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Fumonisin B_1 regulates LDL receptor and ABCA1 expression in an LXR dependent mechanism in liver (HepG2) cells

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ABSTRACT

The metabolic toxicity of Fumonisin B_1 (FB_1) converges at the accumulation of sphingoid bases and reduced ceramide levels. Several studies have alluded to a hypercholesterolemic endpoint after FB_1 exposure, yet the molecular mechanisms remain elusive. Cell surface receptors are important regulators of cholesterol metabolism by regulating influx of lipids and efflux of cholesterol. Western blot analysis showed that FB_1 elevates the expression of ABCA1 (a cholesterol efflux promoter) in an LXR dependent mechanism. We further highlight the potential role of PCSK9 in the degradation of LDL receptor. These data provide important evidence for the mechanism underlying hypercholesterolemia in FB_1 treated models. The disruption of lipid homeostasis by FB_1 is beginning to shift away from canonical ceramide synthase inhibition, and this changed perspective may shed light on diseases caused by dysregulated cholesterol metabolism such as cancer initiation and promotion.

1. Introduction

Metabolomics in toxicological research provides a detailed analysis of altered metabolic pathways that are targeted by harmful chemicals (Heijne et al., 2005; Ramirez et al., 2013). This is especially important when considering the increase in mycotoxigenic fungal infection of staple crops. The Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) have prioritized research focus into mycotoxins because of their major toxicological impact on human and animal health (Medina et al., 2017).

From and African perspective, the infection of agricultural commodities with *Fusarium sp*. is of particular concern as members of this genus are prolific producers of fumonisins which commonly contaminate dietary staples (Misihairabgwi et al., 2019). While several types of fumonisins have been identified, only fumonisins B_1 (FB_1), B_2 (FB_2) and B_3 (FB_3) are commonly detected in agricultural commodities intended for human and animal consumption (Kamle et al., 2019). Exposure to these fungal metabolites are pathologically linked to equine leukoencephalomalacia (Wilson et al., 1990; Thiel et al., 1991; Vendruscolo et al., 2016), porcine pulmonary edema (Osweiler et al., 1992; Harrison et al., 1990) and liver and kidney toxicity in lambs (Henry and Wyatt, 2001). The fumonisins have also demonstrated cancer initiating potential *in vivo* and *in vitro* (Gelderblom et al., 1992, 2008), and exposure to these toxins in high quantities is considered an etiological risk factor for the development of esophageal cancer in countries like China (Wang et al., 2008), Iran (Alizadeh et al., 2012) and South Africa (Rheeder et al., 1992; Sydenham et al., 1990). A study by Shepheard et al. (2007) linked high prevalence of esophageal cancer in humans exposed to 8 μg/kg body weight of FB_1 in Centane, a region of South Africa. While other studies have linked FB_1 exposure to hepatocarcinogenesis (Ueno et al., 1997; Gelderblom et al., 2001).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) have assigned a provisional maximum tolerable daily intake (PMTDI) of 2 μg/kg body weight per day for FB_1, FB_2 and FB_3 alone or in combination (Bolger et al., 2001; Shephard et al., 2019). In line with this, the Codex Alimentarius Committee, together with international consensus has set the maximum levels at 4000 μg/kg for raw maize and at 2000 μg/kg for maize flour and maize meal for FB_1 and FB_2 (CAC, 2014; Shephard et al., 2019). Several countries, including South Africa have adopted these maximum levels as part of their food safety regulations (Shephard et al., 2019). Exposure assessments conducted in South African populations found levels of FB_1 to be above the PMTDI and maximum levels as set out in regulations (Shephard et al., 2007, 2019). Furthermore, these exposure levels are likely to be higher in rural communities where subsistence farming makes mycotoxin regulation impractical (Shephard et al., 2019). This has far reaching

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socio-economic implications as millions of people are consuming contaminated maize and maize-based products as part of a staple diet (Shephard et al., 2007).

