been recognized for several decades, such exposures remain widespread in many regions of Asia and sub-Saharan Africa (Benkerroum 2020). As an example, recent analyses of somatic mutations in 163 HCCs from Chinese patients demonstrated that ˜ 10% of these cancers showed a typical signature of AFB_1 (Zhang et al. 2017). The COSMIC data base reports the AFB_1 footprint in 19 out of 493 HCCs (single base substitution (SBS) signature 24) (https://cancer.sanger.ac.uk/signatures/sbs/sbs24/).

Following ingestion, AFB_1 is metabolically activated by microsomal enzymes to an epoxide, which can intercalate into DNA and covalently bind to it, predominantly at the N7 position of guanine (reviewed in (Kensler et al. 2011; McCullough and Lloyd 2019)). This initial cationic*trans* -8,9-dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin B_1 (AFB_1-N7-Gua) adduct is chemically unstable and can either undergo hydrolytic base loss giving rise to an apurinic (AP) site, or interact with a hydroxyl to produce the imidazole ring-opened*trans* -8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl-formamido)-9-hydroxyaflatoxin B_1 (AFB_1-FapyGua) (**Figure 1**) (Croy and Wogan 1981). Based on numerous observations summarized below, the AFB_1-FapyGua adduct is implicated as a major contributor to AFB_1-induced mutagenesis.

In contrast to the initial AFB_1-N7-Gua adduct that rapidly disappears from cellular DNA, AFB_1-FapyGua persists much longer. Specifically, while it comprised ˜80% of the AFB_1 adducts detected in rat liver DNA 72 h post intraperitoneal injection (Croy and Wogan 1981), in mice, it represented ˜95% of the total AFB_1 adducts at 48 h post injection (Vartanian et al. 2017; Coskun et al. 2019). Additionally, prior investigations, using site-specifically modified DNA demonstrated that AFB_1-FapyGua was highly mutagenic in *Escherichia coli* and primate cells (reviewed in (McCullough and Lloyd 2019)). When analyzed in primate (COS7) cells using a SV40-based shuttle vectors, it induced ˜84% G to T transversions, 8% G to A transitions, 2.5% G to C transversions, and 2.5% single nucleotide deletions, with only ˜3% of progeny vectors containing no mutations at the target site (Lin et al. 2014). Furthermore, there is a correlation between spectrum of AFB_1-FapyGua-induced base substitutions (Lin et al. 2014) and the mutagenic properties of AFB_1. These have been investigated by a variety of methods, ranging from the assessment of mutations in vector DNA that was pretreated with activated AFB_1 and replicated in cells, to sequencing of genomic DNA isolated from exposed animals (reviewed in (McCullough and Lloyd 2019)). Consistently throughout these investigations, the data

implicated DNA damage at guanines as a major contributor to mutagenesis, with G > T transversions being the predominant type.

Beyond the overall mutagenic G > T signature, AFB_1-induced base substitutions showed non-random, sequence context-dependent distribution (Chawanthayatham et al. 2017; Huang et al. 2017; Zhang et al. 2017; Fedeles and Essigmann 2018; Kucab et al. 2019; Alexandrov et al. 2020; Volkova et al. 2020). When frequencies of AFB_1-induced base substitutions were normalized against frequencies of trinucleotide sequences, the dominant G > T transversions concentrated in the CGC and CGG sequence contexts (Fedeles and Essigmann 2018). The molecular mechanisms accounting for difference with respect to sequence contexts are currently unknown. The present study addressed the question whether mutational signature of AFB_1 can be explained by differential, sequence-dependent fidelity of replication past AFB_1-FapyGua. Site-specifically modified vectors containing this adduct in four different local sequence contexts were constructed, replicated in COS7 cells, and progeny DNA analyzed for mutations.

MATERIAL AND METHODS

2.1 Preparation of AFB_1-FapyGua-containing oligodeoxynucleotides

The 11-mer oligodeoxynucleotides site-specifically modified with AFB_1-FapyGua were prepared from unmodified oligodeoxynucleotides containing the unique N7-guanine alkylation site (5'-CCAT*AGC* TACC-3', 5'-CCAT*CGC* TACC-3', 5'-CCAT*TGC* TACC-3', or 5'-CCAT*CGA* TACC-3', where the target and neighboring sites are underlined). These were annealed with the corresponding 6-mer oligodeoxynucleotides (5'-TAGCTA-3', 5'-TAGCGA-3', 5'-TAGCAA-3', and 5'-TATCGA-3') to form partially double-stranded DNA structures and reacted with AFB_1 *exo* -8,9-epoxide. The conditions for chemical modifications, purification by reverse-phase HPLC, and characterization by mass spectrometry were previously described (Li et al. 2015; Tomar et al. 2021).

2.2 Construction of single-stranded DNA vectors containing site-specific AFB_1-FapyGua

To evaluate the mutagenic potential of AFB_1-FapyGua in different sequence contexts, we utilized the pSBL vector system, with the AFB_1-FapyGua-containing 11-mer oligodeoxynucleotides inserted between the *Eco* RV sites in a stem region of single-stranded pSBL, using the same conditions as previously described (Minko et al. 2017). Vectors were purified using Amicon Ultra 100K centrifugal filters (Merck Millipore, LTD) with TE buffer (10 mM Tris-HCl (pH 7.4) and 1 mM EDTA).

