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Differential modulation of the lipid metabolism as a model for cellular resistance to fumonisin B_1–induced cytotoxic effects *in vitro*

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Differential sensitivity of primary hepatocytes and Chang cells to the cancer promoter fumonisin B_1 (FB_1) -induced cytotoxic effects were investigated in relation to changes in membrane lipid distribution. In contrast to primary hepatocytes, Chang cells were resistant to FB_1-induced cytotoxic effects. This was associated with a high cholesterol (Chol) and sphingomyelin (SM) and low phosphatidylcholine (PC) content, resulting in a significant (Po 0.05) decrease in phosphatidylethanolamine (PE)/PC ratio, increased Chol/total phosphoglyceride (TPG) ratios and low total polyunsaturated fatty acids (PUFA) content in PC and PE, suggesting a more rigid membrane structure. High levels of C18:1 and reduced polyunsaturated fatty acid (PUFA) levels are likely to provide selective resistance to FB_1-induced oxidative stress. FB_1-associated lipid changes included decreases in SM and Chol, increases in sphinganine (Sa) and PE with the increases in key saturated, monounsaturated, and PUFAs in PE as key role players in the differential responses to FB_1-induced cell growth responses in cells.

1. Introduction

Cellular resistance to apoptotic cell death and uncontrolled cell proliferation are some of the hallmarks of carcinogenesis and represent targets for cancer prevention [1]. A prerequisite for sustaining proliferation is readily available macromolecular building blocks, such as lipids and proteins, as well as an adequate supply of energy. Fatty acid (FA) synthesis is likely to increase in order to sustain the demand for membrane components and it was shown that highly proliferating cells contain much lower levels of polyunsaturated fatty acids (PUFA), which was also linked to lower cellular oxidative status [2–4]. Proliferating pre-cancerous tissues and tumor cells were shown to sustain low oxidative stress levels through limiting of peroxidisable substrates [5,6] presumably via defective function of PUFA desaturation by delta-5 and delta-6 desaturases [7].

Modulation of PUFA content is likely to influence the integrity and function of membranes as well as lipid mediators regulating cell signaling pathways [2]. Low PUFA content and an increased 18:1ω9 level, suggested to possess antioxidant properties [8], are associated with a lower oxidative status in rat liver nodules, which could favor cell proliferation similar to the condition in cancerous lesions [9,10]. Distinct changes in lipid content were detected in pre-cancerous and cancerous tissue in animal models and humans compared to surrounding normal tissue [11–14]. In the liver, these changes included an increase in cholesterol (Chol), phosphatidylethanolamine (PE) and the saturated fatty acid (SFA) 16:0 as well as a decrease in the phosphatidylcholine (PC)/PE ratio, 20:4ω6-PC/PE ratio and other long-chain PUFA [9,11,15,16].

The induction of a resistant phenotype in a number of hepatocytes during cancer initiation and the ability to expand into focal lesions and nodules during cancer promotion is one of the first events during hepatocarcinogenesis [17]. A selective growth stimulus via the process of mitoinhibition is introduced during

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cancer promotion, allowing resistant hepatocytes to proliferate dynamically, while inhibiting the growth of the majority of the surrounding normal hepatocytes [18]. Fumonisin B_1 (FB_1) is a common maize contaminant exhibiting cancer promoting activity in rat liver [19]. FB_1 exhibits mitoinhibitory properties in primary hepatocytes and in rat liver *in vivo* which has been proposed to be the underlying mechanism for cancer promotion [20–22]. Altered lipid metabolism, involving distinct changes in specific FA, chol, phosphoglycerides and sphingolipids affected by FB_1 and other liver cancer promoters, have been implicated in the growth selection of pre-neoplastic lesion in rat liver *in vivo* [16,20,22–24].