Fumonisin B_1 is the most prevalent and well investigated of the fumonisins with limited studies conducted on the toxicity of FB_2, FB_3, and fumonisin B combinations. A recent study found that combinational treatment with fumonisins decreased cell viability, increased cell death and induced endoplasmic reticulum (ER) stress. Furthermore, the authors were able to rank the toxicity of fumonisins ($FB_1 > FB_2 » FB_3$) and showed that synergism occurred at low concentrations while antagonism was observed at high concentrations in human gastric epithelial cells. Although the exact mechanisms of toxicity is not well established for FB_2 and FB_3, toxicity can be inferred from FB_1 which has similar chemical structure and well-known mechanisms of toxicity (Yu et al., 2020).

Fumonisin B_1 is classified as a Group 2B carcinogen (Ostry et al., 2017). The cellular mechanisms of toxicity associated with FB_1 include the induction of oxidative stress, apoptosis, alterations in cytokine expression and epigenetic dysregulation (Stockmann-Juvala and Savolainen, 2008; Arumugam et al., 2019, 2020; Chuturgoon et al., 2014, 2015). At the metabolic level, the clear structural similarity of FB_1 to the cellular sphingolipids has been shown to competitively inhibit ceramide synthase resulting in the accumulation of sphingosine, sphinganine and its derivatives (Soriano et al., 2005; Zitomer et al., 2009; Merrill et al., 1993; Riley and Merrill, 2019). While other studies have demonstrated the multifaceted effects and implications of lipid dysregulation by this toxin as alternate mechanisms for liver tumorigenesis (Burger et al., 2018; Riedel et al., 2016). Fumonisin B_1 was shown to impair biosynthesis and modify the fatty acid profile, by increasing the amount of saturated and polyunsaturated fatty acids and decreasing the level of monounsaturated fatty acids (Szabó et al., 2019). Hypercholesterolemia is an observed and established effect of FB_1 exposure (Vilà et al., 2011; Fincham et al., 1992); however the hypercholesterolemic mechanism remains elusive.

Hypercholesterolemia and related disorders often result from defects in the feedback systems that regulate the low-density lipoprotein receptor (LDLR) pathway. LDLR expression is tightly regulated by Liver X receptors (LXRs) at the transcriptional level and by proprotein convertase subtilisin/kexin type 9 (PCSK9) at the posttranscriptional level (Zhang et al., 2016). Activation of LXRs downregulates lipoprotein receptor expression at the cell surface and enhances the expression of ATP-binding cassette transporter (ABCA1), thereby lowering cholesterol uptake and promoting its cellular efflux (Endo-Umeda and Makishima, 2019).

The liver plays a key role in lipid metabolism and is considered as the hub of fatty acid synthesis and lipid circulation (Nguyen et al., 2008). The liver derived HepG2 cell line is an established *in vitro* toxicity model and expresses many of the genes required for regulating cholesterol synthesis and efflux (McNutt et al., 2009; Di et al., 2012), thus presents a suitable model for determining the effects of FB_1 on these pathways. Dysregulated signalling hubs controlling lipid metabolism in the liver have been revealed as potential mechanisms for inducing hepatocellular carcinoma (Lee et al., 2016; Ranjpour et al., 2019) and hypercholesterolemic events (Zhang et al., 2012a; Erickson et al., 2003).

The molecular mechanisms surrounding the hypercholesterolemic effects of FB_1 remains largely unknown. In the present study, to clarify a mechanism for FB_1-induced development of hypercholesterolemia, we investigated the overall changes in expression of proteins responsible for regulating lipid/cholesterol influx and efflux in HepG2 liver cells. Our results provide novel insights into the roles and mechanisms of FB_1 in cholesterol efflux as an alternative mechanism to the established disruption of sphingolipid metabolism. This data will add to the growing body of evidence supporting the metabolic toxicity of FB_1.

2. Materials and methods

2.1. Cell culture and treatment protocol

The HepG2 cell line (Highveld Biologicals, Johannesburg, South Africa) used in this study was maintained in Eagle's Minimum Essential Medium (EMEM, Invitrogen) supplemented with 10% fetal bovine serum, 5 mM L-glutamine and 1% penstrep-fungizone in a humidified incubator (37 °C, 5% CO_2).

