Aflatoxin B₁ inhibits the type 1 interferon response pathway via STAT1 suggesting another mechanism of hepatocellular carcinoma

Patrick W. Narkwa 1, David J. Blackbourn 2 and Mohamed Mutocheluh 1*

Abstract

Background: Aflatoxin B₁ (AFB₁) contamination of food is very high in most sub-Saharan African countries. AFB₁ is known to cause hepatocellular carcinoma (HCC) by inducing mutation in the tumour suppressor gene TP53. The number of new HCC cases is high in West Africa with an accompanying high mortality. The type I interferon (IFN) pathway of the innate immune system limits viral infections and exerts its anti-cancer property by up-regulating tumour suppressor activities and pro-apoptotic pathways. Indeed, IFN-α is reported to show significant protective effects against hepatic fibrogenesis and carcinogenesis. However, the mechanism behind AFB₁ deregulation of the type I interferon (IFN) signalling pathway, with consequent HCC is largely unknown. This current study seeks to test the hypothesis that AFB₁ inhibits the type I IFN response by directly interfering with key signalling proteins and thus increase the risk of HCC in humans.

Methods: We evaluated the effects of AFB₁ on the type I IFN signalling pathway using IFN stimulated response element (ISRE)-based luciferase reporter gene assay. In addition, the effects of AFB₁ on the transcript levels of JAK1, STAT1 and OAS3 were assessed by real-time quantitative polymerase chain reaction (RT-qPCR) and confirmed by immunoblot assay.

Results: Our results indicated that AFB₁ inhibited the type I IFN signalling pathway in human hepatoma cell line HepG2 cells by suppressing the transcript levels of JAK1, STAT1 and OAS3. AFB₁ also decreased the accumulation of STAT1 protein.

Conclusion: The inhibition of the type I IFN anti-cancer response pathway by AFB₁ suggest a novel mechanism by which AFB₁ may induce hepatocellular carcinoma in humans.

Keywords: Aflatoxin B₁, Hepatocellular carcinoma, STAT1, Type I interferon pathway, HepG2 cells, JAK1, ISRE

Background

The innate immune response is activated within few hours upon exposure of the human system to infectious agents and other toxic chemical compounds such as mycotoxins and works to protect the individual against the harmful effects of the chemical agents and the disease causing microorganisms. One component of the innate immune system that plays a key role in the first line of defence in eliminating pathogens and tumour cells is the IFN system. The type I IFNs for example in addition to their antiviral properties have been employed in the treatment of certain cancers such as Hairy cell Leukemia, AIDS-related Kaposi’s sarcoma and other malignancies [1]. It has been reported that treatment of cells with IFN leads to the activation of the tumour suppressor gene p53 which plays a central role in the apoptosis of some tumour cells [1]. Indeed, Aziz and co-workers showed in their study that IFN-α has a significant protective effects against hepatic fibrogenesis and carcinogenesis [2]. Therefore any substance being
component of pathogen or chemical produced by microorganisms which tend to inhibit or suppress the type I IFN will weaken the innate immune system and predispose individuals to infections and cancers.

AFB₁, a lethal mycotoxin produced by Aspergillus flavus and Aspergillus parasiticus is a potent hepatocarcinogen in humans [3, 4] and in view of that it has been classified as group 1 human carcinogen by the International Agency for Research on Cancer (IARC) [5]. AFB₁ contamination of diet coupled with subsequent prolonged heavy exposure is a major risk factor for the development of HCC. Food meant for human and animal consumption have been reported to contain high levels of aflatoxins in some West African countries such as Ghana, Togo, Nigeria and Benin [6–8] largely due to sub-optimal farming practices, high humidity and poor storage conditions. For example in Ghana 83.3% of wean-mix, food prepared locally from maize and groundnut for children have been reported to have aflatoxin levels higher than the national acceptable levels of 15 ppb [9]. Cereal based foods are staple in Ghana in particular and sub-Saharan Africa in general, it means many more people are exposed to high levels of aflatoxins and thus increasing their risk of HCC.

The incidence of HCC in the West African sub region is high with an annual death rate of about 200,000 [10]. In fact West Africa is ranked second, aside Eastern Asia as region affected most with HCC [10]. In West Africa, the death rate of HCC is almost equal to its incidence with most HCC sufferers dying within weeks of their diagnosis indicating the aggressive and dangerous nature of HCC [10, 11]. In addition to AFB₁, other risk factors that contribute to HCC include chronic HBV/HCV infection and heavy alcohol consumption. Information available indicates that the risk of HCC developing is amplified through the synergistic effects of aflatoxin ingestion and HBV infection. The risk of HCC in people with chronic HBV infection and also exposed to aflatoxin is up to 30 times greater in individual exposed to either of the two factors only [12–14]. These two risk factors (aflatoxin and HBV) are common in underdeveloped countries of the world including Ghana [6, 15] suggesting that the risk of HCC is likely to be high in Ghana.

