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Cellular Responses to Aflatoxin-Associated DNA Adducts

Michael Fasullo

Abstract

Aflatoxin B1 (AFB1) is the most potent known hepatocarcinogen. The signature p53 mutation (p53 249^{ser}) that is found in AFB1-associated liver cancer suggests that AFB1 is a potent genotoxin. AFB1 is not genotoxic *per se* but is metabolically activated by cytochrome P450 enzymes that convert the promutagen into a highly reactive epoxide, which primarily reacts with the N⁷ group of guanine, forming 8,9-dihydro-8-(N⁷-guanyl)-9-hydroxyaflatoxin B1 (AFB1-N⁷-dG). While this primary adduct is unstable, the subsequent trans-8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimidin-5-yl-formamido)-9-hydroxy aflatoxin B1 (AFB1-Fapy)-derived adducts are stable and are mutagenic. Studies have revealed that nucleotide excision repair (NER), base excision repair (BER), recombinational repair, and DNA replication bypass are all involved in conferring AFB1 resistance. To minimize the genotoxicity of AFB1, pathways function to detoxify the metabolically active intermediate, excise resulting DNA adducts, bypass unrepaired adducts, and repair secondary DNA breaks. How these repair pathways functionally cooperate to minimize AFB1-associated genetic instability phenotypes is not well understood. Insights can be gained from epidemiological research and model organisms. Gene profiling and next-generation sequencing are facilitating how pathways and tissue-specific differences are induced. This review will encompass studies concerning human genetic susceptibility to AFB1 and pathways that repair and tolerate AFB1-associated DNA damage.

Keywords: aflatoxin B1, liver carcinogenesis, DNA damage tolerance, oncogenes, p53

1. Introduction

The mycotoxin aflatoxin B1 (AFB1) is the most potent known liver carcinogen [1] and is also a lung [2] and esophageal carcinogen [3]. The International Agency for Research and Cancer (IARC) has classified AFB1 as a Group 1 human carcinogen [1]. AFB1 was discovered as the causative chemical agent in Turkey X disease, so named after a 1960 occurrence where 100,000 turkeys in Great Britain died after feeding on contaminated peanut meal imported from Brazil [4]. Its notoriety is underscored by its persistence in grain supplies, ground nuts and animal feed, which must be continually monitored [5]. Produced by *aspergillus parasiticus* and *aspergillus flavus*, the mycotoxin is a particular problem in subtropical areas of China, and in tropical areas of Southeast Asia and Africa [6, 7]. In temperate climates, such as in North America, high levels of AFB1 contamination have been found in corn and nuts, such as almonds and pistachios [8]. To minimize health risks in humans, the Food and Drug Administration (FDA) mandates that the

human food supply contain no more than 20 ppb AFB1 [5]. While human food supply is relatively protected in developed countries, outbreaks of acute mycotoxin contamination have been noted in third world countries and among animals, as recently as 2006 [5]. Although the incidence of acute aflatoxicosis is rare, it is estimated that a large fraction of the population in the developing world are chronically exposed to AFB1 and thus at a higher risk for aflatoxin-associated cancer, especially liver cancer [6].

Liver cancer ranks third in all worldwide cancer mortalities [9–11] and ninth in cancer mortalities in the United States [12, 13]. 4–28% of cancer cases are related to AFB1 exposure [2]. Most liver cancer is characterized as hepatocellular carcinoma (HCC). HCC is highest where there is both a high rate of hepatitis B (HBV) [14, 15] and C virus (HCV) infection [15–17] and high levels of AFB1 contamination in the human food supply, especially in areas of Southeast Asia, China and Africa [6]. Interestingly, the incidence for liver cancer is higher in men than women, regardless of whether the cancer is associated with AFB1 exposure [18]. Because diagnosis is often late and there is no effective treatment for late-stage cancer, the five year survival rate is low in both men and women [12, 13]. The carcinogenic potency of AFB1 is correlated with AFB1 being a strong genotoxin, the signature p53 mutation, p53 249^{ser} [19, 20], is found in 40–60% of all liver cancer derived from patients in heavily contaminated areas [2]. Animal studies have further strengthened the idea that AFB1 carcinogenicity is associated with its genotoxicity; AFB1-associated DNA adduct levels are directly proportional to the number of the animals stricken with liver cancer [21, 22].

Observations that HCC incidence is correlated to AFB1 exposure continues to motivate biomedical researchers to study the repair and toleration of AFB1-associated DNA adducts, the cellular response to these DNA adducts, and associated factors that may enhance or mitigate the high mutagenicity of the DNA adducts in humans. This review will address (1) associated risk factors that enhance or synergize with AFB-associated DNA adducts that increase liver cancer incidence, (2) genetic instability phenotypes associated with AFB1-associated DNA adducts, and (3) repair mechanisms that have been elucidated in model organisms and conserved in humans, (4) cellular responses that enhance repair mechanisms, and (5) future directions in understanding the contributions of genes in AFB1-associated DNA repair. In particular, novel research that addresses epigenetic factors that can alter the repair of AFB1-associated genotoxic damage will be addressed.

2. Progression of HCC

Liver cancer progression is slow and the median age of onset is 60–65 years [11]; populations in areas that are at high risk for environmental and life-style factors are exceptions. For example, the incidence of liver cancer in the Qidong province of China peaks at 45 years [9, 11]. HCC generally develops as a consequence of liver injury, whether caused by chronic hepatitis or cirrhosis, which leads to chronic inflammation and deposition of connective tissue. Chronic hepatitis leads to upregulation of mitogenic pathways, partially through epigenetic mechanisms [23]. Monoclonal populations of dysplastic hepatocytes may exhibit telomere erosion and re-expression of telomerase to maintain viability. Eventual malignant cells accumulate irreversible genetic alterations [23]. As the transformed phenotype advances, the rate in the accumulation of genetic alterations increases [24]. The exact threshold for the number of mutations or alterations present in liver cancer has not been established. Thus, the progression of liver cancer is associated and is accelerated with the accumulation of genetic mutations and altered gene expression patterns.

2.1 Mutations that contribute to liver cancer

Understanding which HCC-associated genetic changes are associated with AFB1 exposure requires a comparison of the genomic alterations that occur in sporadic HCC or HCC associated with other causes. For sporadic HCC, similar to solid tumors, there is both a multiplicity and heterogeneity in genetic alterations in HCC [23–25]. In general, these genetic alterations can be grouped into those that result in loss of function and those that result in gain of function. Genetic alterations that result in loss of function include dominant negative mutations and recessive mutations, which are expressed after loss of heterozygosity (LOH).

Among sporadic tumors, both loss of heterozygosity (LOH) and mutations have been found in HCC tumors. Among 363 patients, The Cancer Genome Atlas Research Network [25], report that the most heavily mutated gene was TP53 (31%), encoding p53, followed by WNT pathway member CTNNB1 (27%), encoding β -catenin, and AXIN (8%), encoding a WNT signaling scaffolding protein, and chromatin remodeling genes (12%) [25]. In greater than 10% of HCC, mutations are found in CDH1, TP53, IGF2H, RB1, CDKN2A, PTEN, KLC, TP73, EXT, MLH1, THRB, THRA, E2F5, and CTNNB1 [23]. Whether these mutations occur early or late in the etiology of liver cancer is still not understood. While the p53 gene functions in controlling the DNA damage response and apoptosis, the WNT pathway is important in controlling cell proliferation [23]. Many of the mutagenic events result from G to T transversions, unlike events found other tumors. The strong bias for G to T transversions suggests that these genetic alterations likely result from chemical DNA damaging agents, rather than spontaneous events, such as cytosine deamination [23]. While the heterogeneity in genetic mutations may reflect multiple mechanisms for liver cell transformation, identifying alterations in HCC are informative in understanding the etiology and possible treatment of individual cancer cases. For example, β -catenin defective liver cancer may be easier to treat than liver cancer resulting from multiple mutations [23–25].

In addition to mutation and LOH events, gain-of-function genetic alterations may confer higher levels of oncogene expression and thereby accelerate carcinogenesis [26–29]. Such alterations could include gene amplification events, such as c-N-methyl-N'-nitro-N-nitroso-guanidine HOS transforming gene (c-MET) and cyclin D (CCND1) [27]. Other gains of function mutations include mutations in the promoter for telomerases reverse transcriptase (TERT) promoter. TERT mutations frequently were shown to be among the earliest and most prevalent neoplastic events in HCC [28, 30].