FB_1 stock solution (5 mM) was prepared and subsequently diluted in EMEM to final concentrations of 5, 50 and 100 μM. These concentrations were selected based on the effects of FB_1 on metabolic activity (WST-1 assay) and cell toxicity (cell biomass) data. The selected concentrations also fit in well with the PMTDI set out in regulations. By applying a range of concentrations, a holistic view for the mechanism of FB_1 toxicity (low dose *vs* high dose) could be observed.

Cells were incubated for 6 h with the selected concentrations. This time period was carefully chosen based on the relatively short half-life of this toxin and its efficient uptake by the liver (Tachampa et al., 2008) as well as lipidomic disruption peaking at between 4 and 8 h after FB_1 exposure (Enongene et al., 2000).

It was hypothesized that the selected concentrations and time period would fit in well with the toxicodynamic and toxicokinetic profiles of FB_1.

2.2. Assessment of cellular biomass – cell viability

HepG2 cells were cultured in 96-well plates (20,000 cells/well). After treatment with FB_1 (0–500 μM) media was decanted and biomass stained by incubating attached cells with crystal violet solution [(0.5% w/v) crystal violet/20% (v/v)] Methanol) for 10 min. After washing with sterile deionized water to eliminate excess dye, the plate was left to dry overnight. The dye was then solubilised with ethanol (100 μL/well) and the absorbance quantified at 595 nm using a spectrophotometer (Bio-Tek uQuant, Winooski, VT, USA).

2.3. Cell membrane disruption – lactate dehydrogenase (LDH) leakage assay

The extracellular LDH detection kit (Roche) was used to measure enzyme activity in cell culture medium as an indicator of cell membrane damage. Supernatant (100 μL) was dispensed into microtitre plates in triplicate. Thereafter, substrate mixture (100 μL) containing catalyst (diaphorase/NAD^+) and dye solution (INT/sodium lactate) was added to sample and allowed to react at room temperature for 20 min. Optical density of the resulting formazan product was measured at 500 nm with a spectrophotometer (Bio-Tek Quant).

2.4. Measurement of cellular metabolic activity by WST-1 assay

Cell metabolic activity was determined by the WST-1 method (Roche). The HepG2 cells were plated in quadruplicate (20,000 cells/well). After incubation for 6 h with varying concentrations of FB_1 (0–500 μM), the cells were incubated for an additional 2 h in medium containing 10% WST-1. The absorbance of the colorimetric reaction was measured at a wavelength of 450 nm with a reference wavelength of 620 nm using a spectrophotometer (Bio-Tek uQuant, Winooski, VT, USA). The percentage of cell viability was calculated in relation to the absorbance at 0 μM FB_1 (control).

2.5. Protein quantification and western blot

Protein expression profiles were determined using western blots. Protein was extracted using Cytobuster Reagent (250 μL) (Novagen, San Diego, CA, USA, catalogue no. 71009) and mechanical lysis. Protein samples were quantified with the bicinchoninic acid (BCA) assay and

Table 1
Antibodies used in this study.

Name	Catalogue #
LDL Receptor α	#ab30532, Abcam
Fatty Acid Synthase	#3180, Cell Signalling Technology
Liver X Receptor α	#ab24532, Abcam
PCSK9	#ab31762, Abcam
ABCA1	#ab7360, Abcam

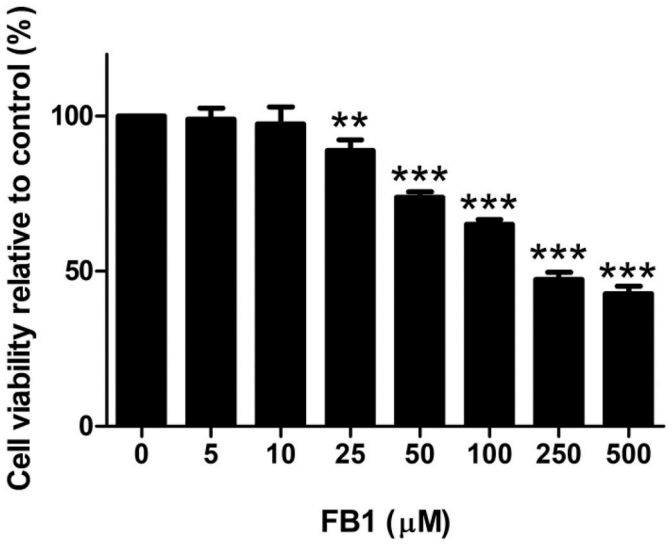

Fig. 1. Percentage of HepG2 cell biomass normalized to the control 6 h FB_1 exposure. Data shown are means with SD. **p < 0.01, ***p < 0.001.