The role of AFB₁ in the pathogenesis of HCC, via mutation in the tumour suppressor gene p53 has been well established. However, information on how AFB₁ could deregulate other anti-cancer pathways such as the type I IFN signalling pathway as a way of causing HCC is very limited. This study was carried out to test the hypothesis that AFB₁ inhibits the type I IFN response pathway thus contributing to the pathogenesis of HCC. Results from this study could influence future therapeutic intervention for AFB₁-induced HCC and also broaden our knowledge of the role of AFB₁ in HCC immunobiology.

Methods
Reagents and chemicals
The AFB₁ used in the study was purchased from Sigma-Aldrich, USA (cat no A6636) and dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 3200 μM. The AFB₁ stock solution was divided into aliquots, wrapped in aluminium foil and stored frozen at −20 °C until used. The AFB₁ stock solution was diluted to the desired concentration in normal growth medium when necessary. The foetal bovine serum (FBS) was purchased from Sigma-Aldrich, USA. Dulbecco’s Modified Eagles Medium (DMEM) (high glucose, L-glutamine, sodium pyruvate and 25 mM HEPES) was purchased from Gibco by Life Technologies, UK (cat no 11668-019). The human recombinant interferon-alpha 2 (rIFN-α2) was purchased from PBL interferon source (cat no 11115-1). The stock solution of the human recombinant interferon-alpha 2 was diluted to working concentration using phosphate buffered saline containing 0.1% bovine serum albumen as a diluent. The STAT1 (cat no PA5-34504) and GAPDH (cat no QE 212271) primary antibodies were purchased from Thermo Scientific, USA. The secondary antibody conjugated to horse-radish peroxidase (cat no 31430) was purchased from Thermo Scientific, USA.

Cell culture
The cells used in this study were kindly donated by Professor David J. Blackbourn of the University of Surrey, UK. The cell lines used were human hepatoma cell line HepG2 (ECACC 85011430) and mouse fibroblast cell line L929 (NCTC) (ECACC 85103115). The HepG2 and the L929 cells were grown in DMEM high glucose containing L-glutamine, sodium pyruvate and HEPES supplemented with 10% v/v heat inactivated FBS, 1% v/v MEM non-essential amino acids, 100 IU/ml of penicillin and 100 μg/ml of streptomycin. The cultures were maintained at 37 °C in 5% carbon dioxide (CO₂) under humidified condition.

Cytotoxicity assay
HepG2 cells were grown to about 60% confluence and then treated with increasing concentrations of AFB₁ (0–3200 μM). Twenty four hours later, the AFB₁ containing medium was removed and fresh medium without AFB₁ was added. The cytotoxic effects was evaluated by an MTS based assay using Cell Titre 96 AQueous One Solution reagent (Promega, USA, cat no G358C) following the manufacturer’s instruction.
**Transient transfections and luciferase assays in HepG2 cells**

Dual luciferase assays were performed according to our previous study [16]. Briefly, the cells were grown in duplicate wells of the 96-well plate until they reached about 80% confluence. The plasmids used in this study were kind gifts from Professor David J. Blackbourn (University of Surrey, UK). The DNA, pISRE-luc used in the study was extracted from E.coli strain HD5α using EndoFree Maxi Prep Kit (Qiagen, USA) following the manufacturer’s instruction. The pISRE-luc expresses the firefly luciferase protein while pRLSV40 plasmid expresses the Renilla luciferase. The Renilla was included as internal control to which the pISRE-luc activity was normalized. A transfection mixture was prepared by diluting the plasmids DNA (pISRE-luc 500 ng; pRLSV40 1 ng) in serum and antibiotic free media and incubated at room temperature for 5 min. In addition, the Lipofectamine 2000 was also diluted in serum and antibiotic free media. After 5 min of incubation, the diluted DNA and Lipofectamine 2000 were mixed and incubated at room temperature for 20 min and then added to the designated wells and incubated at 37 °C in 5% CO₂ under humidified condition for 24 h.

For the experiment that involved the determination of the minimum concentration of rIFN-α that would induce the maximum activity of IFN-α-inducible pISRE-luc activity, the transfected cells were stimulated with increasing concentrations of IFN-α (100-400 IU/ml). Twenty four hours later, luciferase assays were performed using the dual luciferase reporter assay system (Promega, USA, cat no E1960) following the manufacturer’s protocol. After preparing the cell lysates, 20 μl of the aliquot was employed for luminescence measurement using Berthold Orion luminometer (Berthold Detection Systems, Germany).