The disruption of ceramide synthase and the resultant elevated levels of sphingoid bases, especially sphinganine (Sa), the altered Sa/sphingosine (So) ratio and depletion of complex sphingolipids have been ascribed as the mechanism of action for the toxic and carcinogenic effects of the fumonisins [23–25]. Although Sa and So are not mitogenic in primary hepatocytes [21], So-1-phosphate (So-1P) is known to induce cell proliferation [26]. FB_1-induced cytotoxicity in primary hepatocytes and some secondary mammalian cell lines is not an acute event and, depending on the dose, occurs after incubation times of more than 24 h [21,27–29]. On the other hand, the arachidonic acid cascade is activated within minutes of exposure to FB_1 in human epithelial cell cultures [30], which could therefore play a key role in the modulation of cell survival. The mitoinhibitory properties of FB_1 in primary hepatocytes appear to occur via the disruption of the epidermal growth factor (EGF) mitogenic response which coincided with decreased 20:4ω6 content [31]. As the addition of prostaglandin E_2 (PGE_2) counteracted the mitogenic response while Sa and So lacked any effects, the disruption of 20:4ω6 metabolism seems to be important in the inhibition of cell proliferation by FB_1. These and other studies highlight that FB_1 has the potential to induce opposing effects regarding the arachidonic acid cascade, i.e. depending on cell type and cell signals, FB_1 can induce either apoptosis or cell proliferation, which is of importance during cancer promotion [22,31–33]. Therefore, alterations in lipid biosynthesis could subsequently trigger critical changes in signal transduction pathways in the liver, resulting in the genesis and development of pre-neoplastic lesions [20].

In vitro studies reported that FB_1 induced necrosis [21,28,34] and apoptosis in different cells [35–37]. In contrast, FB_1 induces mitogenesis in Swiss 3T3 fibroblasts [38] and in normal oesophageal epithelial cells [39], indicating differential effects on cell proliferative indices dependent on the cell type. In the current study, Chang cells served as a model for initiated cells since they represent a mitotically active cell population in culture and presumably display resistance to FB_1-induced toxic effects. On the other hand, primary hepatocytes provided a model reflecting non-proliferating normal cells, known to display sensitivity to cytotoxic and mitoinhibitory effects of FB_1 [20]. The present study aimed to characterise FB_1-induced lipid associated changes in Chang cells compared to primary hepatocytes in order to characterise specific responses related to cellular resistance to FB_1-induced toxicity, thereby further elucidate the mechanisms of cancer promotion by FB_1 in the liver.

2. Material and methods

2.1. Chemicals and reagents

Fumonisin B_1 was isolated according to the method described by Cawood et al. [40] to a purity of approximately 95%. Williams E medium, insulin, HEPES buffer, proteinase K and RNase A were obtained from Sigma Chemical Co., Johannesburg, South Africa. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin were from Bio-Whittaker, Walkersville, MD, USA. Triton-X-100 and NADH for the cytotoxicity assay, cholesteroloxidase and -esterase as well as the cholesterol standards and Tween 20 were purchased from Boehringer Mannheim (Roche Applied Science, Randburg, South Africa). Butylated hydroxytoluene (BHT), 2,2′-(2,5-Thienediyl)bis[5-(2-methyl-2-propanyl)-1,3-benzoxazole] (BBOT), staurosporine, the caspase-3 activity kit (#Casp-3-C) and the free fatty acid standards were acquired from Sigma Chemical Corporation (Johannesburg, South Africa). The Caspase-Glo® 3/7 assay kit and lysis buffer (Promega, Madison, USA) were obtained from Whitehead Scientific (Cape Town, South Africa). The 5-Bromo-2′-deoxyuridine (BrdU) ELISA kit was obtained from Roche, Applied Sciences, Randburg, South Africa. Silica gel 60 thin layer chromatography (TLC) plates, ethylenediamine tetraacetic acid (EDTA) and all other analytical grade chemicals and solvents for lipid analysis were obtained from Merck (Cape Town, South Africa). Organic solvents (methanol, chloroform and hexane) used for lipid extractions and analysis were glass distilled prior to use. All glassware was cleaned with a phosphate free soap (Contrad concentrate, Merck, Cape Town, South Africa) and rinsed with glass-distilled methanol before use.

2.2. Studies in Chang cells

The Chang cells were a gift from Prof. M. De Kock, University of Western Cape, Bellville, South Africa and were maintained in DMEM containing 10% FBS, L-glutamine (2 mM), and penicillin/streptomycin (100 U/ml and 100 mg/ml). For the experiments, viable (≥95%) cells ($2\ 10^4$ per dish) were plated in the aforementioned medium in 60 mm culture dishes and incubated for 72 h. The medium was replaced by DMEM containing 0.5% FBS, L-glutamine (2 mM), penicillin/streptomycin (100 mg/ml and 100 U/ml) before adding FB_1.