Both epidemiological and molecular pathology studies have facilitated the identification of which genetic alterations are likely to be associated AFB1 exposure. Mutated genes found in HCC from areas with high AFB1 exposure include p53 and β -catenin [30]. The p53 249^{ser} mutation shows a strong correlation with HCC associated with AFB1 exposure, while is less frequent or absent in HCC from localities where there is little AFB1 exposure [19, 20]. For example, among have HCCs from southern Guangxi province of China, an area of high AFB1 exposure, the p53 249^{ser} mutation was found in 36% of tumors [30]. CTNNB1 mutations and β -catenin protein accumulation in human hepatocellular carcinomas is also associated with high exposure to AFB1, although it is less clear whether these mutations directly result from AFB1-associated DNA adducts [30]. Whether these mutations must occur early or late in cancer progression is still unclear. One hypothesis is that initial mutations confer a higher level genetic instability that is aggravated by further exposure to genotoxic agents.

To determine whether mutations found in HCC confer higher levels of genetic instability and a higher probability of liver cancer when present in a non-cancerous

liver, scientists have constructed transgenic mice that exhibit similar genotypes found in human cancer. Ghebranious and Sell [31] constructed transgenic mice that were both homozygous and heterozygous for the p53ser246 gene, equivalent to the human p53 249^{ser} mutations. Male mice expressing p53ser246 increased the incidence of AFB1-associated high-grade tumors to 14%, compared to 0% exhibited by p53+ (wild type) mice [31]. These studies indicate that the mutant p53 249^{ser} may also be a driver of AFB1-associated liver cancer.

The role of inflammation in liver cancer has led to insights into the gender bias of its incidence. Men are afflicted more than women in nearly all age groups; however the prognosis of liver cancer in either sex is about the same [9]. Naugler et al. [18], have shown that inflammatory cytokines, such as IL-6, are more prevalent in men than women, estrogen having a negative effect on IL-6 production. This gender difference is not only true for humans [32, 33] but also for rodents, including mice and rats [31]. The gender bias underscores the notion that inflammatory responses play a role in liver cancer etiology.

2.2 Associated risk factors that accelerate AFB1-associated liver cancer: role of HBV and HCC virus

The incidence of HCC synergistically increases when individuals are both exposed to AFB1 and infected with either HBV or HCV virus. Interestingly, the incidence of high grade tumors in p53ser246 transgenic mice that are HBsAg-positive is 100% [31]. The common molecular mechanisms by which HBV and HCV infection stimulates AFB1-associated genetic instability phenotypes are still not completely understood; HBV is a DNA virus that replicates by reverse transcription while HCC is a RNA virus that replicates by RNA replication and encodes a single polycistronic message [34, 35]. While 257 million individuals are estimated to be infected with HBV, 140 million individuals are estimated to be infected with HCV; and chronic HBV and HCV infection is the leading cause for 60–70% of HCC [35, 36]. Although HCC contains no oncogenes *per se*, HCV-associated carcinogenesis is associated with increase in reactive oxygen species (ROS), ROS-associated genetic instability, inflammation, and hepatocyte proliferations [36]. Similarly, HBV-associated HCC is associated with inflammation and necro-inflammatory liver damage [16, 36]. Both viruses are not cytopathic *per se*; liver damage caused by HCV and HBV is likely induced by viral-specific CD8⁺ T and natural killer cells (NK) [35, 36]. Thus, both HCV and HBV create an inflammatory cellular environment that stimulates repopulation of hepatocytes, enhancing AFB1-associated genetic instability.

However, different pathologies of HBV and HCV infection may accelerate HCC progression at different rates. While the median onset age for HBV-associated HCC is 55 years that of HCV is 65 years [11]. HBV can chronically infect children after transmission from the mother [11, 36]. Once HBV is stably integrated into the host genome, HBV can promote chromosomal rearrangements and mutations in cancer-associated genes and interfere with checkpoint controls [37, 38]. For example, HBV integration can occur in TERT promoters, stimulating expression of telomerase, and near LINE sequences [39]. The HBV-encoded oncogene HBx can activate both Src and Ras signaling and is essential for viral DNA (cccDNA) replication. To facilitate replication, HBx mediates chromatin changes by recruiting histone acetyltransferases to acetylate histone H3. HBx is also thought to interact with p53 249^{ser}, and attenuate DNA repair and apoptosis [20].

Besides stimulating host cell replication, HBx may also interfere with the host cell's DNA repair pathways and promote genetic instability and replication [38, 40–44]. HBx binds to DNA damage binding protein 1 (DDB1) and cullin-4 (Cul4), which form a ubiquitinase complex, and can perturb the stability of structural

maintenance of chromosome proteins 5 and 6 (Smc5/6) and thus affect DNA replication and DNA damage tolerance [35]. HBx may also interfere with nucleotide excision repair (NER) of AFB1-associated DNA adducts [40–42, 44]. Although less substantiated, HBx is also thought to interfere with PARP1 and decrease excision repair of DNA adducts. Thus HBx drives carcinogenesis by multiple mechanisms that accelerate carcinogenesis.

2.3 Gene polymorphisms associated with AFB1-associated liver cancer

While HBV and HCV infections are the primary factors that aggravate the risk for AFB1-associated HCC, genetic risk factors have also been postulated [45]. With the advent of technologies that accelerate genome sequencing, such as next generation sequencing (NGS), epidemiologists have identified candidate polymorphic genes that increase the risk for aflatoxin-associated liver cancer. Single nucleotide polymorphisms (SNPs) may be located in the amino acid coding region, the introns, or the promoter regions of the candidate genes. Risk factors generally can be grouped into those that (1) are associated with AFB1 metabolic activation and detoxification and (2) that function in DNA repair or DNA damage tolerance genes.

To understand genetic risk factors that affect metabolic activation and detoxification of AFB1 it is necessary to identify genes involved in these pathways. AFB1 is activated by cytochrome P450 enzymes that hydroxylate AFB1 so that the metabolized carcinogen can be rendered hydrophilic and effectively excreted; for review, see [46–48]. Referred to as phase I enzymes and monooxygenases, the cytochrome P450 enzymes contain a heme group at their active sites and catalyze the transfer of single oxygen to specific sites on the target molecule [46]. Cytochrome P450 enzymes require NADPH oxidoreductase (POR) to maintain activity [46]. The P450 enzymes are located in the endoplasmic reticulum in the vicinity of the POR [46]. Of the characterized enzymes expressed by 57 CYP450 genes, CYP1A2 is liver specific and has a high affinity for AFB1, while CYP3A4 constitutes approximately 50% of the hepatic P450 activity. While there have been disagreements over which cytochrome P450 enzymes is chiefly responsible for AFB1 activation in the liver [46, 49, 50], several reports favored CYP3A4 [50, 51], while another report suggested that CYP3A5 has the highest catalytic activity [52]. Among extrahepatic CYPs, CYP2A13 activates AFB1 in the lung, while CYP1A1 catalyzes the formation of AFM1, a hydroxylated AFB1 derivative that can be excreted in milk, which is still carcinogenic [2]. A transient intermediate in the hydroxylation pathway is a highly reactive epoxide, referred to as AFB1-8,9-exo-epoxide (AFBO) (**Figure 1**). This epoxide can be effectively detoxified by either epoxide hydrolases (EHs) or glutathione S-transferases (GSTs), referred to as phase II enzymes [47, 48]. While multiple cytochrome P450s can activate AFB1, the highly reactive epoxide is thought to be the predominant reactive intermediate in all P450 reactions. Thus, gene polymorphisms that increase the risk of HCC could: (1) increase P450 enzyme levels or activation, (2) downregulate phase II enzymes, (3) decrease the repair of DNA existing lesions, and (4) channel the repair of the DNA lesions into mutagenic pathways.