For the experiment that involved the determination of the effects of AFB1 on the type 1 IFN signalling pathway, the transfected cells were stimulated with rIFN-α (400 IU/ml) and simultaneously treated with increasing concentrations of AFB1 (0.8–32 μM). Twenty four hours later, dual luciferase reporter gene assay was performed as described above.

**Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)**

The cultured HepG2 cells were treated with AFB1 and simultaneously stimulated with the rIFN-α for 24 h as described above. Total RNA was then extracted using Gene JET RNA purification kit (Thermo Scientific, Germany) following the instructions of the manufacturer. The quantity and the purity of the total RNA was verified by spectroscopy (Nano Drop 1000, Thermo Scientific). The purity was later confirmed by 1% agarose gel electrophoresis using ethidium bromide as stain.

Prior to the cDNA synthesis, any traces of genomic DNA present in the total RNA was removed by treating the total RNAs with double stranded (ds) DNase (Thermo Scientific, Germany) at 37 °C for 2 min followed by maintenance of the mixture on ice. The total RNAs were converted to cDNA using Moloney Murine Leukemia virus (M-Mul V) reverse transcriptase, oligo (dT) and random hexamer primers in a final reaction volume of 20 μl. The mixture was first incubated for 10 min at room temperature followed by further 15 min of incubation at 50 °C. The entire cDNA synthesis reaction was stopped by heating the mixture at 85 °C for 5 min. The cDNA was stored frozen at −80 °C until used in the qPCR.

JAK1, STAT1 and OAS3 target genes were amplified using the Maxima Probe/Rox qPCR master mix (Thermo Scientific, Germany). The primers and probes used were designed and synthesized by Biomers, Germany (Table 1).

The primers and probes of the target genes and the endogenous control (GAPDH) were labelled with different fluorescent reporter dyes at the 5’ end and quencher dyes at the 3’ end and this allowed the target genes to be amplified in the same tube in a duplex qPCR reaction.

After optimizing the primer and probe PCR conditions, a duplex qPCR was performed in a 25 μl reaction volume that contained 0.3 μM forward and reverse primers of the target genes, 0.2 μM of the target probes, 0.1 μM forward and reverse primers of the GAPDH, 0.2 μM of the GAPDH probe and 2.5 μl of 1:10 dilution

| Table 1 Sequences of probes and primers |
| Name of gene | Sequence of primers and probes | Fluorophores |
|--------------|---------------------------------|-------------|
| JAK1         | Probe: 5′AGAGCATCAGTGGGCG       | 5′ FAM- 3′ BHQ-1 |
|              | TCAATCTCC-3′                     |             |
|              | Forward primer 5′- CAATGGGCAT    |             |
|              | GGAACCACCGAC-3′                  |             |
|              | Reverse primer 5′- CAAATCATACT   |             |
|              | GTCCCTGAGCAAAC-3′                |             |
| STAT1        | Probe: 5′CGGCTCTGCTCTCCGC       | 5′ FAM- 3′ BHQ-1 |
|              | TTCCACTCC-3′                     |             |
|              | Forward primer 5′- GTTGCTGAATGT  |             |
|              | EACCTGAATCC-3′                   |             |
|              | Reverse primer 5′- AGCTGATCCAA   |             |
|              | GCAAGCATGGG-3′                   |             |
| OAS3         | Probe: 5′ AGCTGTTGCGCTCCTTC     | 5′ FAM- 3′ BHQ-1 |
|              | AATGTC-3′                        |             |
|              | Forward primer 5′- CTCGCGTACA    |             |
|              | TCCGTAGATC-3′                    |             |
|              | Reverse primer 5′- TCCTGCGAGCT   |             |
|              | CTGTGAAG-3′                      |             |
| GAPDH        | Probe: 5′ CCGTTGACTCGAGCCCTC    | 5′HEX- 3′ TAMRA |
|              | ACCCTCC-3′                       |             |
|              | Forward primer 5′- AGCCACATCGCT  |             |
|              | CAGACACC-3′                      |             |
|              | Reverse primer 5′- TGACAGAGGACC  |             |
|              | CATAACG-3′                       |             |
of the cDNA samples. The qPCR cycling conditions were as follows: 95 °C for 10 min for the first cycle (initial denaturation), 95 °C for 15 s for 40 cycles (denaturation) and 60 °C for 60 s for 40 cycles (annealing/extension). The qPCR reaction products were analyzed using Bio-Rad CFX 96 manager software (Bio-Rad, USA). The relative quantification of the target genes was calculated using the comparative CT method. The relative quantities of \( JAK1 \) and \( \beta-actin \) were determined using \( 2^{-\Delta\Delta CT} \) as previously described [17].