2.3 Mutagenesis assays

Transfection of COS7 cells with modified vectors, isolation of replication products, and obtaining the individual clones using *E. coli* cells were performed according to the published procedures (Minko et al. 2017). The plasmids were sequenced by DNA Sequencing Core, Vollum Institute, Oregon Health & Science University.

2.4 Statistical Analyses

The mean frequencies of base substitutions with standard errors were calculated from three repeats of mutagenesis assays. To evaluate statistical significance of the differences observed, the *p*values were calculated by the Student's *t* -test, using KaleidaGraph 4.1 software (Synergy Software, Reading, PA).

RESULTS AND DISCUSSION

3.1 Mutagenic properties of AFB_1-FapyGua in different sequence contexts

The single-stranded pSBL vectors containing AFB_1-FapyGua in the AXC, CXC, TXC, and CXA (X = AFB_1-FapyGua) sequence contexts were constructed and replicated in COS7 cells. The CGC sequence has been identified as a hot-spot for AFB_1-induced G > T transversions, while the AGC, TGC sequences and especially the CGA sequence were less prone to these substitutions (Fedeles and Essigmann 2018). Following isolation of the progeny DNAs, individual clones were obtained by transformation of*E. coli* DH5α cells and subjected to Sanger sequencing. The experiments were conducted in triplicate, beginning from transfection of COS7 cells. The sample size was fixed in advance at the level of ten clones per DNA isolate, excluding

clones that either did not contain the insert sequences (˜ 20%) or had alterations others than point mutations (**Table 1**).

These data revealed that regardless of the sequence contexts, AFB_1-FapyGua was highly mutagenic, inducing base substitutions at frequencies of ˜ 80-90% (**Figure 2**). The difference between sequence contexts with regard to non-mutagenic replication was insignificant. Similar to results of the prior study, in which the TXA sequence was utilized (Lin et al. 2014), mutational spectra were dominated by G > T transversions. In addition, G > A transitions and G > C transversions were observed in each of the four sequences tested. The levels of G > A transitions were higher in the AXC and CXA sequences relative to the CXC and TXC sequences, with difference observed for the AXC versus CXC sequence approaching significant level ($p = 0.057$). The level of G > C transversions was elevated in the CXA sequence; relative to the AXC sequence, the difference was significant ($p = 0.047$).

3.2 Mutagenic potential of AFB_1-FapyGua in relation to mutational signature of AFB_1

High levels of base substitutions at the C/G sites, predominantly C/G > A/T transversions, is a common feature of DNA isolated from AFB_1-exposed cells or animals and human AFB_1-driven cancers (COSMIC signature SBS24) (Chawanthayatham et al. 2017; Huang et al. 2017; Zhang et al. 2017; Fedeles and Essigmann 2018; Kucab et al. 2019; Alexandrov et al. 2020; Volkova et al. 2020). AFB_1-FapyGua has been implicated as a major contributor to AFB_1-induced mutagenesis and carcinogenesis (reviewed in (McCullough and Lloyd 2019)) particularly, based on observed correlation between the mutational spectra of AFB_1 and the miscoding properties of AFB_1-FapyGua in replication bypass reactions (Lin et al. 2016) and cultured primate cells (Lin et al. 2014). However, a limitation of these site-specific studies was that the analyses were restricted to a single, TXA, local sequence context. Here, we substantiated the hypothesis about a major role of AFB_1-FapyGua in AFB_1-induced mutagenesis and carcinogenesis by demonstrating its high potential for inducing base substitutions, including high frequency G > T transversions, in four additional sequence contexts (Figure 2).

The G > T transversions that constitute signature SBS24 are not distributed randomly, but preferentially arise in a limited subset of trinucleotide sequences (Chawanthayatham et al. 2017; Huang et al. 2017; Zhang et al. 2017; Fedeles and Essigmann 2018; Kucab et al. 2019; Alexandrov et al. 2020; Volkova et al. 2020). Several mechanisms have been considered through which this signature is manifested, including sequence-dependent DNA adduct formation, stability of the initial AFB_1-N7-dG adduct, rates of repair, and fidelity of replication bypass. The present data, in combination with previous analyses (Lin et al. 2014), strongly suggest that differential fidelity of replication bypass of AFB_1-FapyGua does not account for the occurrence of these mutational hot-spots since the adduct was highly mutagenic in all five sequence contexts. The difference between the hot-spot CGC sequence and three other sequences analyzed in the present study was insignificant (Figure 2). Thus, mechanism(s) that define sequence-dependent occurrence of AFB_1-induced mutations likely operates prior to replication. Consistent with such possibility, we recently have demonstrated that sequence context modulates the catalytic efficiency of human glycosylase NEIL1 acting on AFB_1-FapyGua, with rates of reactions being inversely correlated with thermal stability of the adduct-containing DNA (Tomar et al. 2021). We speculate that AFB_1-FapyGua in a more thermally-stable local environment would be more likely to escape repair not only by NEIL1, but also by nucleotide excision repair and thus, largely contribute to the final mutational signature of AFB_1. In support for a role of pre-replication events in shaping the final mutational signature of AFB_1 is the distribution of AFB_1-induced mutations throughout genome: these are enriched in the intergenic and late-replicating regions and the signature has a transcriptional strand asymmetry (https://cancer.sanger.ac.uk/signatures/sbs/sbs24/).