One source of polymorphic enzymes that can influence the fate of AFB1 is glutathione S-transferases that are present in the liver [53]. In the mouse, knock-out of GSTa3 confers extreme AFB1-associated toxicity [54] and GSTa3 expression levels correlate with AFB1-associated liver cancer in young mice [55]. In humans, HCC risk is dramatically increased by SNPs in glutathione S transferase mu1 (GSTM1) and (glutathione-S-transferase theta1) (GSTT1) [56]. Expression of epoxide hydrolase in yeast also leads to detoxification of AFBO [57]; however, polymorphisms associated with epoxide hydrolase only have a weak association with liver

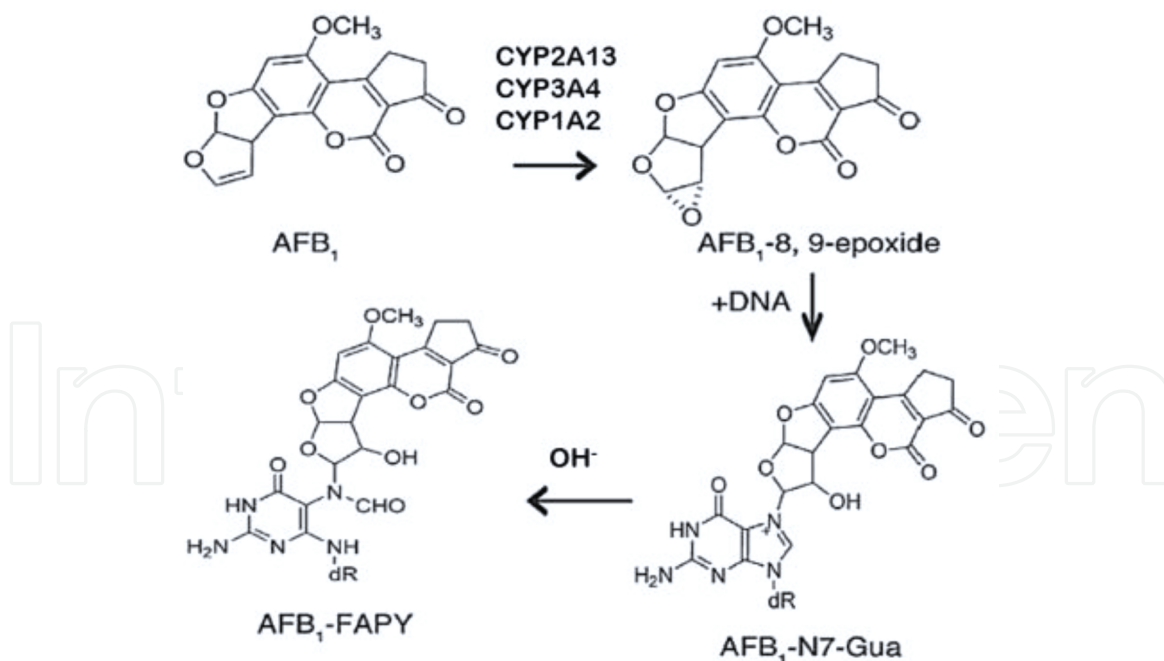


Figure 1.

CYP-mediated metabolic activation of AFB₁ to the activated AFB₁-8,9 epoxide and adduct formation and conversion to AFB₁-Fapy. Adapted from Ref. [130].

cancer [45]. These studies support the idea that detoxification of the highly reactive epoxide is critical in reducing AFB₁ toxicity.

While diminished ability to detoxify AFBO is a risk factor for AFB₁-associated liver cancer, higher or altered P450 activity could also increase HCC risk. HBx activates the pregnane receptor (PXR) and stimulates expression of CYP3A4 [58]. Particular CYP3A5 alleles, such as CYP3A5*3, are correlated with higher levels of expression and aflatoxin-protein adducts in individuals from Gambia, Africa [59]. CYP3A5*3 is present in a high percentage of individuals in Gambia but not in the Caucasian population [59]; the allele found in the Caucasian population confers an altered spliced mRNA, which is poorly expressed [60]. However, establishing correlations between HCC and increased expression of other P450 genes is complicated by the multiple interactions between P450 enzymes.

Genetic risk factors have also been identified among polymorphic alleles of DNA repair and cell cycle checkpoint genes, which may increase chromosomal instability in cells chronically exposed to AFB₁. These risk factors have been found in p53, XRCC1, XRCC3, and ERCC1. The combination of p53 codon 72 Arg72Pro and MDM2 (mouse double minute 2 homolog) SNP309 (T>G) increases the risk of HCC in individuals infected with HBV [61]; p53 codon 72 Arg72Pro affects the frequency of double strand breaks and is associated with hyper-methylation of promoters in tumor suppressor genes [61]. XRCC3 (X-ray complementing defective repair in Chinese hamster cells) encodes a Rad51 paralog which is involved in double-strand break repair and could be involved in error-free bypass of AFB₁-associated DNA lesions. The XRCC3 rs861539 allele (codon Thr241Met polymorphism) is a risk factor for HCC, and the risk is aggravated if individuals are exposed to AFB₁ [62–64]. Other alleles that have been associated with higher risk for HCC include those participating in the base excision repair (BER) and NER pathways, such as XRCC1 rs25487 polymorphism (codon Arg399Gln polymorphism) [65, 66] and XPD rs25487 polymorphism, respectively [67]. These studies reinforce the idea that AFB₁-associated genotoxicity can accelerate HCC progression. To understand the genotoxicity in more detail it is important to understand the nature of the AFB₁-associated DNA adducts.

2.4 AFB₁-associated DNA adducts and cellular targets

AFB₁-associated DNA adducts have been characterized *in vitro* and isolated from organisms that were exposed *in vivo*. DNA exposed to synthesized AFBO reacts predominately with the N⁷ group of guanine bases forming 8,9-dihydro-8-(N⁷-guanyl)-9-hydroxyaflatoxin B₁ (AFB₁-N⁷-Gua), as identified by mass spectrometry analysis. In the presence of hydroxyl ions (base), N⁷-guanine DNA adduct is unstable and decays into an apurinic site and a AFB₁ formamidopyrimidine (Fapy, **Figure 2**) DNA adduct; for review, see [68]. It is unclear whether both apurinic sites and AFB₁-Fapy DNA adducts are equally generated; based on mutations generated by DNA lesions constructed *in vitro*, it has been suggested that AFB₁-Fapy DNA adducts are the primary source of genetic mutations [69, 70], especially G to T transversion mutations that are found in AFB₁-associated liver cancer [71, 72]. The AFB₁-Fapy adduct is stable and can be present in two anomer forms; the alpha and the beta forms. While the beta form is highly mutagenic in *Escherichia coli* [69], the alpha form can stabilize the duplex helix and interfere with DNA replication [70]. In the rat liver, the half-life for AFB₁-N⁷-Gua is 7.5 h, while that for AFB₁-Fapy is at least 24 h [68]. While the AFB₁-N⁷-Gua is unstable, the accumulation of AFB₁-Fapy in the rat liver may also result from differential repair of the two types of DNA adducts.

AFB₁ exposure also generates oxidative stress (ROS) in exposed cultured cells *in vitro* and in the liver and lung *in vivo* [73]. Multiple factors may contribute to

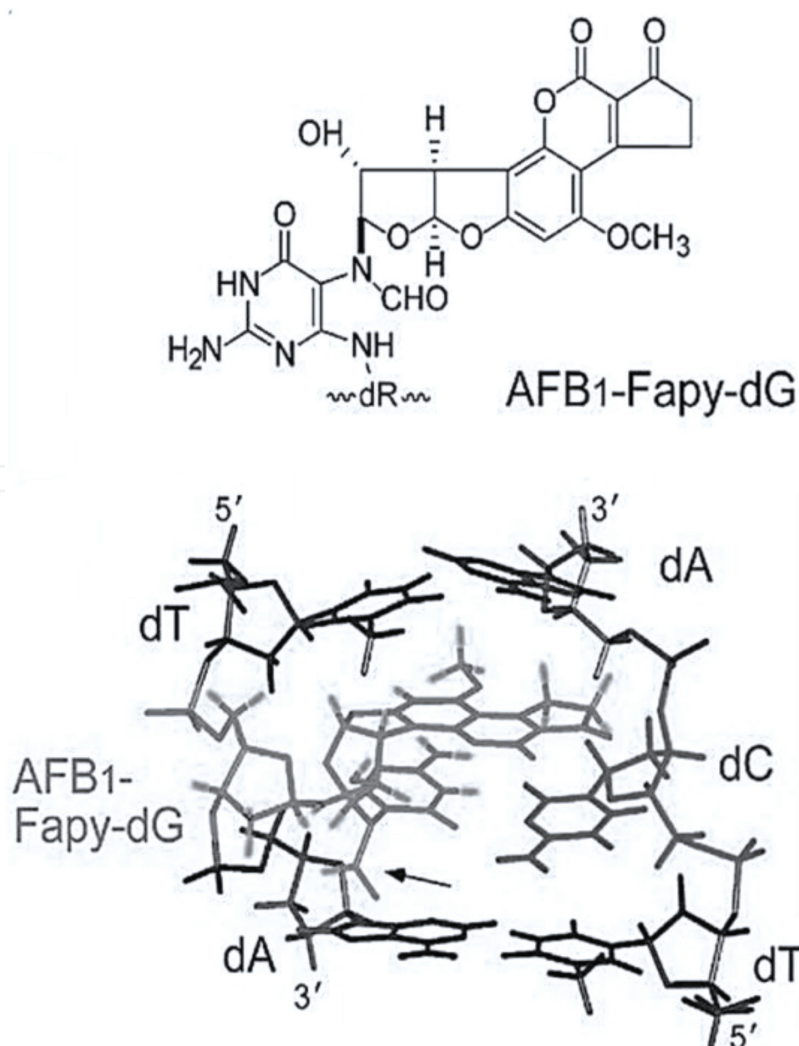


Figure 2.
 Intercalation of the AFB₁-Fapy-dG in duplex DNA. The adduct is in lighter tone; adapted from Ref. [121].