2.3. Studies in primary hepatocyte cultures

The use of primary hepatocytes in this experiment was approved by the Medical Research Council's (MRC) Ethics Committee for Research on Animals and the policies and standards described in the MRC's principles and guidelines for use of animals in biomedical research as well as the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) were followed. Male Fischer 344 rats were provided by the Primate Unit of the MRC, Tygerberg, South Africa, and were housed in a controlled environment with free access to feed and water until they reached a body weight of approximately 200 g. The hepatocytes were prepared according to the collagenase perfusion technique described by Hayes et al. [41]. Viable (≥90%) cells, determined by Trypan blue exclusion, were plated in 60 mm culture dishes ($6\ 10^5$ cells per dish) in Williams E medium and cultured for 4 h in a humidified incubator at 37 °C with 5% carbon dioxide. The Williams E medium was supplemented with 10% heat inactivated FBS, insulin (20 U/ml), L-glutamine (2 mM), HEPES (10 mM), streptomycin (100 mg/ml) and penicillin (100 U/ml). Cells were washed with Hanks balanced salts buffer solution and for the FB_1 exposure experiment a modified Williams E medium containing 0.5% FBS, insulin (20 U/ml), L-glutamine (2 mM), HEPES (10 mM), streptomycin (100 mg/ml), penicillin (100 U/ml) as well as L-proline (2 mM) and sodium pyruvate (10 mM) was used.

2.4. FB_1 treatments and determination of cell growth indices

A stock solution was prepared by dissolving FB_1 (21.6 mg/ml) in saline (0.9% sodium chloride in double distilled water). The

solution was filter sterilised and aliquots (50 µl) of each dilution added to obtain the respective 25 mM, 75 mM, 150 mM, 250 mM and 500 mM FB_1 concentrations. For each of the assays performed, Chang cells and primary hepatocytes were exposed to the different FB_1 concentrations for 48 and 44 h, respectively. Saline was added to control dishes.

2.4.1. Cytotoxicity assay

Samples (100 µl of the supernatant) were collected prior to harvesting the cells for lipid analyses after 44 (primary hepatocytes) and 48 h (Chang cells), respectively and mixed with an equal volume of Triton X-100 (2% in 10 mM potassium phosphate buffer, pH 7.4) for cytotoxicity analyses [41,42]. The activity of lactate dehydrogenase (LDH) released into the culture medium was measured spectrophotometrically (Uvikon 923 spectrophotometer, BioTek Instruments, Winooski, VT, USA) using sodium pyruvate as substrate and NADH as substrate and co-factor respectively. LDH activity was expressed as percentage of the total LDH release in the control dishes after cell lysis with Triton X-100 (final concentration 1%) and used as the index of cytotoxicity.

2.4.2. Cell proliferation assay

Cell proliferation in Chang cells was determined by the incorporation of 5-Bromo-2′-deoxyuridine (BrdU) into cellular DNA using an ELISA kit. Chang cells (3 10^2 cells per well) were grown in black (opaque bottom) 96 well microtiter plates and treated with 25 mM, 75 mM, 150 mM, 250 mM and 500 mM FB_1 as described above. The BrdU labelling solution (diluted in DMEM) was added 2 h before the end of the treatment period (48 h). Cells were fixed and the assay performed according to the manufacturer's instructions. Chemiluminescence was recorded using a Veritas Microplate luminometer (Turner BioSystems, Sunnyvale, USA) and expressed as % of proliferating cells compared to control which was taken as 100%. Experiment was repeated twice with 5 replications per treatment. The effect of FB_1 on cell proliferation in primary hepatocytes has been reported previously [21].

2.4.3. Apoptosis

2.4.3.1. Caspase-3 assays. Primary hepatocytes were incubated with 150 and 250 mM FB_1 for 44 h, while the Chang cells were incubated for 48 h in the presence of 3 different (150, 250 and 500 µM) FB_1 concentrations as described above. A colorimetric kit (#Casp-3-C) was used for determination caspase-3 activity in cell lysates and the manufacturer's instructions were followed utilising the 96 well microtiter plate method. A standard curve was constructed using p-nitroaniline (pNA) and each sample was analysed with and without a caspase-3 inhibitor supplied with the kit. Cell lysates were prepared by scraping cells from 3 culture dishes (60 mm) into the medium and centrifuging at 300 g for 10 min The pellet was re-suspended in 50 µl lysis buffer (provided with the kit), incubated for 20 min on ice followed by centrifugation at 18,000 g for 15 min The supernatant was stored in aliquots at 80 °C prior to analysis of caspase-3 activity and protein content. Activity was expressed as pmol pNA/min/mg protein. Apoptosis in Chang cells was also monitored utilising the Caspase 3/7-Glo kit. Cells (3 10^2 cells/well) were incubated in 96 well microtitre plates in the presence of 250 mM FB_1 as indicated above and lysed using a reporter assay lysis buffer after 3, 6, 24 and 48 h of incubation. Equal amounts of cell lysates and Caspase 3/7 Glo reagent were added to white walled 96 well plates, incubated at room temperature for 2 h and the luminescence was measured using a Veritas Microplate luminometer (Turner BioSystems, Sunnyvale, USA). Data were expressed as fold increase compared to the control. Two independent experiments were conducted with five replications for each treatment.