3.3 Replication bypass of AFB_1-FapyGua: modulation by sequence context and proposed mechanisms

Although AFB_1-FapyGua was nearly equally mutagenic in all sequence contexts tested, the spectra of mutations were partially affected by the identities of neighboring nucleotides. Specifically, there was a trend for elevated frequencies of G > A transitions in the AXC sequence context. The frequency of G > C transversions

was increased in the CXA context. These observations suggest that replication bypass of AFB_1-FapyGua can proceed via the primer-template misalignment/realignment mechanism, with the 5' adjacent nucleotide being utilized as a template instead of modified nucleotide. It is possible that at least a fraction of G > T transversions generated in either the TXC context or the previously tested TXA context (Lin et al. 2014), is formed via a similar mechanism.

Our data demonstrate that even in the absence of a T at neighboring positions, dAMP is preferentially inserted opposite AFB_1-FapyGua in primate cells. The mechanism accounting for such a mutagenic event is currently unknown. It could be formation of a mispair between the modified nucleotide and incoming dATP, as was proposed for unsubstituted FapyGua (Gehrke et al. 2013), or non-templated dAMP incorporation, according to the "A rule" (Strauss 2002), as previously proposed for α-anomers of nitrogen mustard-induced FapyGua (Minko et al. 2017). With regard to the later proposal, AFB_1-FapyGua exists as a complex mixture of various isomeric species that include interconvertible α- and β-anomers (Brown et al. 2009; Li et al. 2015). The fraction of α-anomeric species is substantial in single-stranded DNA (Brown et al. 2009).

The identity of DNA polymerase(s) responsible for bypass of AFB_1-FapyGua has been addressed by biochemical studies with recombinant human DNA polymerases (Lin et al. 2014). The majority of these enzymes could not synthesize DNA past AFB_1-FapyGua. The only polymerase that could, pol ζ, did it in an extremely error-prone manner by preferentially inserting a dAMP opposite the adduct and extending exclusively from this mispair. Further validation of the role of pol ζ in the bypass of the AFB_1-FapyGua adduct was obtained from differential cytotoxicity, cell cycle progression, and chromosomal abnormalities when cells proficient or deficient in pol ζ were challenged with the AFB_1 epoxide (Lin et al. 2016).

3.4 Mutagenic potential of AFB_1-FapyGua: COS7 cells versus *E. coli*

Investigations on mutagenic properties of AFB_1-FapyGua in *E. coli* utilized the CXA sequence context (Smela et al. 2002). Comparison of these data with results of the present study demonstrated dramatically lower fidelity of replication bypass of AFB_1-FapyGua in primate cells. While only three clones out of 30 analyzed had no mutations in this sequence context in our data set (**Figure 2**), sequences with the correct G at the target site were generated in *E. coli* at frequencies of ˜90% under normal conditions or ˜60% following induction of SOS response.

CONCLUSION

The mutagenic properties of the major AFB_1-induced FapyGua adduct were assessed in primate cells using site-specifically modified vectors with the adduct being positioned in four different sequence contexts. Regardless of the context, AFB_1-FapyGua was highly mutagenic, predominantly causing G > T transversions. These data provide further evidence for a primary role of this adduct in AFB_1-induced mutagenesis. No correlation was apparent between the frequencies of AFB_1-FapyGua-induced G > T transversions and the relative occurrences of these mutations in corresponding trinucleotide sequences following AFB_1 exposure. Collectively, these data suggest a role of pre-replicative events in defining the mutational signature of AFB_1.

AUTHOR CONTRIBUTIONS

Irina G. Minko: data collection and manuscript writing; Andrew H. Kellum Jr.: preparation of modified oligodeoxynucleotides; Michael P. Stone and R. Stephen Lloyd: study design and manuscript editing. All authors edited the manuscript and approved its submission.

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Table 1. Complex mutation and targeted deletion in clones isolated following replication of AFB_1-FapyGua-containing vectors in COS7 cells.

Sequence context	Identity of mutation
CXC	Deletion starting at the -1 position relative to the lesion site: 5'-CCATCXCTACC-3' to 5'-(˜ 600 nt Δ)TACC-3'
CXC	Complex mutation: 5'-*C*C*AT*CX*C*T*A*CC-3' to 5'-*GC GG*CG*AT G*CC-3'

Figure legends

Figure 1. Structure of AFB_1-FapyGua.

Figure 2. Spectra of base substitutions induced by AFB_1-FapyGua following replication of site-specifically modified vectors in COS7 cells. The data were collected from three biological replicates. The uncertainties are standard errors.