Western blotting

To examine the effects of AFB1 on the protein accumulation of STAT1, the cultured HepG2 cells were treated with AFB1 and simultaneously stimulated with the rIFN-\( \alpha \) for 24 h as described above. The cells were later harvested and lysed to extract total proteins using cold Radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific, USA) containing freshly added protease and phosphatase inhibitor cocktails and ethylenediaminetetraacetic acid (EDTA) (Thermo Scientific, Germany) following a standard protocol. Aliquots of the protein samples were mixed with 2X sample buffer containing 2-beta mercaptoethanol and the mixture was heated for 5 min at 95 °C to denature the proteins. The proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then blotted onto 0.45 \( \mu \)m pore size polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories). The PVDF membranes were blocked in 5% non-fat dried milk in 1X Tris buffered saline with Tween-20 (TBST) and then blotted onto 0.45 \( \mu \)m pore size polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories). The membranes were then incubated with the primary antibodies at 4 °C overnight. The secondary antibodies conjugated to horseradish peroxidase (Thermo Scientific, USA) were used at a dilution of 1:5000. The membranes were probed with the secondary antibody at room temperature with gentle shaking for 1 h after which bands were visualized by performing enhanced chemiluminescence using Pierce enhanced chemiluminescence (ECL) western blotting substrate (Thermo Scientific, USA). The images were captured with a camera (Li-COR Bioscience, USA) and analyzed using image J software.

Results

Cytotoxic effects of AFB1 and IFN concentration course

The AFB1 is an extremely toxic compound which could not have been used directly on the cells. Therefore, the concentration of AFB1 that was not toxic to the HepG2 cells was determined using MTS based assay as described above. As shown in Fig. 1a, AFB1 killed the HepG2 cells in a dose-dependent fashion after exposure to concentrations up to 3200 \( \mu \)M for 24 h followed by maintenance of cells in AFB1 free media for 24, 48 and 72 h. The experiment established up to 10 \( \mu \)M of AFB1 was not toxic to the cells and was therefore used in subsequent experiments. Next the concentration of rIFN-\( \alpha \) required to induce maximal activity of the type I IFN response pathway was determined. It was observed that the pISRE-luc activity (a measure of the type I interferon activity) increased with increasing concentration of rIFN-\( \alpha \) and peaked at concentrations of 200-400 IU/ml (Fig. 1b).

AFB1 suppresses IFN-\( \alpha \) induced ISRE signalling

To measure the effect of AFB1 on the anti-cancer activity of the type I IFN response pathway, a luciferase reporter gene expressing pISRE-luc was employed. The pathway activation was induced with rIFN-\( \alpha \) (400 IU/ml) through the transactivation of interferon stimulated response elements (ISRE). It is already established that the pathway is activated following the binding of IFN-\( \alpha \) to the IFN-\( \alpha \) receptors R1 and R2 on cells to trigger cascades of events leading to the transactivation of the ISRE as reviewed in Randall and Goodbourn [18].

The cultured HepG2 cells were stimulated with rIFN-\( \alpha \) and simultaneously treated with AFB1 as described above. As shown in Fig. 2, the activated pathway peaked at about five-fold above background or basal levels (see fourth bar; Fig. 2). It was noted that AFB1 did not influence the pathway activity in anyway because cells treated with AFB1 alone showed same background level of pathway activity as those cells in which the pathway was not activated; the so called ‘none treated cells’ (see first bar; Fig. 2).

The addition of AFB1 to the cells in which the type I IFN response pathway was activated saw AFB1 dose dependent inhibition of the pathway activity up to 54.8% in cells treated with 10 \( \mu \)M of AFB1 and 68.2% in cells treated with 32 \( \mu \)M respectively (Fig. 2). The pathway activity was measured by the firefly luciferase pISRE-luc activities (Fig. 2).

The concentration of AFB1 and rIFN-\( \alpha \) used in the experiment were chosen based on the following: (i) at 10 \( \mu \)M of AFB1 \( \geq 90\% \) of the cells survived (Fig. 1a), (ii) between 200 and 400 IU/ml of rIFN-\( \alpha \) induced the maximum activity of IFN-\( \alpha \) inducible ISRE promoter i.e. a measure of the type I IFN response pathway (Fig. 1b). In the subsequent experiments, 10 \( \mu \)M of AFB1 was used and that concentration was selected based on the fact it had the capacity to significantly inhibit the type I IFN induced signalling in HepG2 cells as measured by the firefly luciferase pISRE-luc activities (p-value \( \leq 0.047 \)) (Fig. 2).
AFB1 inhibits transcripts expression of JAK1, STAT1 and OAS3 genes

Having demonstrated at the luciferase reporter gene assay level that AFB1 inhibits the type I IFN response signalling pathway, the next task was to test our hypothesis that AFB1 would inhibit the transcripts of key signalling elements of the pathway. The JAK-STAT-ISRE arm of the type I IFN response pathway was chosen for the study because when activated it leads to the activation of interferon responsive genes such as OAS3 whose inhibition by AFB1 was hypothesized in the current study.