standardized to 1 mg/mL. The samples were then boiled (5 min) in Laemmli Sample buffer [dH20, 0.5 M Tris-HCl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol, 1% bromophenol blue]. Prepared protein samples were electrophoresed, transferred and immunoprobed as per Chuturgoon et al. (2014). Membranes were incubated with primary antibody (1:1000 dilutions, Table 1) overnight (4 °C). The membranes were incubated (RT) with HRP-conjugated secondary antibody for 2 h (1:5000 dilution). Protein bands were identified using the Clarity western ECL chemiluminescent substrate and images were captured with the ChemiDocTM XRS + Molecular Imaging System (Bio-Rad). Protein expression was analyzed using Image Lab Software version 5.0 (Bio-Rad) and the results were expressed as relative band density (RBD).

2.6. Statistical analysis

All assays were conducted in triplicate as three independent experiments Statistical analyses were carried out by 1-way ANOVA followed by Bonferroni post tests using GraphPad Prism 5. Data are presented as means and standard deviation (SD) unless otherwise stated. A p-value of <0.05 was taken as statistically significant.

3. Results

3.1. HepG2 cell biomass is decreased by FB_1

We quantified HepG2 cell biomass as a measure of cell viability after exposure to FB_1. After 6 h, biomass was significantly decreased with the two highest concentrations (250 μM and 500 μM) accounting for a 50% loss (Fig. 1). Thus, treatment with this mycotoxin was found to be acutely toxic and results in extreme loss of cell viability only at exceptionally high concentrations.

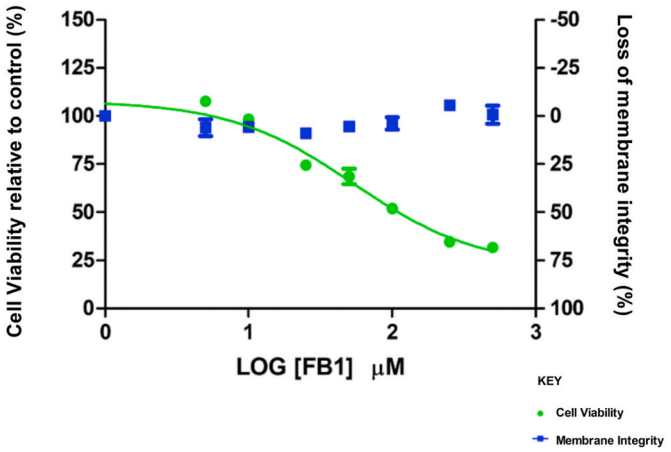

Fig. 2. Results of WST-1 assay reveal a dose dependent decrease in cell viability without overt membrane damage in HepG2 cells exposed to FB_1.

3.2. HepG2 cells exhibited decreased metabolic activity without membrane disruption following FB_1 exposure

The metabolic activity is capable of being estimated quantitatively by adding tetrazolium salts to cells. In the WST-1 assay, a dose dependent decrease in absorbance after treatment with FB_1 was observed (green). Further we noted no drastic changes to cell membrane integrity as indicated by no significant detection of LDH leakage from experimental cells when compared to the control (Fig. 2).

3.3. FB_1 promotes cholesterol efflux in HepG2 cells

In order to clarify the mechanism responsible for the hypercholesterolemia associated with FB_1 exposure (Vilà et al., 2011; Fincham et al., 1992), we examined the expression levels of cholesterol influx and efflux proteins. All tested concentrations of FB_1 decreased LDL receptor expression in comparison with control (untreated) cells (Fig. 3A). We observed that the lowest concentration (5 μM) had the greatest inhibitory effect on LDL receptor expression. These data may suggest a hormetic response to FB_1 exposure as the cell tries to compensate for shifts in the intracellular lipidomic profiles. We further showed that ABCA1 expression was significantly enhanced by treatment with all tested concentrations of FB_1 (Fig. 3B). The data collectively indicate inhibited lipid influx and promotion of cholesterol efflux.