AFB1-associated oxidative stress including cytochrome P450 activity that involves iron-catalyzed reactions and Kupffer cells [68]. Oxidative stress generates hydroxyl radicals that form 8'-hydroxy-2'-deoxyguanosine (8oxodG) DNA damage. AFB1 exposure increases the 8OH-dG in the livers of ducks [74] and rats [75] and cultured woodchuck hepatocytes [68].

Interestingly, Niranjan et al. [76] observed that in rats, AFB1 bound to mitochondrial DNA exceeded the amount that was bound to the nuclear DNA and persisted for a longer period of time [76]. Furthermore, the persistence of mitochondrial DNA adducts correlated with a longer delay in expression of mitochondrial proteins, compared to that of nuclear-encoded proteins. The authors speculated that the persistence of AFB1 in the mitochondria may result from the lack of NER in the mitochondria. These studies support the notion that mitochondria are a prime target for acute effects of AFB1 exposure, and oxidative stress associated with AFB1-exposure could be indirect due to damage to mitochondria and the generation of superoxide.

To further elucidate the pathological consequences of AFB1-associated mitochondrial DNA lesions, Liu and Wang [77] measured AFB1-associated mitochondrial damage in primary broiler hepatocytes by monitoring mitochondrial membrane potential (MMP), ROS generation, apoptosis, and nuclear factor erythroid 2-like factor 2 (Nrf2)-related signal pathway. They observed mitochondrial ROS generation, decreased MMP and induced apoptosis. The increase in apoptotic cells correlated with an increase expression of caspase-9 and caspase-3. They concluded that AFB1 exposure results in a disruption of mitochondrial functions, generating more ROS, and consequently inducing apoptosis while triggering the Nrf2 signaling pathway [77].

2.5 Epigenetic changes associated with AFB1-associated damage

While genetic instability associated with AFB1 have been described, less well known are epigenetic changes. Epigenetic changes are inheritable changes that result in phenotypic changes without affecting the DNA sequence. Epigenetic changes can result from DNA methylation (hypermethylation) or demethylation (hypomethylation), histone modifications, and changes in microRNA (miRNA) expression [78]. AFB1-associated epigenetic changes have been observed in cell cultures, animal studies, and human tumors (**Table 1**). Hypomethylation has been observed to increase the expression of oncogenes and repetitive sequences, while hypermethylation may decrease expression of DNA repair and tumor suppressor genes (**Table 1**). Zhang et al. [79] observed global hypomethylation in AFB1-associated cancers, where particular genomic repetitive elements, such as LINE-1 elements, were hypomethylated; correlating with increased retro transposition and genetic instability [80]. Hypomethylation also correlated with increased expression of the oncogene c-MET, which is associated with accelerated liver cancer progression and poor prognosis [81]. Hyper-methylated genes include the DNA repair gene methylguanine methyl transferase (MGMT) and p16, which have a negative effect on DNA repair and apoptosis [82]. Thus, methylation patterns may possibly serve as biomarker that can indicate increased risk for HCC [83–85].

Additional biomarkers that indicate AFB1 exposure include alterations in miRNA expression. miRNAs are small noncoding RNAs that are generally 19–25 nucleotides in length and regulate gene express at the post-transcriptional level. They are important factors in regulating HCC development in mammalian organisms [87, 88], and a list of miRNAs that correlate with AFB1 exposure is shown in **Table 1**. This comprises a partial group of total miRNAs that have been associated with HCC. Several miRNAs upregulated after rats or liver cell lines are exposed to

Epigenetic change	Gene functions affected	Consequence	Context/ref.
<i>DNA methylation</i>			
DNA hyper methylation	MGMT expression decreased	DNA Repair downregulated	Human tumor tissue [82]
DNA hypo methylation	c-MET, RAB27A, TXNRD1 expression increased LINE 1	Growth and metastasis increased, decreased expression of GSTs, LINE1 transposition increased	Human tumor tissue [79, 81, 82]
<i>miRNA expression</i>			
miR-429 ↓	Downregulates Rab23	Metastasis increased when miR-429 decreased	HCC tumor tissue [83, 85]
miR-4-34a ↑	Downregulation of WNT/β-catenin pathway	Tumor suppressor effect; p53 enhances its expression	HepG2 cell lines [83]
miR-33a ↑	Downregulation of WNT/β-catenin pathway	Tumor suppressor effect; p53 enhances its expression	HepG2 and normal cell lines [83]
miR-24 ↑	Inhibition of apoptosis	Larger tumor size	HCC tumor tissue [83]
miR-34a-5p ↑	c-MET, CCND1, CCNE2 suppressed	Cell cycle arrest	<i>In vivo</i> rat livers [83]
miR-122 ↓	CUTL1 suppressed in mice	Required for tumor differentiation	Human studies [83, 88]
miR-138-1* ↓	PDK1 and indirectly PI3K/PDK/Akt	Inhibits colony formation, migration, invasiveness	P450-B-2A13 human cell culture [83]
<i>Histone modification</i>			
H3K9me3 ↑	Repression of gene expression (multiple)	Reprogramming of pluripotency	Porcine oocytes [83]
H3K27me3 ↓	Repression of gene expression (multiple)	Developmental gene programing in stem cell differentiation	Porcine oocytes [83]
H3K4me2 ↓	Activating gene expression (multiple)	Developmental gene programing in stem cell differentiation	Porcine oocytes [83]
↑, up arrow designates upregulation; ↓, down arrow designates down regulation.			

Table 1.
Epigenetic changes associated with AFB1 exposure.

AFB1 may be protective by downregulating cell proliferation, while upregulated miRNAs found in AFB1-associated HCC may promote tumor size or carcinogenesis. While an individual microRNA may target multiple genes, the expression of individual microRNA can be influenced by multiple transcriptional and epigenetic factors, as well as by genomic changes. These factors include CpG methylation, c-Met signaling, and gene copy number.

Among HCC tumor cells associated with AFB1 exposure, upregulation of several miRNAs, such as miR-429 and miR-24 [86], are associated with larger tumor size [83]. In human bronchial epithelial cells that express CYP2A13 (P50-B-2a13 CELLS), AFB1 exposure induces malignant transformation of immortalized cells [89]. Among transformed cells, one downregulated miRNA was miR-138-1, observed to inhibit proliferation, colony formation, and transformation of P50-B-2a13 CELLS [89]. This miRNA preferentially inhibits 3-phosphoinositide dependent protein kinase-1 (PDK1), which lowers the expression of the P13K/PDK/Akt pathway [89]. These studies indicate that changes in miRNA expression in AFB1-associated HCC may promote carcinogenesis.

HBV infection also upregulates the expression of miRNAs in hepatocytes and may promote HBV-associated HCC. The expression of miR106b-25 is upregulated in HCC patients in general, and in HCC patients infected with HBV [90]. Hep 3B cells transformed with an HBx expression plasmid also express higher levels of miR106b-25, compared to cells that do not express HBx. The miR106b-25 is a member of a cluster of miRNAs in MCM7 that downregulate the expression of several tumor suppressors, including p21, E2F, BIM, and pTEN [91]. Thus, HBV infection may not only interfere with DNA repair mechanisms but also epigenetically silence tumor suppressor genes and accelerate HCC progression.