RT-qPCR analysis of the transcripts levels of JAK1, STAT1 and OAS3 genes in the cultured HepG2 cells stimulated with or without rIFN-α (400 IU/ml) and simultaneously treated with or without AFB1 (10 μM) was performed.

The cells in which the pathway was activated showed about 3-fold increase in the transcripts levels of JAK1 compared to background or basal levels. However, when AFB1 was added to the cells in which the pathway was activated the transcripts levels of JAK1 reduced to almost half (49.1%, p-value ≤ 0.0001).

**Fig. 1** Establishing the maximum non-toxic concentration of AFB1 and maximum inducible concentration of rIFN-α (a) HepG2 cells were cultured at density of 5 × 10⁴ cells per well of the 96-well plate until they reached 60% confluence. The cells were then treated with or without increasing amount of AFB1 (0–3200 μM) for 24 h after which the AFB1 containing media were replaced with fresh media. Cytotoxicity was evaluated by MTS-based assay at 24, 48 and 72 h. The viability of cells was calculated as ratio between AFB1 treated cells and non-treated cells. Data are presented as mean and standard deviation of three independent experiments each performed in duplicate wells, p-value ≤ 0.977 as determined by one-way ANOVA. b Establishing the maximal IFN-α induction of ISRE driven luciferase reporter gene activity. HepG2 cells were cultured at density of 5 × 10⁴ cells per well of the 96-well plate until they reached 80% confluence. The cells were transiently co-transfected with pISRE-luc (500 ng) and pRLSV40 (1 ng). At 24 h post-transfection, the cells were treated with increasing concentration of rIFN-α. Luciferase activity was measured 24 h later. The data are presented as mean and the standard deviation of three independent experiments each conducted in duplicate wells. There was no significant difference in pISRE-luc activity of cells treated with 300 and 400 IU/ml of rIFN-α (p-value ≤ 0.7527)

**Fig. 2** AFB1 inhibits IFN-α induced ISRE signalling in a dose dependent fashion. HepG2 cells were cultured at density of 5 × 10⁴ cells per well of the 96-well plate until they reached 80% confluence. The cells were transiently co-transfected with pISRE-luc (500 ng) and pRLSV40 (1 ng). The pRL40-luc which constitutively expresses the Renilla luciferase was included as internal control to which pISRE-luc activity was normalized. At 24 h post-transfection, the cells were stimulated with or without rIFN-α (400 IU/ml) and simultaneously treated with or without AFB1 (10 μM) and rIFN-α (400 IU/ml). The data are presented as mean normalized pISRE-luc activity and the standard deviation of three independent experiments each conducted in duplicate wells. There was a significant difference in pISRE-luc activity of cells stimulated with rIFN-α alone compared to cells stimulated with rIFN-α and simultaneously treated with 10 μM of AFB1 (p-value ≤ 0.047)
Although the pathway activities in those experiments to assess the effect of STAT1 and OAS3 transcripts levels showed over 10-fold above background levels, similar pattern of results were seen for STAT1 and OAS3 because the transcripts levels were reduced by AFB1 to 47% (p-value ≤ 0.03) and 39% (p-value ≤ 0.05) respectively (Fig. 3b & c).

Taken together, it was observed that AFB1 significantly inhibited the mRNA expression levels of JAK1, STAT1 and OAS3 genes (Fig. 3). To be sure that HepG2 cells were responding to rIFN-α treatment and that the ISRE was functioning, a parallel experiment in which HepG2 cells were transiently transfected with pISRE-luc and pRLSV40 as described in Fig. 3 were assayed for dual luciferase activity and the inhibition of ISRE activity was confirmed as described in Fig. 2 (data not shown).

**AFB1 inhibits STAT1 protein synthesis**

Different post-transcriptional events could be involved in translating mRNAs into proteins [19] suggesting that lower mRNA levels might not necessarily corresponds to lower protein expression and vice versa. Therefore, western blot assay was employed to ascertain whether the inhibition of the mRNA expression level of STAT1 by AFB1 would ultimately affect its translation into proteins as well (see Fig. 3).