3.4. FB_1 increases PCSK9 and LXR expression to regulate LDLR and ABCA1

PCSK9 is a proprotein convertase that regulates the post-transcriptional degradation of the LDL receptor to reduce cholesterol uptake (Zhang et al., 2016). To determine whether FB_1 regulates cholesterol metabolism in HepG2 cells in a PCSK9-dependent process, we analyzed its protein expression along with the expression of its transcriptional activator, LXR. Our data show that PCSK9 was increased in FB_1 treated cells when compared to untreated cells (Fig. 4A). Concomitantly, LXR was also upregulated by FB_1 treatment (Fig. 4B).

4. Discussion

The physiological consequence of FB_1 toxicity is canonically attributed to dysregulated lipid metabolism and lipidomic profiles which can disrupt membrane integrity and lipid raft formation (Burger et al., 2018; Riedel et al., 2016; Merrill et al., 1993). Fumonisin B_1 inhibits ceramide synthase with a consequential accumulation of sphingosine, sphinganine and their 1-phosphate derivatives, while depleting complex sphingolipids (Merrill et al., 1993; Riedel et al., 2016; Riley and Merrill,

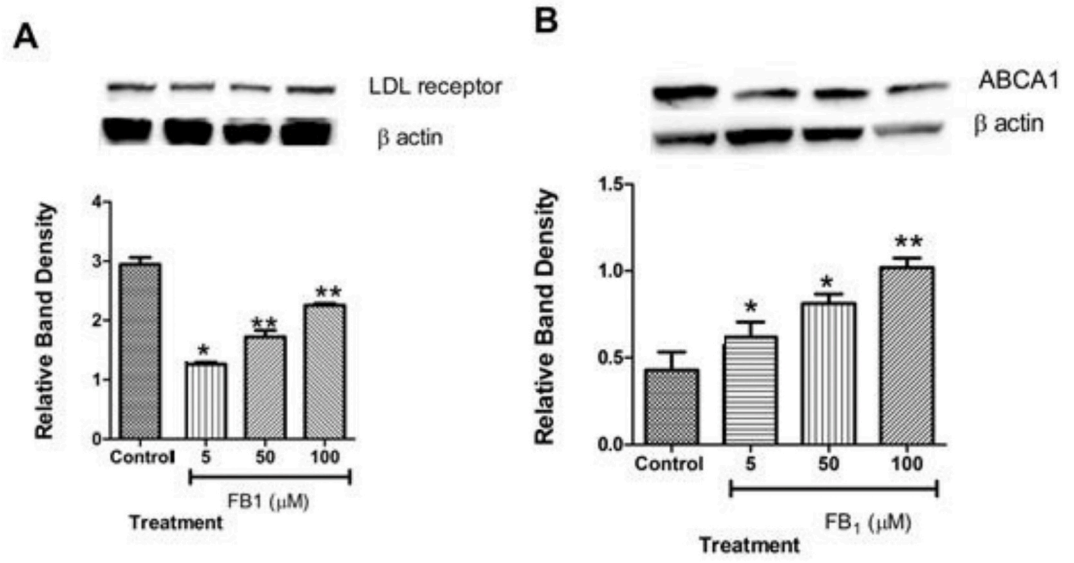

Fig. 3. In HepG2 cells FB_1 inhibits lipid uptake (A) but promotes cholesterol efflux (B). Data shown are means with SD. *p < 0.5, **p < 0.01.