2.6 Mutagenic signatures associated with AFB1-associated DNA adducts

Mutation signatures are useful biomarkers to determine AFB1 exposure and HCC progression. AFB1 is known to induce mutations in *E. coli*, *Saccharomyces cerevisiae* (budding yeast), and in mammalian cells. AFB1 was one of the original carcinogens published in the Ames assay [92, 93]. While G to T transversions are considered associated with chronic AFB1 exposure in humans [19, 20, 68, 71], in *E. coli*, carcinogen-induced transversion mutations require over-expression of expression of MucAB, which encodes the polV error-prone polymerase [94]. In budding yeast expressing either human CYP1A2 or CYP1A1, AFB1 has been shown to increase mutation frequencies at a *CAN1*, *LYS2*, and *URA3*; however the mutagenic signature of AFB1 in yeast has yet to be identified [95, 96]. The mutagenicity of AFB1 in yeast, however, is low compared to many alkylating agents, such as ethyl methane sulfonate (EMS) [95].

While AFB1 is well-known to cause G to T transversion mutation in mammalian cells, other nucleotide substitutions occur, some of which are in the vicinity of the AFB1-DNA adduct. Investigators have used two approaches to determine the DNA sequence context of AFB1-associated mutations; one technique utilizes PCR (QPCR) and ligation-mediated PCR (LMPCR), and the second technique utilizes whole genome sequencing. Using the first technique, Denissenko et al. [97] mapped total AFB1 adducts in genomic DNA treated with AFB₁-8,9-epoxide. In a second experiment, Denissenko et al. [97] mapped total AFB adducts in hepatocytes exposed to either AFB1 activated by rat liver microsomes or AFB1 activated by human liver microsomal preparations. The p53 gene-specific adduct frequencies in DNA, modified in cells with 40–400 μ M AFB1, were 0.07–0.74 adducts per kilobase (kb). *In vitro* modification with 1–4 ng AFB₁-8,9-epoxide per microgram DNA produced 0.03–0.58 lesions per kb. The adduct patterns obtained with the epoxide

and the different microsomal systems were virtually identical indicating that AFB1 adducts share similar sequence-specificity whether occurring *in vitro* and *in vivo*.

With the advent of next generation sequencing (NGS) [98], investigators have studied the entire genome and determine whether particular mutation signatures. Huang et al. [99] determine whole genome sequencing data to determine the position of >40,000 mutations in two human cell lines, and in liver tumors from wild type mice and a transgenic mouse carrying the hepatitis B surface antigen. The mutational signature from all four experimental systems was remarkably similar and compared well with experimental mutational signatures derived from sequenced HCCs from Qidong County in China, an area of high AFB1 exposure [100]. The Catalog of Somatic Mutations in Cancer (COSMIC) mutational signature 24 [101], previously associated with AFB1-associated liver cancer, was confirmed and also shown to be present in a high proportion (16%) in HCC from Hong Kong, but in 1% or less from HCC from Japan or North America. The COSMIC mutation signature 24 indicates guanine damage with a very strong transcriptional strand bias for C>A mutations. Additional studies being performed by multiple research groups [25, 102] confirm the presence of signature 24 in human HCC tumors and in tumors induced by AFB1 in mice. In addition to signature 24, investigators have also noted the presence of transition mutations that might also occur in the context of oxidative stress. It has not been determined which of these minor mutation classes drive HCC.

2.7 AFB1 is a potent recombinagen

In budding yeast expressing CYP1A2, AFB1 is potent recombinagen but a poor mutagen [95]. Exposure to AFB1 stimulates homologous recombination between sister chromatids (sister chromatid exchange or SCE), chromosome homologs, and repeated sequences located on non-homologous chromosomes. Using a recombination assay involving truncated fragments of *his3* [103] positioned on non-homologous chromosomes, Sengstag et al. [95] showed that homologous recombination could be stimulated 50-fold in contrast to a less than 10-fold stimulation of mutations. AFB1 concentrations as low as 5 μ M were shown to be effective at stimulating the formation of reciprocal translocations, and the karyotypes were confirmed by pulse field gel electrophoresis [103]. AFB1 is also a recombinagen in human and Chinese hamster ovary (CHO) cells and can increase the frequencies of SCE [104–107]. It is unclear whether the same AFB1-associated DNA lesions can stimulate both mutations and recombination. For example, it could be possible that particular lesions that stall DNA replication and generate breaks generate more recombination events while other lesions that can be bypassed by DNA polymerases generate more mutagenic events. These studies thus demonstrate that the genotoxicity of AFB1 extends beyond making mutations and involves stimulating chromosomal rearrangements in model eukaryotic organisms and in humans.

2.8 Repair of AFB1-associated DNA damage

Considering the genotoxicity of AFB1-associated DNA adducts and possible hindrance of DNA replication, it is important to identify which DNA repair pathways and which replication bypass mechanisms are used to tolerate the most persistent AFB1-associated DNA adducts. There are several pathways that are involved in repairing AFB1-associated DNA damage. Among these repair pathways are nucleotide excision repair NER, BER, and recombinational repair; for a general review see [108]. Post-replication repair pathways to bypass DNA adducts involve (1) either error-prone or error-free DNA polymerases, or (2) template switch mechanisms. The later

mechanism involves DNA recombination mechanisms, which are utilized in tolerating UV-induced DNA damage and alkylated DNA bases. While in some organisms there are preferred pathways, a general theme in DNA repair is that organisms have evolved redundant DNA repair mechanisms. A prediction of redundant DNA repair pathways is that eliminating genes in two or more repair pathways should effectively lead to a synergistic decrease in AFB1 resistance, while eliminating genes in the same pathway should confer no greater sensitivity than the most sensitive mutant.

Nucleotide excision repair (NER) involves the recognition of the DNA adduct, the opening of the helix at the DNA damage site, the excision of the DNA adduct and the re-synthesis of DNA using the non-damaged DNA strand as a template. In general, 12–13 nucleotides are excised in prokaryotes (for review see [109]) while 24–32 nucleotides are excised in eukaryotes. Global genome repair (GGR) can occur on either the transcribed or non-transcribed strand. Transcription-coupled repair (TCR) does discriminate and preferentially repairs the transcribed strand. The mechanistic difference between the two pathways is how the DNA adduct is recognized; in GGR specific proteins recognize the DNA helical distortion while in TCR, the RNA polymerase stalled complex is recognized; for general review see [110]. In eukaryotes and prokaryotes, both mechanisms are used. While the mechanism is widely conserved among eukaryotes, the mechanism differs between prokaryotes and eukaryotes in the amount of DNA that is excised.

NER is likely to be the predominant mechanism for the repair of AFB1-associated DNA damage in many eukaryotic and prokaryotic organisms [68]. The AFB1-N⁷-guanine adduct is fairly unstable while the AFB1-Fapy DNA adduct can insert between the base pairs of the DNA double helix [111]. UvrABC from *E. coli* can effectively excise both DNA adducts, although the AFB1-Fapy adduct appears to be more chemically stable [68, 112]. The excision of the DNA adducts does not depend on the SOS response; thus, basal levels of the DNA repair enzymes appear to be adequate in repairing the DNA lesions. In *E. coli*, both AFB1-N⁷-Gua and AFB1-Fapy adduct appear to be excised at a similar rate. One explanation is that the UvrABC complex does not rely on helix distortion to repair the DNA adduct, but rather size and structure of the aromatic rings [68, 112].

Other insights from model organism yeast revealed that the NER genes are required to excise AFB1-associated DNA adducts [96, 113, 114]. *RAD14* (XPA) and *RAD1-RAD10* (XPF-ERCC1) are required for AFB1 resistance. Failure to repair the DNA adducts in a *rad4* (XPC) haploid mutant results in S phase arrest, supporting the notion that particular AFB1-associated DNA adducts interfere with DNA replication [113, 114]. In addition, in *rad4* mutants the level of AFB1-N⁷-Gua DNA adducts was reported to increase three fold [114]. These studies support the notion that the yeast NER pathway recognizes and repairs AFB1-N⁷-Gua DNA adducts.

In mammalian cells, the NER pathway preferential repairs AFB1-N⁷-Gua DNA adducts but still participates in the repair of AFB1-Fapy DNA adducts [115, 116]. In XPA human fibroblast cells, the loss of AFB1-N⁷-Gua DNA is much slower and the accumulation of the AFB1-Fapy DNA adducts is greater compared to wild type cells [116]. XPA^{-/-} deficient mice are also more susceptible to AFB1-associated tumorigenesis compared to wild-type mice [117]. Since the accumulation of DNA adducts correlate with the increased carcinogenicity of the DNA adducts [71, 72, 118], it is likely that the burden of AFB1-associated DNA adducts increases the frequencies of carcinogen-associated mutations in the XPA deficient mice.