Again the cultured HepG2 cells were stimulated and treated as described in Fig. 3. Protein extracts of HepG2 cells stimulated with or without rIFN-α and treated with AFB1 (10 μM) were analysed by western blotting for STAT1 using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control. Briefly, cell lysates were prepared and thereafter, protein samples were separated on SDS-PAGE and immunoblotted with anti STAT1 and anti-GAPDH antibodies respectively. In order to ensure that the cells were functionally responding to stimulation and treatment at the time when the lysates were prepared, a parallel experiment in which the cells were transiently transfected with pISRE-luc and pRLSV40 as described in Fig. 3 were assayed for dual luciferase activity and the inhibition of the type I IFN response pathway activity by AFB1 was confirmed (data not shown).

Consistent with the results shown in Fig. 3, STAT1 accumulation peaked in cells in which the type I IFN pathway was activated in the absence of AFB1 (see Fig. 4; band 2 from right). However, on the contrary the STAT1 accumulation was substantially reduced in cells in which the pathway was activated in the presence of AFB1 (see Fig. 4; band 1 from right). Again, cells in which the type I IFN pathway was not activated showed low background levels of STAT1 presence (see Fig. 4; bands 1 and 2 from left). Also consistent with Fig. 3 the protein levels of GAPDH were not affected by AFB1 as their levels were shown to be equal in all cells regardless of the AFB1 treatment or stimulation with rIFN-α.

**Discussion**

AFB1 is a potent hepatocarcinogen [3, 4] which when ingested is metabolized by CYP450 class of enzymes in the liver to AFB1, 8-9 exo epoxide [3, 4]. The AFB1 8-9 exo epoxide binds DNA and induces AGG to AGT transversion mutation at codon 249 in the tumour...
suppressor gene p53 [20, 21]. This mutation leads to the inhibition of the p53 mediated transcription and underlines the mechanism by which AFB1 causes HCC [22]. It must be stated however that, the mechanism by which AFB1 causes HCC may not be limited to p53 mutation alone and that AFB1 may also induce cancers by deregulating other anticancer signalling pathways. For example Ubagai et al. [23] demonstrated that AFB1 may also induce tumourigenesis by deregulating the insulin-like growth factor 1 receptor (IGF-IR) signalling pathway suggesting that AFB1 may also induce tumourigenesis by deregulating other anticancer pathways such as the type I IFN signalling pathway. In this study we tested the hypothesis that AFB1 would suppress/inhibit the type I IFN signalling pathway and thus provide another mechanism by which AFB1 may cause cancer. Findings from the current study in which AFB1 was shown to inhibit the type I IFN response pathway by targeting the key signalling elements are consistent with that of Jiang et al. [24] who reported that AFB1 inhibited the mRNA expression levels of IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ and TNF-α in the small intestines of broilers treated with AFB1. Moreover, AFB1 has also been reported to inhibit mRNA and protein expression levels of IL-4, IL-6, IL-10 in the peritoneal macrophages and splenic lymphocyte cell lines [25, 26] and STAT5A gene mRNA expression levels in bovine mammary epithelial cells [27]. One way by which the type I IFN signalling response exerts its anti-cancer and antiviral response is through the activation of the JAK-STAT-ISRE arm of the pathway. One component of the JAK-STAT-ISRE signalling pathway considered to have tumour suppressor function is STAT1 [28]. When activated, STAT1 suppresses tumour development by inducing apoptosis [29] and also inhibit tumour angiogenesis [30]. Therefore the suppression of STAT1 by AFB1 at the both transcription and translational levels as demonstrated in the current study will impair the ability of STAT1 in orchestrating the expression of myriad of genes which are required to promote apoptosis, inhibit cell proliferation and angiogenesis in response to AFB1.

The activation of the JAK-STAT-ISRE arm of the type I IFN signalling pathway results in the activation of the so called interferon responsive elements including the OAS3, PKR, Mx etc as reviewed in Randall and Goodbourn [18]. The OAS3 pathway when activated leads to the establishment of antiviral state by inhibiting protein synthesis which culminates in the destruction of both viruses and infected cells [31, 32]. The OAS3 has also been reported to play a role in inhibiting tumour development by inducing apoptosis and anti-proliferative responses [33, 34]. The activation of the JAK-STAT-ISRE signalling pathway starts upon the phosphorylation and activation of JAK1 and TYK2 when the correct ligands...
bind to the IFNAR (Fig. 5). The activated JAK1 and TYK2 in turn phosphorylate STAT1 and STAT2 setting in motion a cascade of events which finally initiates the transcription of IFN-inducible genes switching on the anti-cancer and anti-viral effects as reviewed in Randall and Goodbourn [18]. Therefore, any stimulus which deregulates the expression of either JAK1 or TYK2 could potentially deregulate the entire type I IFN response signalling pathway and thereby weaken the immune system and thus predispose individuals to infections and or cancer.