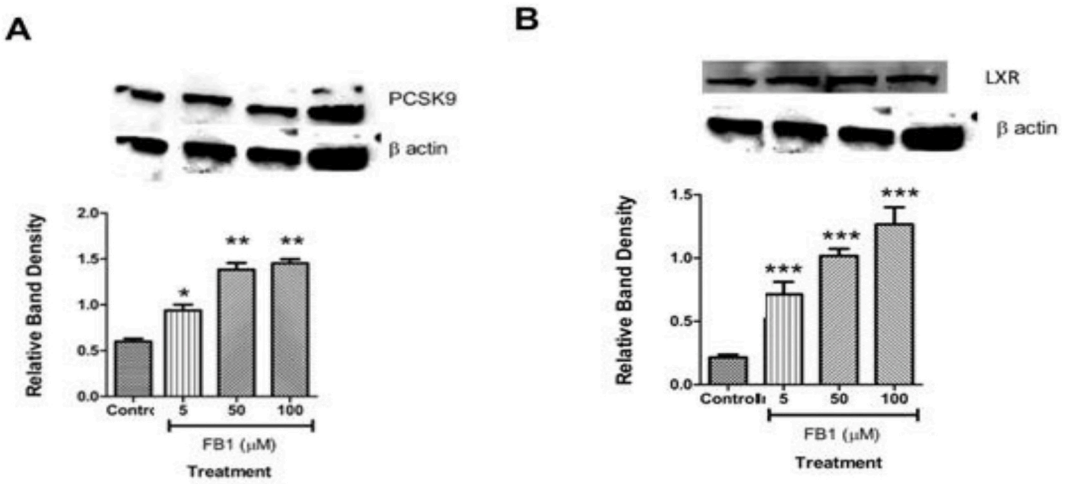

Fig. 4. FB_1 regulates cholesterol cellular fluxes in a PCSK9 dependent manner in HepG2 cells by increasing its expression (A) along with elevated expression of its transcriptional activator (B). Data shown are means with SD. *p < 0.5, **p < 0.01, ***p < 0.001.

2019). Moreover, *in vivo* and *in vitro* studies in rat liver and primary hepatocyte models indicated that FB_1 increased circulating cholesterol levels (Vilà et al., 2011; Burger et al., 2018), yet the mechanisms underlying these hypercholesterolemic events are not fully elucidated. In the present study we provide evidence to support a PCSK9 and LXR dependent pathway in which FB_1 prevents lipid influx and promotes cholesterol efflux in the HepG2 liver cell line.

The liver is an important modulator of cholesterol homeostasis (Takeyama et al., 2017) as it is the major organ responsible for the turnover of LDL from circulation (Harkes and Van Berkel, 1984; Horton et al., 1993). Thus, hepatic LDLR is central to maintaining cholesterol homeostasis and reduced LDLR expression results in decreased LDL catabolism, leading to elevated levels of plasma LDL-choleterol. Cellular cholesterol levels are controlled by the influx of lipoprotein into cells and efflux from cells through stringent, multi-level regulation of cell surface transport proteins. (Goedeke et al., 2015). It is well established that disruption of the cell membrane also impacts on membrane associated protein receptors and related signalling events (Head et al., 2014). Moreover, in lipid rafts of dendrites of cultured hippocampal neurons, the FB_1-induced depletion of cholesterol and sphingolipids resulted in the instability of surface receptors and a loss of synapses and dendritic spines (Hering et al., 2003). The LDH leakage assay is sensitive to compounds that directly damage cellular membranes (Ivanova and Uhlig, 2008). We show that membrane integrity was not compromised after exposure to all tested concentrations of FB_1 for 6 h as evidenced by no significant release of LDH (Fig. 2). These contrasting data suggest that FB_1 induced loss of membrane integrity is dependent on a host of factors such as cell/tissue type, concentration and duration of exposure. Under our selected study conditions FB_1 has a minimal effect on membrane integrity and does not affect the transport protein expression profiles observed.

By investigating the potential molecular mechanism for the hypercholesterolaemic effect of FB_1, we revealed that FB_1 significantly altered the levels of cell surface LDLR and ABCA1 protein without affecting membrane integrity in HepG2 cells. Transcriptional and post-translational regulation is critically involved in the FB_1-mediated decrease of cell surface LDLR and increase of ABCA1, which in turn promotes cholesterol efflux but inhibits cholesterol influx in HepG2 cells exposed to FB_1 (Fig. 3).