2.4.3.2. DNA fragmentation by agarose gel electrophoresis. Both the primary hepatocytes and Chang cells were incubated for 24 h with FB_1 (250 µM) as described above when conducting the caspase-3 assay. Cells from 4 dishes were lysed in a buffer consisting of 10 mM Tris–HCl (pH 7.5), 10 mM NaCl, 10 mM EDTA, 0.5% sodium dodecyl sulphate and incubated with 100 mg/ml proteinase K (Sigma Chemical Co, Johannesburg, S.A.) for 60 min at 50 °C on a heating block and the DNA extracted as described previously [43]. DNA was dissolved in Tris–HCl (10 mM)/EDTA (1 mM) buffer and treated with RNase A (0.1 mg/ml) for 60 min at 37 °C. Purity and concentration of the DNA was confirmed by measuring the absorbance at 260 and 280 nm spectrophotometrically and 20 mg of DNA was subjected to electrophoresis on a 1.5% agarose gel containing ethidium bromide for 2.5 h at 100 V in Tris-acetate-EDTA buffer (40 mM Tris–HCl, 20 mM acetic acid, 10 mM EDTA). Gels were documented using a Kodak 1D Image analysis software (Version 3.0, Scientific Imaging Systems, Eastman Kodak Company, Rochester, NY, USA).

2.5. Lipid analyses

2.5.1. Sphingolipid analysis

Sample preparation: For the sphingolipid analyses the Chang cells were treated with FB_1 for 48 h, using FB_1 concentrations of 1, 25, 150, 250 and 500 µM. Cells of two 60 mm petri dishes were washed twice with ice-cold 50 mM potassium phosphate buffer (pH 7.0) and combined by scraping into 3 500 µl buffer. The cell suspensions were centrifuged (1000 g, 4 °C, 10 min), supernatant discarded and the cell pellet suspended in 300 µl phosphate buffer (50 mM, pH 7.0). An aliquot was removed for protein analysis and the remaining cell suspension was stored at 80 °C for HPLC analysis. The sphingoid base content in primary rat hepatocytes has been published previously [21] but was included in Fig. 3B for comparative purposes.

HPLC analysis: Sphingoid bases, Sa and So, were determined according to the method described by Castegnaro et al. [44]. The sphingoid bases were extracted from the cell suspensions by adding 10 µl internal standard (C_{20}-sphinganine, gift from Professor A.H. Merrill, Jr, Department of Biochemistry, Emory University, School of Medicine, Atlanta, GA, USA), 1.5 ml 0.8% KCl (w/v), 50 µl 1 M KOH and 4 ml ethyl acetate to 100 µl of the cell homogenate in a screw capped glass tubes as described previously [45]. Samples and standards were separated using a HPLC system equipped with a Rheodyne injector (200 µl sample loop, model 7725i, Rheodyne, Rohnert Park, CA, USA), a Waters automated gradient controller (Millipore, Billerica, MA, USA) connected to 2 Waters Model 510 HPLC pumps, a Phenomenex Synergi 4 µ Max-RP column (75 4.6 mm internal diameter) and a Waters 474 scanning fluorescence detector (excitation at 335 nm and emission at 440 nm). The mobile phase consisted of 20% methanol in 5 mM potassium phosphate buffer (pH 3.35, mobile phase A) and 100% methanol as mobile phase B. Separation was accomplished with 80% mobile phase A and 20% mobile phase B for 36 min followed by 100% mobile phase B for 5 min at a flow rate of 1 ml/min. Data were expressed as pmol So or Sa/mg protein of three replications per analyses.