Taken together, the current study has revealed for the first time the inhibition of the type I IFN response pathway by AFB1 via the inhibition of the transcripts of JAK1, STAT1 and OAS3 and also inhibit the protein accumulation of STAT1. The findings of the current study could be another mechanism by which AFB1 may cause HCC (Fig. 5).

**Conclusions**

In conclusion, the current study has shown that AFB1 down-regulated the type I IFN response pathway by significantly inhibiting the key signalling elements such as JAK1, STAT1 and OAS3 and also the STAT1 protein. Findings from this study reveals the negative effects of AFB1 on the health of people who consume AFB1 contaminated food as evidenced by its ability to inhibit and or deregulate the innate immune response. In view of this it is recommended that public education is intensified in Ghana and other developing nations of the world where AFB1 contaminated food form a greater portion of the diets so as to reduce HCC and its associated deaths.

**Abbreviations**

AFB1: Aflatoxin B1; AIDS: Acquired immunodeficiency syndrome; CYP450: Cytochrome p450; DMEM: Dulbecco’s modified eagles’ medium; EDTA: Ethylenediaminetetraacetic acid; FBS: Foetal bovine serum; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IARC: International agency for research on cancer; IFN: Interferon; IFN-α: Interferon-alpha; IFN-β: Interferon beta; IGF-IR: Insulin-like growth factor 1 receptor; IL: Interleukin; IRF-9: Interferon regulatory factor 9; ISGF-3: Interferon stimulated gene factor 3; Jak1: Janus activated kinase 1; MEM: Minimum essential medium; OAS: Oligo adenylate synthetase 3; PBL: Peska Biomedical Laboratories; ppb: Part per billion; PVDF: Polyvinylidene difluoride; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; STAT1: Signal transducer and activator of transcription 1; TNF-α: Tumour necrosis factor-alpha

**Acknowledgments**

The authors sincerely thank the Leverhulme-Royal Society Africa Award II Scheme for making funds available for this work. The authors also thank...
Professor Ellis Owusu-Dabo, Dr. Augustine Annan and the entire staff at the Kumasi Centre for Collaborative Research (KCCR) for their technical support during the RT-qPCR work. The authors also thank everyone who contributed to this work in one way or the other.

**Funding**

This work was supported with funds from the Leverhulme-Royal Society Africa Award II grant won by both Professor David Blackbourn and Dr. Mohamed Mutocheuhl.

**Availability of data and materials**

The datasets on which the conclusions of the current study were made are available in the Kwame Nkrumah University of Science and Technology (KNUST) space repository (ir.knust.edu.gh/handle/123456789/9321). In addition, the raw datasets analyzed during the current study will be available from the corresponding author on reasonable request.

**Authors’ contributions**

MM and DJB conceived the idea and designed the experiments. DJB provided the cell lines, and the plasmids used in the study. PWN and MM performed the experiments, analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

**Competing interest**

The authors declare that they have no competing interests.

**Consent for publication**

Not applicable

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Publisher’s Note**

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Author details**

1Department of Clinical Microbiology, School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. 2Department of Microbial and Cellular Sciences, School of Biosciences and Medicine, University of Surrey, Surrey GU2 7XH, UK.