The second major pathway to repair DNA involves the BER pathway (for review, see Fortini and Dogliotti [119]). As in NER, the DNA damage base is excised and new DNA is synthesized using the undamaged DNA as template for repair (**Figure 3**). In BER, the modified DNA base is recognized and excised by a specific enzyme that generally referred to as a glycosylase. Subsequently, a apurinic endonuclease (APE1)

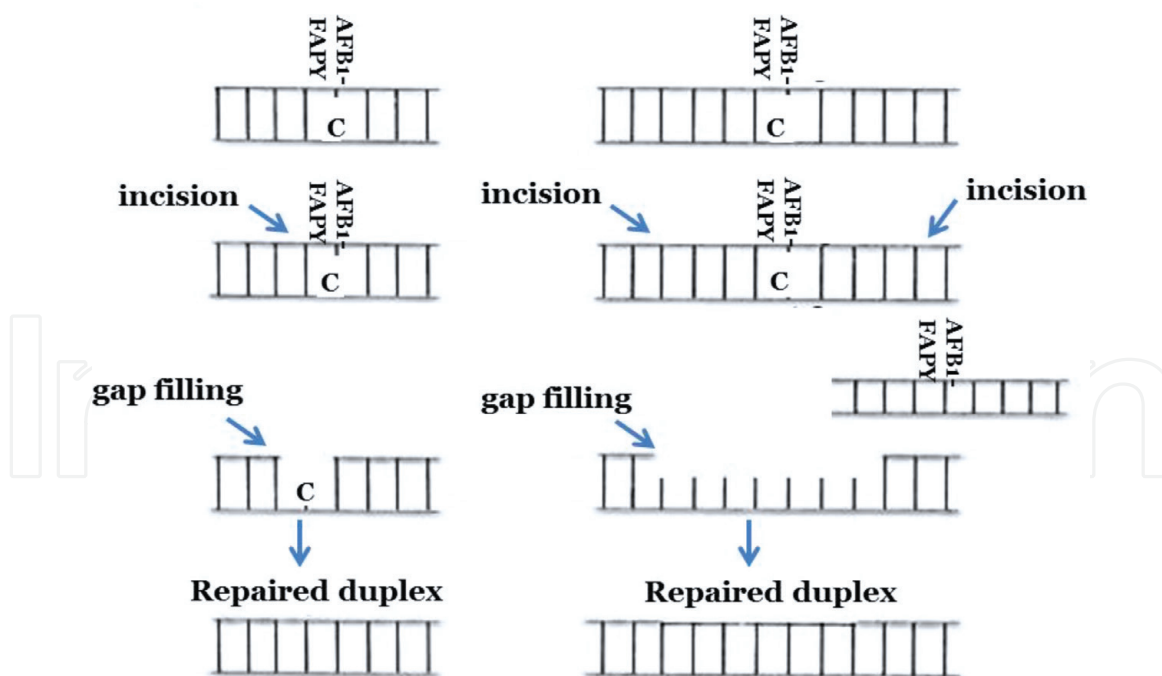


Figure 3.
BER (left) and NER (right) mechanisms to repair the AFB1-Fapy adduct. Both mechanisms involve incision and excision of the damage base, followed by unscheduled DNA synthesis.

generates a 3'OH for primer recognition and new DNA synthesis. In mammalian cells, polymerase β synthesizes new DNA across the gap and removes the deoxyribose residue, and XRCC1/Ligase III cooperate to seal the nick. An alternative pathway that does not involve APE1, employs endonuclease VIII like-1 (NEIL1). Following excision of the damaged base by a $\beta\delta$ excision mechanism, the 3' phosphate is excised by polynucleotide kinase (PNK) to yield a 3'OH for primer recognition and new DNA synthesis. For long patch repair, DNA polymerase δ /PCNA/RFC synthesizes across the gap, the displaced oligonucleotide is excised by FEN1, and the nick is sealed by Ligase I [119]. Poly(ADP-ribose) polymerase PARP1 generally protects the single-strand gap from being subjected to further cleavage or from serving as a substrate for recombinational repair proteins although additional pathways have been proposed [120].

Interestingly, while BER mechanisms have been thought to play a minor role for DNA repair of some AFB1-associated DNA adducts in yeast, BER mechanisms for AFB1-associated DNA adducts can occur in mammalian cells. In budding yeast, the *apn1/apn2* haploid double mutant is no more AFB1 sensitive than the haploid wild type [96]. However, AFB1-associated mutagenesis is lower in the *apn1/apn2* haploid double mutant compared to wild type [96], suggesting that either Apn1 or Apn2 still function in processing the AFB1-associated adducts for post-replication repair. One interpretation of these results is that there is redundancy in both NER and BER mechanisms for conferring AFB1 resistance, while another interpretation is that budding yeast lack the BER enzymes, such as NEIL1, which may actively participate in the repair of AFB1-associated DNA adducts.

In mice, the NEIL1 gene has been isolated and knock-out of the gene leads to higher levels of AFB1-associated DNA adducts and AFB1-associated HCC [121]. The NEIL1 enzyme recognizes and excises AFB1-Fapy-dG adducts in "bubble" DNA structures, such as the one described by Brown et al. [70, 111]. One idea is that AFB1-Fapy-dG adducts may stably intercalate in the helix and be recognized by NEIL1-dependent BER pathway but not by the NER pathway; the repair pathway may thus depend on the DNA sequence context of the AFB1-Fapy-dG adduct. Knock-out of NEIL1 in mice leads to an increase of AFB1-associated tumors and

an accumulation of Fapy-adducts [121]. Vartanian et al. [121] assert that the AFB1-associated carcinogenicity in *Neil1*^{-/-} mice is as high if not higher than that observed in *Xpa*^{-/-} mice, noting that both the size and number of tumors are greater in the *Neil1*^{-/-} mice compared to the *Xpa*^{-/-} mice. However, the investigators indicate that spontaneous tumors arise at a much higher frequency in *Xpa*^{-/-} mice, so that the increase in AFB1-associated tumors were measured until the mice were 11 months in age and not when the mice were 15 months in age.

AFB1 exposure is also associated with oxidative stress, as evident by the accumulation of 8-oxodG lesions. It is particularly interesting whether 8-oxodG accumulates in particular DNA repair mutants and contributes to genotoxicity and the etiology of liver cancer. The contribution of 8-oxodG to overall AFB1-associated genotoxicity is unclear; *Ogg1*^{-/-}/*Ogg1*^{+/+} transgenic mice do not exhibit more AFB1-associated lung tumors than those that are wild type, but do exhibit increased weight loss and mortality [122]. However, *Ogg1*^{-/-} null mice succumb to other cancers after being exposed to oxidizing agents and carcinogens [123]. These studies suggest that AFB1-associated 8-oxodG lesions are not the causative lesions in liver or lung cancer.

The third major pathway in cells that function in AFB1-associated DNA damage is recombination repair. Knocking out *RAD51* in either *rad14* or *rad4* cells leads to a synergistic increase in AFB1 sensitivity in yeast [96, 113]. There are two different explanations. One explanation is that some AFB1-associated DNA lesions that accumulate in *rad4* cells are converted into single or double-strand breaks and require recombinational repair. A single double-strand break has previously been shown to be lethal in strains defective in homologous recombination [124]. An alternative explanation is that cells require *RAD51* to bypass the DNA lesions and accumulate stalled replication forks. Studies have shown that *RAD51* is required for DNA damage-associated SCE [125], which likely occur by replication bypass mechanisms. This second reason is also supported by the notion that *rad4* cells tend to arrest in a small budded stage upon entry into the cell cycle.