PCSK9, is an important protein involved in the post-transcriptional

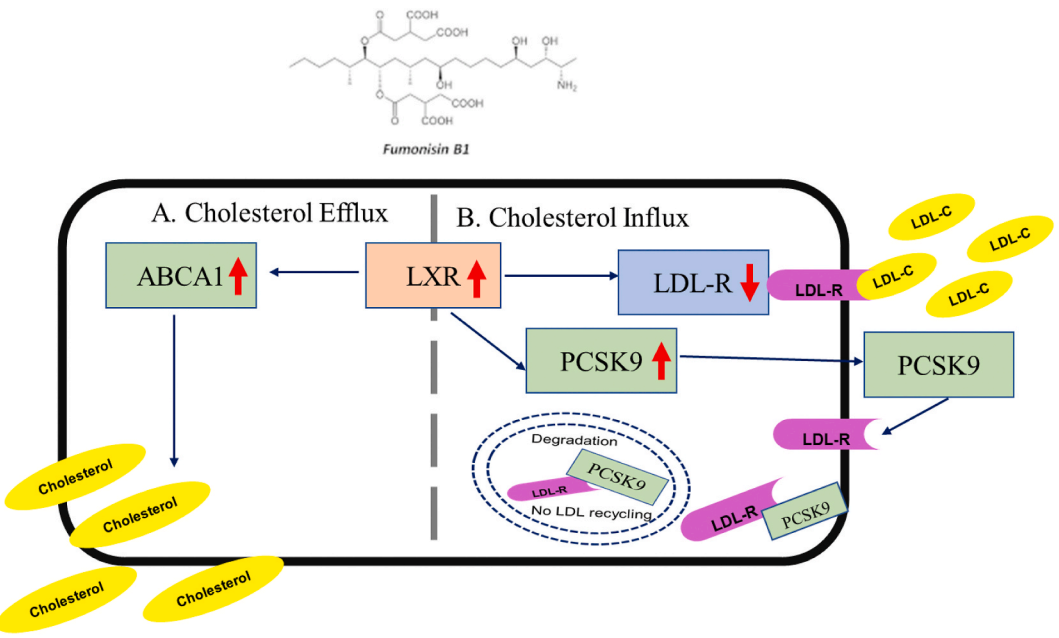

Fig. 5. FB_1 elevates the expression of LXR. LXR increases the expression of ABCA1 to promote cholesterol efflux from the cell. LXR inhibits cholesterol influx (B) by directly inhibiting the expression of LDL-R and enhancing this receptors degradation by elevating PCSK9 (a post transcriptional regulator of LDL-R). Both pathways may contribute to observed hypercholesterolemic effects of FB_1.

degradation of LDLR thereby restricting its recycling to the cell surface (Athavale et al., 2018). PCSK9 is expressed in hepatocytes and studies have validated that PCSK9 overexpression or its knockout in mice models has an inverse relationship with LDLR levels, thus suggesting that PCSK9 expression correlates with plasma cholesterol levels (Le May et al., 2009; Maxwell et al., 2005; Stoekenbroek et al., 2019). Our results suggested that FB_1 upregulated PCSK9 protein expression, consequently decreasing the cell surface LDLR, and inhibiting cholesterol influx. Furthermore, LDLR is regulated at the post transcriptional level by a pathway involving LXR. LXR maintains cholesterol homeostasis through upregulation of the E3 ubiquitin ligase inducible degrader of LDLR (IDOL), which activates the ubiquitination and degradation of LDLR, and preventing reuptake of LDL (Zelcer et al., 2009). We show that LXR was significanlty elevated by FB_1, lending support to a loss of LDLR via both pathways. Although PCSK9 and IDOL share the same LDLR substrate, the underlying mechanisms are independent and distinct (Zhang et al., 2012b; Sorrentino and Zelcer, 2012). Moreover, LXR induces the expression of genes involved in cholesterol efflux, such as ABCA1 (Goedeke et al., 2015). Here we show that FB_1 directly manipulates cholesterol influx and efflux by downregulating LDLR and upregulating ABCA1 expression (Fig. 3) which is dependant on transcriptional activation by LXR (Fig. 4B). In line with this, Regnier and co-workers (2019) suggested a prominent role of LXRs in mediating the toxic effects of FB_1 independent of its putative effects on sphingolipid metabolism (Régnier et al., 2019). These observations together with ours may explain the hypercholesterolemic role of FB_1 by increasing efflux of cellular cholesterol via increased ABCA1 expression and reduced uptake via supressed LDLR expression.