2.5.2. Cholesterol, phosphoglyceride, sphingomyelin and fatty acid analyses

The treated Chang cells and primary hepatocytes were washed with ice cold saline and stored on ice during the harvesting process. Five independent experiments were conducted and cells of 5 petri dishes per cell line were combined, representing one sample, by scraping in 3 0.5 ml ice cold saline. An aliquot of the cell suspension (100 µl) was stored at 20 °C for protein determination, while the remainder was extracted with chloroform/

methanol (CM, 2:1, v/v, containing 0.01% BHT as antioxidant) ensuring a CM:saline ratio of 20:1 for a total of three times as described previously [31,46]. Lipid extracts were stored in chloroform/methanol/saline (CMS, 86:14:1, v/v/v, containing 0.01% BHT as antioxidant) under nitrogen at 4 °C for lipid analyses.

The phosphoglyceride fractions PE, PC and SM were separated using thin layer chromatography [47]. The running solvent contained BBOT for visualising phosphoglycerides and SM under UV light at 366 nm. The inorganic phosphate content of each phosphoglyceride and SM fraction was determined spectrophotometrically [48] and expressed as µg inorganic phosphate (P_i) per mg protein. For FA analyses of PC and PE, the respective spots were scraped off and transmethylated with methanol-1.8 M sulphuric acid (95:5, v/v) at 70 °C for 2 h as described by Smuts et al. [49]. The FA methyl ester content was analysed on a Varian model 3700 Gas Chromatograph equipped with fused silica megabore DB-225 columns (J&W scientific, Cat No. 125-2232). A standard mixture consisting of free FA (C14:0 to C22:6) was used to identify the FA methyl esters in the samples by comparison of retention times, while C17:0 served as internal standard for quantification. Results were expressed in mg FA per mg protein as well as percentage of the total FA content identified in the respective phospholipid fraction.

The Chol content was determined after solubilising the lipid extract in Triton-X 100 (1% in double distilled water). The assay was based on the enzymatic (cholesterol oxidase and cholesterol esterase preparation) iodine method with an external standard as described by Richmond [50]. Results were expressed as µg Chol per mg protein.

2.6. Protein determination

Cell suspensions were diluted in a 2% sodium dodecyl sulphate solution containing EDTA (2 mM) and sodium bicarbonate (20 mM) buffer and protein content determined using a method described by Kaushal and Barnes [51] based on the reaction of bicinchoninic acid with proteins in the presence of an alkaline copper solution. Bovine serum albumin was used for the standard curve and data were processed with the Lowry programme [52].

2.7. Statistical analyses

The means of at least 3 independent experiments with duplicate treatments, unless stated otherwise, were subjected to the standard One-Way analysis of variance (ANOVA) in combination with the Tukey test to determine significant differences between treatment groups using SAS (version 9.3). Normal distribution of the data was determined using the Kolmogorov-Smirnov test. Differences were considered significant when $P \leq 0.05$.

3. Results

3.1. Cell survival indices

3.1.1. Cytotoxicity and cell proliferation

No significant cytotoxicity was induced by FB_1 in Chang cells after a 48 h incubation period at concentrations of up to 500 µM (Fig. 1A). In primary rat hepatocyte cultures, the LDH release was significantly ($P \leq 0.05$) increased dose-dependently from 75 mM FB_1 up to 500 mM after a 44 h incubation period. No significant effect of FB_1 on cell proliferation in Chang cells at any of the concentrations tested was recorded. The % proliferation recorded was: control (100±7.8%); 25 mM (86.4±7.1); 75 mM (80.7±8.8); 150 mM (91.3±8.7%); 250 mM FB_1 (88.5±14.5%) and 500 mM (84.4±11.5%). The anti-proliferative effect of FB_1 in primary hepatocytes, utilising similar FB_1 concentrations, has been reported previously [21].