2.9 DNA damage tolerance and AFB1-associated DNA damage

DNA damage tolerance pathways allow cellular replication mechanisms to bypass blocking DNA adducts, such as the AFB1-Fapy DNA adduct, resulting in persistence of the DNA adduct in the divided cells. These mechanisms are divided into error-free mechanisms where the original “correct” base is opposite the modified base and error-prone mechanisms where an “incorrect” base is inserted opposite the damaged base, thereby generating mutations. The insertion of the “incorrect” base is generally accomplished by substituting a “high fidelity” polymerase with a lower fidelity polymerase that also has lower processivity. The polymerase switch mechanism is accomplished by a series of ubiquitination reactions on PCNA, which is the processivity factor for DNA polymerase on the DNA template; for review, see [126, 127]. The first ubiquitination reaction of PCNA is a monoubiquitination reaction catalyzed by Rad18/Rad6. Subsequent polyubiquitination of PCNA by Rad5/Ubc13/Mms2 is required for error-free by-pass mechanisms, which includes template-switch mechanisms. Both *RAD18/RAD6* and *RAD5* genes are well conserved in eukaryotes.

The function of replication bypass in conferring AFB1 resistance has been validated in model organisms. In budding yeast, *RAD18*, *RAD5*, *REV1*, and *REV7/REV3* are required for AFB1 resistance [96]. These genes are also required for AFB1-associated mutagenesis [96]. These results indicate that all three translesion polymerases are required for AFB1 resistance, while it is unclear which gene is required for replication bypass of individual AFB1-associated DNA adduct.

While there are only three translesion DNA polymerases in budding yeast, in humans, there are at least 11 translesion polymerases, forming the majority of the 15 DNA template-dependent DNA polymerases [128]. Both the AFB1-N7-Guanine and

the AFB1-Fapy DNA adducts can be bypassed by translesion polymerases [129, 130]. Of the translesion polymerases, DNA polymerase ζ , also referred to as Rev3L the Rev3 homolog in humans, limits chromosomal damage and promotes cell survival following AFB1 exposure [131]. The authors suggest that Rev3 is required for progression through S phase since mouse embryonic fibroblasts, derived from *Rev3L*^{-/-} knock down mice, arrest in S/G2 after AFB1 exposure [131]. These cells also exhibit an increase in gamma-H2AX foci, micronuclei, and chromosomal aberrations; the kinetics of micronuclei formation support a replication-dependent mechanism that results in the accumulation of unrepaired DSBs in. The Rev3 requirement for DNA replication of an AFB1-associated DNA adduct was also demonstrated for a single lesion present on a replicating plasmid in HEK293 cells [131]. Considering the number of mammalian translesion polymerases [128], the Rev3 requirement for replication bypass may reflect the efficiency by which AFB1-associated DNA adducts block other polymerases or Rev3's ability to minimize detrimental chromosomal damage [132].

2.10 Template-switch mechanisms as an alternative mechanism for tolerating DNA damage

Exposure to AFB1 stimulates SCE in multiple organisms. One possible mechanism is that in post-replication repair, processing of AFB1-associated DNA damage generates apurinic sites and/or subsequent DNA single-strand gaps, which initiate SCE by serving as substrates for DNA recombination proteins. Template switch mechanisms are another mechanism (**Figure 4**) that avoid the necessity of using error-prone polymerase for replication bypass. In support of the role of template switching in AFB1-associated SCE, studies have been performed in budding yeast indicating that *rad51* null mutants, deficient in DNA damage-associated SCE [125], exhibit higher frequencies of AFB1-associated mutations [96, 133]. In addition, Rad51 foci appear as cells enter S phase [114] and not in G2, suggesting that the appearance of Rad51 foci are replication-dependent and not associated with double- or single-strand breaks after replication. However, it is possible that multiple mechanisms are involved.

AFB1-associated SCE are also observed in human and mammalian cells. SCEs have been detected in human lymphocytes, Chinese hamster V79 cells, rat and mouse hepatocyte cell lines [104–107]. It has not yet been determined whether mammalian cells defective in homologous recombination exhibit more AFB1-associated mutations. Nonetheless, it is interesting that polymorphisms of XRCC3 [62–64], which functions in homologous recombination, are a risk factor for HCC.

2.11 Tissue specificity of DNA damage repair of AFB1-associated DNA adducts

Since AFB1-associated DNA adducts are found in different tissues, the question can be asked whether there are tissue-specific differences in repair mechanisms. Mudler et al. [134] addressed the question whether oxidative damage caused by AFB1, 8-oxodG, was repaired more efficiently in the mouse lung compared to the mouse liver. They exposed mice to a low chronic amount of AFB1 (0.2 or 1.0 ppm AFB1) and then assayed for the amount of dGTP incorporation. Interestingly, they found that although Ogg1 was present in both the lung and the liver, there was a lower repair efficiency in the liver after exposure to 1.0 ppm AFB1. The lower efficiency of the repair in the liver did not correspond to AFB1-associated cytotoxic effects, and they speculated that the differences could result from AFB1 directly inhibiting Ogg1 [134].

Bedard et al. [135] asked the question whether AFB1-N⁷-Gua and AFB1-Fapy DNA adducts were repaired more efficiently in the mouse liver or lung. They also compared the efficiency of repair in the rat liver and the mouse liver. After exposing mice to 50 mg/kg AFB1, extracts were obtained from the various tissues and used to determine the repair of plasmid DNA AFB1-N⁷-guanine or AFB1-Fapy adducts as

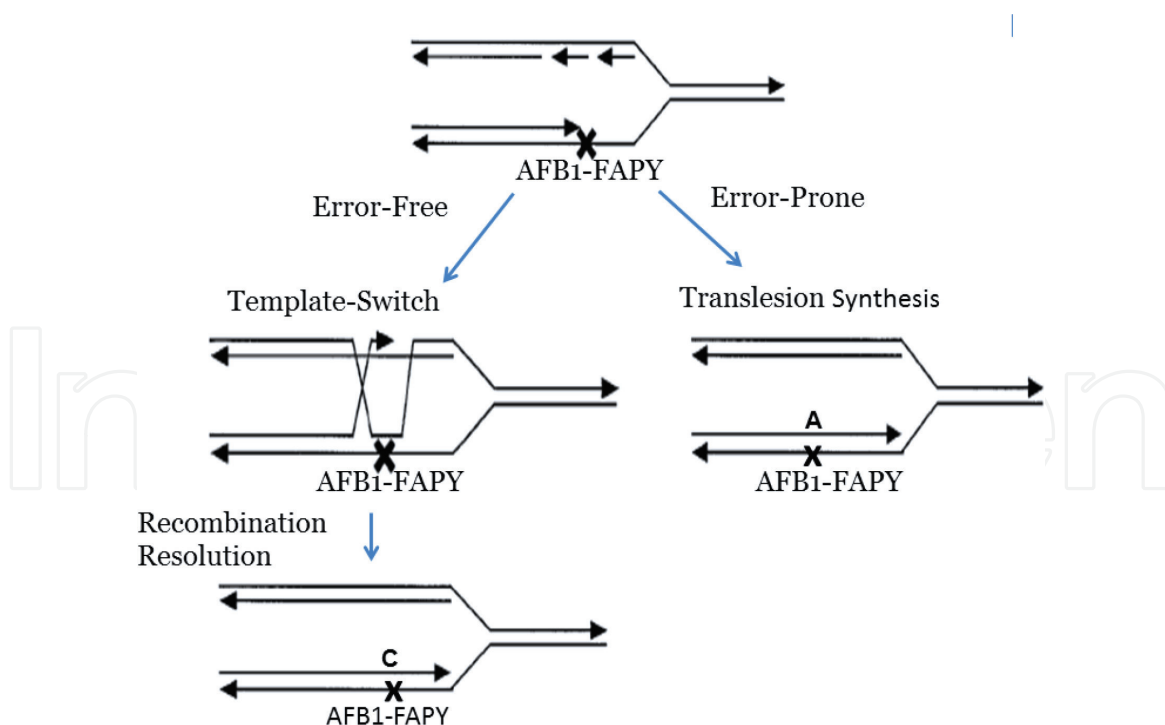


Figure 4.

DNA damage tolerance mechanisms used to bypass a AFB1-Fapy DNA adduct blocking the leading strand polymerase on a growing replication fork. Error-free (left) bypass uses a template switch mechanism while error-prone (right) bypass uses a low fidelity DNA polymerase, resulting in the insertion of an A opposite the DNA adduct.

substrates. Mouse liver extracts repaired AFB1-N⁷-guanine and AFB1-Fapy adducts 5- and 30-fold more effectively, respectively, than did extracts from the mouse lung. Mouse liver extracts also repaired the adducts 6-fold and 4-fold more effectively, respectively, than did liver extracts from rats. They conclude that there is a tissue-specific induction in repair in the mouse liver that renders the mouse liver more resistant to AFB1-associated carcinogenesis. However, further studies are needed to determine which NER and BER enzymes are preferentially induced in the liver.