**Received:** 9 November 2016 **Accepted:** 10 March 2017

**Published online:** 20 March 2017

**References**

1. Bekisz J, et al. Anti-proliferative properties of type I and type II Interferon. Pharmaceutica. 2010:2;994–1015.
2. Aiz T, Azz MA, et al. Interferon-alpha gene therapy prevents aflatoxin and carbon tetrachloride promoted hepatic carcinogenesis in rats. Int J Mol Med. 2005;15(1):21–6.
3. Sudakil D. Dietary exposure and chemoprevention of cancer: a clinical review. J Toxicol Clin Toxicol. 2003;41:195–204.
4. Wild CP, Gong YY. Mycotoxins and human disease: a largely ignored global health issue. Carcinogenesis. 2010;31:71–82.
5. IARC, In Parkinson DM, editor. Cancer Incidence in five continents. IARC scientific publications, vol. VIII (No. 155). Lyon: IARC Press; 2002.
6. Awuah RT, Kpodo KA. High incidence of Aspergillus flavus and aflatoxins in stored groundnut in Ghana and the use of a microbial assay to assess the inhibitory effects of plant extracts on aflatoxin synthesis. Mycopathologia. 1996;134(2):109–14.
7. Kpodo KA, Thane U, Hald B. Fusaria and fumonisin in maize from Ghana and their co-occurrence with aflatoxins. Int J Food Microbiol. 2000;51:147–57.
8. Oyelami OA, et al. Aflatoxins in the autopsy brain tissue of children in Nigeria. Mycopathologia. 1996;132:35–8.
9. Kum J, et al. Aflatoxins and fumonisin contamination of home-made food (Wearinia) from cereal-legume blends for children. Ghana Med J. 2014;48(3):121–6.
10. Ladep NG, et al. Problem of hepatocellular carcinoma in West Africa. World J Hepatol. 2014;6(11):783–92.
11. Jemal A, et al. Global cancer statistics. CA Cancer J Clin. 2011;61:69.
12. Groopman JD, Kensler TW, Wild CP. Protective interventions to prevent aflatoxin-induced carcinogenesis in developing countries. Annu Rev Public Health. 2008;29:187–203.
13. Liu Y, Wu F. Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. Environ Health Perspect. 2010;118:218–24.
14. Wu F, Khlangwiset P. Health economic impacts and cost-effectiveness of aflatoxin reduction strategies in Africa: case studies in biocontrol and postharvest interventions. Food Addit Contam Part A. 2012;27:496.
15. Alain JP, et al. The risk of hepatitis B virus infection by transfusion in Kumasi, Ghana. Blood. 2003;101(6):2419–25.
16. Mutocheuhl M, et al. Kaposis sarcoma-associated herpesvirus viral interferon regulatory factor-2 inhibits type I interferon signalling by targeting interferon-stimulated gene factor-3. J Gen Virol. 2011;92(10):2394–8.
17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCt method. Methods. 2001;25:402–8.
18. Randall RE, Goodbourn S. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J Gen Virol. 2008;89(Pt 1):1–47.
19. Racolsy R, Heilenb M, Friedman M. Low levels of aflatoxin B1, rnc, and milk enhance recombinant protein production in mammalian cells. PLoS One. 2013;8(8):e71682.
20. Agular F, Hussain SP, Cerutti P. Aflatoxin B1 induces the transversion of G→T in codon 249 of the p53 tumor suppressor gene in human hepatocytes. Proc Natl Acad Sci U S A. 1993;90(8):856–90.
21. Hsu IC, et al. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. Nature. 1991;350:427–8.
22. Martin I, Dufour JP. Tumor suppressor and hepatocellular carcinoma. World J Gastroenterol. 2008;14(17):220–33.
23. Ubajia T, et al. Aflatoxin B1 modulates the insulin-like growth factor-2 dependent signalling axis. Toxicol In Vitro. 2010;24:783–9.
24. Jiang M, et al. Effects of aflatoxin B1 on T-cell subsets and mRNA expression of cytokines in the intestine of broilers. Int J Mol Sci. 2015;16:6945–59.
25. Bruneau JC, et al. Aflatoxins B1, B2 and G1 modulate cytokine secretion and cell surface marker expression in J774A. 1 murine macrophages. Toxicol In Vitro. 2012;26:686–93.
26. Marin D, et al. Changes in performance, blood parameters, humoral and cellular immune responses in weanling piglets exposed to low doses of aflatoxin. J Anim Sci. 2002;80:1250–7.
27. Furoihemehr A, Karkhezdad H, Qasemi-Panahi B. Evaluation of STAT5A gene expression in aflatoxin B1 treated bovine mammary epithelial cells. Adv Pharm Bull. 2013;3(2):461–4.
28. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunomodulation. Nat Rev Immunol. 2006;6:836–48.
29. Khodarev NN, Roizman B, Weichselbaum RR. Molecular pathways: interferon/ STAT1 and STAT3 in tumorigenesis: two sides of the same coin. Clin Cancer Res. 2013;19(11):3015–21.
30. Pensa S, et al. STAT1 and STAT3 in tumorigenesis: two sides of the same coin. In: Stephanou A, editor. JAK-STAT pathway in disease. Austin: Landes Bioscience; 2008. p. 100–21.
31. Muller U, et al. Functional role of type I and type II interferons in antiviral defense. Science. 1994;264:1918–21.
32. Sen GC. Viruses and interferons. Annu Rev Immunol. 1989;7:227–64.
33. Silverman RH, et al. Control of the ppp(a2p)nA system in HeLa cells. Effects of interferon and virus infection. Eur J Biochem. 1982;124:131–8.
34. Stark GR, et al. How cells respond to interferons. Annu Rev Biochem. 1998;67:227–64.

Submit your next manuscript to BioMed Central and we will help you at every step:  
• We accept pre-submission inquiries  
• Our selector tool helps you to find the most relevant journal  
• We provide round the clock customer support  
• Convenient online submission  
• Thorough peer review  
• Inclusion in PubMed and all major indexing services  
• Maximum visibility for your research  

Submit your manuscript at www.biomedcentral.com/submit