Several studies have drawn attention to the advantages imparted by PCSK9 functionality to certain cancer types with its potential to trigger hyerlipidemia shown to promote tumor growth (Athavale et al., 2018; Huang et al., 2016). Clinically, the connection between altered serum lipid level and hepatocellular carcinoma (HCC) has been documented. Intriguingly, the modifying role of FB_1 on lipid metabolism, is proposed to play a regulatory function in promoting survival of preneoplastic liver cell populations, cells exposed to this toxin exhibit a resistant phenotype to apoptosis and the promotion of hepatocarcinogenesis in rats (Gelderblom et al., 1996; Riedel et al., 2016), despite toxicokinetic studies showing a short elimination half life of around 4 h in this organ (Martinez-Larranaga et al., 1999).

In this study we show that FB_1 is acutely toxic to liver cells *in vitro* as evidenced by reduced cell viability after a 6 h incubation. We noted loss of cell viability to below 50% only after exposure to substantially high levels (250–500 μM) which are not likely to be consumed by humans or animals (Fig. 1). The adoption of strict guidelines for FB1 levels in food and feed by national and international public health and governmental authorities (Shephard et al., 2019), along with good agricultural practices and food processing limit mycotoxin levels in the food chain but may not completely eliminate them (Karlovsky et al., 2016), this suggests that low exposure to mycotoxins still remains a challenge and highlights the need for active research rather than reactive research into the mechanisms of toxicity to mitigate the adverse health effects associated with mycotoxin exposure.

We further showed that metabolic activity of treated cells is much more susceptible to FB_1 toxicity (Fig. 2). NADH is a key cytosolic cofactor in WST-1 reduction. Thus, respiratory inhibitors such as FB_1 (Domijan and Abramov, 2011; Sousa et al., 2020) generally stimulate WST-1 reduction due to their sparing effect on cytosolic NADH levels (Berridge and Tan, 1998). Taken together these results suggest disturbances to molecular machinery at low, physiologically relevant doses with FB_1. Such data underwrites the "fumonisin paradox" which propose hypotheses related to toxic mechanisms of low FB_1 exposure - high physioiological impact (Shier, 2000).

The cancer promoting properties of FB_1 (Gelderblom et al., 2001) have recently been linked to changes in lipid raft composition and altered lipidomic profiles (Burger et al., 2018; Riedel et al., 2016). Our data provides a molecular basis for the hypercholesterolemic events observed in FB_1 toxicity by dysregulating the circuitry involved in cholesterol metabolism, particularly with respect to lipid influx and cholesterol efflux from liver cells (Fig. 5). The endpoints and transport protein expression profiles monitored in this work further lend support to the FB_1 paradox hypothesis.

5. Conclusion

A considerable part of life is spent in the postprandial state, therefore

dietary toxins can direct cellular phenotypes and induce a state of metabolic toxicity. This study illustrates the negative effects of FB_1 after a short exposure period on lipid metabolism in the liver. Our results provide novel insights into the molecular pathways disrupted by FB_1 and underscore the toxicological relevance of this toxin in the dysregulation of cholesterol metabolism. We show, for the first time, that FB_1 diminishes cell-surface LDLR levels and promotes ABCA1 expression in an LXR dependent mechanism in HepG2 cells. We further highlight the prominent role of PCSK9 as a post translational regulator of LDL receptor in cells exposed to this toxin. Our data highlights the pathologic potential of this FB_1 in promoting dyslipidemias and related disorders such as cancer. Thus, accurate lipidomic profiling and metabolomic evaluations using *in vivo* and *in vitro* models should form the basis of future studies to further probe and provide mechanistic details of the multifaceted toxic profile of FB_1. Given the ubiquitous exposure of the general population to FB_1, the association between altered metabolism and its tumorigenesis potential warrants further study.

Credit author statement

Naeem Sheik Abdul: Conceptualized study, methodology, writing original draft. **Anil Chuturgoon**: Conceptualized, Supervision, writing: review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2020.12.011.

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