2.12 AFB1-induction of DNA repair and protective mechanisms

The redundancy in repair mechanisms for DNA adducts in yeast and in mammalian organisms provokes the question of which genes are transcriptionally induced after the exposure of AFB1. Two complementary studies have been performed using budding yeast and several studies have been performed in mammalian cells. While studies in yeast utilized microarrays, more recent studies in mammalian cells have used RNAseq and NGS technology. The common genes that are induced have provided clues into which pathways are shared among eukaryotic organisms.

Keller-Seitz et al. [113] determined which budding yeast genes were induced after exposure to AFB1. Essentially, an exponentially grown culture was concentrated to 4×10^8 cells/ml and then exposed to 25 μ M AFB1 in phosphate buffer (pH 7.5). After RNA was extracted, cDNA was synthesized and labeled for analysis on microarrays. Fourteen DNA repair genes were upregulated more than two-fold, with *RAD51* being upregulated more than seven-fold. Among NER genes, *RAD16*, *RAD3*, and *RAD1* were AFB1-inducible. The upregulation of selected genes was verified by RT-PCR. Additional genes that were induced included those involved in mismatch repair and DNA synthesis, while genes participating in NHEJ were downregulated.

A similar study was done by Guo et al. [136], except AFB1-inducible genes were identified in actively growing cultures. Similar to the Keller-Seitz study [113], *RAD51* was upregulated over seven-fold. However, additional genes involved in

regulating dNTP levels were also upregulated including *DUN1*, which encodes a DNA damage-signaling kinase, and *RNR2* and *RNR4*, which are subunits of ribonucleotide reductase. Although the functional significance of the AFB1-associated inducibility is unknown, there is good overlap with a cluster of genes identified as DNA damage-inducible but not generally stress-inducible [137]; DNA damage-inducible genes from multiple studies include *DUN1*, *RAD51*, *RNR2*, and *RNR4*. In contrast to the previous study by Keller-Seitz, NER genes were not upregulated. In both studies, the DNA damage-inducibility of *RAD51* is *MEC1*-dependent; *MEC1* is the ATM/ATR orthologue of yeast. The functional significance of the upregulation was illustrated by showing that the recombination deficiency exhibited by *mec1* mutants could be partially suppressed by over-expression of *RAD51* [113]. Thus, upregulation of particular DNA repair genes could enhance AFB1 genotoxic effects.

Additional genes that were upregulated in both studies included genes involved in cell cycle control, protein transport, DNA metabolism, and ion homeostasis [113, 136]. Although the functional significance of the upregulation of each of these genes is unknown, many of these genes are involved cell cycle regulation. Interestingly, genes involved in histone biosynthesis were downregulated, reflecting a delay in S phase [136]. The delay in S phase may result from the stability of the AFB1-Fapy DNA adduct during the exposure time.

Identification of AFB1-inducible genes in mammalian cells revealed broader classes of upregulated genes, compared to the yeast studies, reflecting the hepatic cell's ability to metabolize and neutralize xenobiotic agents. Merrick et al. [138] performed RNA seq analysis on liver cells after the rat was injected with AFB1. In brief, RNA was obtained from male rats exposed 1 ppm AFB1 in feed for 90 days, and RNA seq analysis was performed using the appropriate number of unexposed rats as controls. 1026 differentially induced transcripts were identified. Genes upregulated more than five-fold relevant to hepatocellular proliferation include follistatin (442-fold), *Aldh3a1* (302-fold), *Mybl2* (21-fold), *Mybl1* (6-fold), and *Sox9* (6-fold). Genes upregulated and involving the E2f1 transcription factor included *Cdk1*, *Mdm2*, *Ect2*, *Mad2L1*, and *Nuf2*. Of those genes that were upregulated, of particular interest are those involved in DNA damage tolerance and repair. A two to four-fold increase was observed for *Mgmt*, *Top2a*, *Rad51*, *Rad18*, *Xrcc6*, *Mnd1*, and *Tynns* [138]. These studies indicate that chronic AFB1 exposure in animals can also induce DNA repair genes that are involved in cell cycle regulation and DNA replication bypass.

2.13 Signal transduction and checkpoint activation

Both studies in yeast and in mammalian cells indicate that AFB1 triggers a checkpoint response that delays cell cycle progression so that DNA damage can be repaired. The mechanism by which the AFB1 DNA adducts are sensed is unknown. However, it is likely that DNA replication stress triggers S phase delay that is associated with *Rad53* (*Chk2* orthologue) phosphorylation [133]. In budding yeast, exposure to 50 μ M AFB1 is sufficient to delay S phase [133, 135]. The *Rad53* phosphorylation is dependent on *MEC1*, the ATM/ATR orthologue. Fasullo et al. [133] observed that the downstream effector of *Rad53*, *DUN1*, was required for both AFB1-associated mutation and AFB1-associated recombination. However, the substrates for the signaling cascade that affect AFB1-associated recombination and mutation are unknown. One possibility is that *Rad55* phosphorylation is important in triggering AFB1-associated recombination.

In mammalian cells, the DNA damage response to AFB1-associated DNA adducts has been addressed by only a few studies. After exposure to AFB1, HepG2 cells exhibit 53BP1 foci and H2AX foci but not *Chk1* or *Chk2* activation [139]. However, other studies [140] in other cell lines suggest a robust stimulation of the checkpoint response. In human bronchial epithelial cells (BEAS-2B) expressing CYP2A13 and exposed to

low concentrations of AFB1, AFB1-DNA adducts and 8oxodG significantly increased, along with phosphorylation of ATR and BRCA1. In addition, Mre11, Rad50 and Rad51 were significantly increased. These studies suggest that similar to yeast, checkpoint activation leads to higher expression of DNA recombination genes in 3BEAS-2B cells.

3. Conclusions

Liver cancer is the third leading cause of cancer deaths, and unfortunately the incidence of liver cancer is increasing in the USA. Environmental and lifestyle factors include AFB1 exposure and infection with HCV and HBV viruses. AFB1 is a potent liver carcinogen because it is a potent genotoxin and AFB1 exposure is correlated to signature mutations found in HCC. Liver injury and inflammation set the stage for regenerative cell proliferation that enhances AFB1-associated genetic instability. As liver cancer progresses, multiple genetic mutations and epigenetic changes accumulate that eventually accelerate an irreversible path toward malignancy and poor prognosis.

Nonetheless, cellular defense mechanisms have evolved to diminish the AFB1 genotoxicity and repair or tolerate AFB1 DNA adducts so that mutations and chromosomal instability are avoided. First, there are multiple pathways to repair AFB1-associated DNA adducts. These include BER repair involving NEIL1 and NER pathways that excise AFB1-associated DNA adducts. However, it is still unclear which pathway is favored in humans and whether they are redundant. Second, there are common repair and checkpoint pathways that are upregulated in both model organisms and in mammalian organisms; these include ATR signaling pathways and recombinational repair pathways. These pathways may suppress chromosomal instability by error-free mechanisms by which DNA adducts can be bypassed by the DNA replication machinery. One error free mechanism involves recombination-mediated template switch mechanisms. Supporting this idea, RAD51 expression is enhanced in yeast and particular polymorphisms XRCC3, a RAD51 paralogue, may be risk factors for HCC. Nonetheless the DNA repair process can be thwarted by HBV virus, where Hepatitis B virus may directly interfere with NER and perpetuate the replication of cells containing damaged DNA.

The studies presented in this chapter point to future directions in elucidating repair mechanisms of AFB1-associated DNA damage and genetic susceptibility to AFB1-associated cancer. The advent of NGS technology has made it possible to profile the yeast and mammalian genomes for AFB1 resistance which will facilitate identifying the most prominent AFB1 resistant genes. This will facilitate epidemiological studies in determining potential gene polymorphisms that may pose the greatest risk for HCC. NGS technology can facilitate characterizing the DNA sequence contexts where AFB1-associated mutations occur. With the advent of NGS it may be possible to determine the temporal and sequence contexts by which AFB1-associated mutations occur. With the accumulation of genetic information, new biomarkers may be available to aid clinicians and epidemiologists to detect individuals most at risk for HCC and to take appropriate prophylactic actions at early signs of HCC progression.

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Conflict of interest

The author declares no conflict of interest.

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