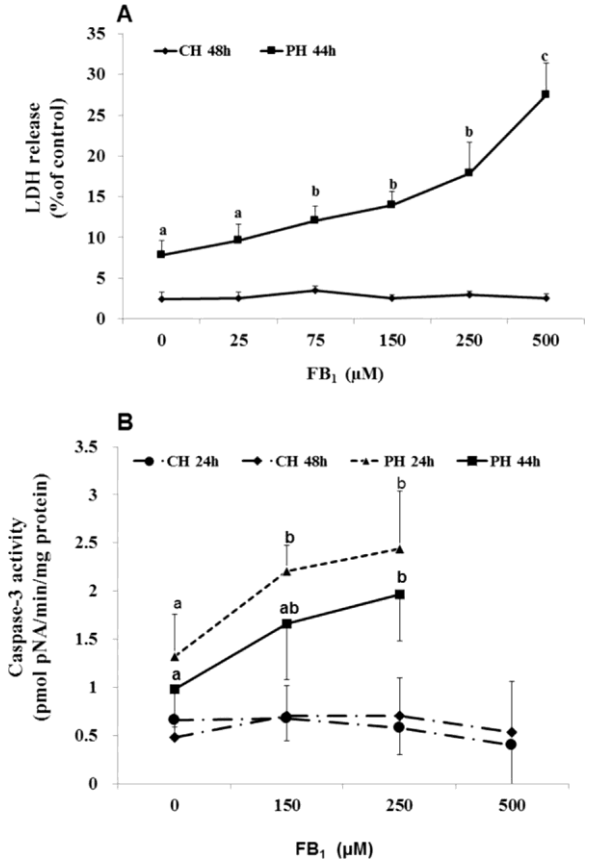

Fig. 1. Effect of fumonisin B_1 on cytotoxicity and apoptosis in Chang cells (48 h) and primary hepatocytes (44 h). A: cytotoxicity measured by the ratio of lactate dehydrogenase activity measured in cell supernatants versus total cell lysates. B: apoptotic effect of FB_1 as measured by caspase 3 activity. Abbreviations: FB_1-fumonisin B_1, CH - Chang cells, pH – primary hepatocytes, LDH - lactate dehydrogenase. Values are means of triplicate determinations ± SD. If letters on error bars differ then $P \leq 0.05$.

3.1.2. Apoptosis

The Chang cells were resistant to the induction of apoptosis since no significant differences in caspase-3 activity were detected after 24 and 48 h at 150 µM, 250 mM and 500 mM treatments (Fig. 1B). No effects were recorded after 3 and 6 h (data not shown). The caspase-3 activity increased significantly ($P \leq 0.05$) at 24 and 44 h in primary rat hepatocytes treated with 150 and 250 µM FB_1. Staurosporine, used as a positive control, significantly increased apoptosis in primary hepatocytes after 24 h (2.53±0.55 pmol pNA/min/mg protein) compared to the control (1.16±0.55 pmol pNA/min/mg protein). The respective results for the Chang cells was 0.26±0.29 for the control and 1.50±0.65 pmol pNA/min/mg protein for the treated cells after 6 h. The lack of FB_1-induced apoptosis in Chang cells was confirmed by the absence of DNA fragmentation, whereas it was observed in primary hepatocytes following treatment with 250 mM FB_1 for 24 h (Fig. 2).

The resistance to apoptosis was also confirmed utilising the cell based luminescence Caspase 3/7-Glo assay. No difference in caspase-3/7 activity was detected between controls (1.0±0.2 fold) and the different FB_1 concentrations (25, 75, 150, 250 and 500 µM FB_1), with the activity ranging between 1.0±0.2 fold to 1.2±0.2 fold after 6, 24 and 48 h incubation. The positive control,

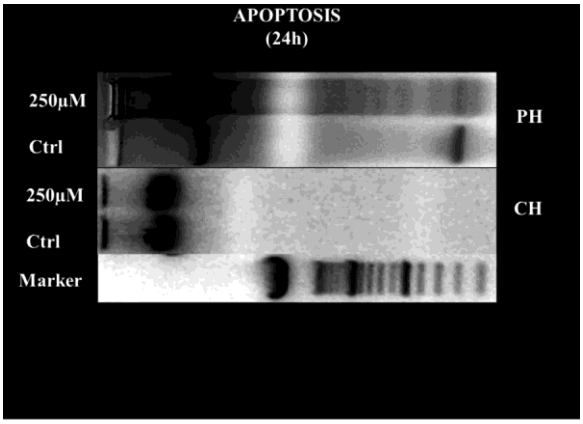

Fig. 2. Apoptotic effects of FB_1 (250 μM) as indicated by induction of DNA fragmentation in Chang cells and primary hepatocytes. Abbreviations: pH – primary hepatocytes, CH – Chang cells; CTRL – control.

staurosporine, exhibited a 5.870.1 fold increase in Chang cells after 6 h as compared to the 1.070.2 fold in the control.

3.2. Lipid analyses

3.2.1. Effect on sphingolipid content

The So content did not change significantly in Chang cells exposed to FB_1 concentrations ranging from 1 to 500 mM, while Sa content increased significantly (Po0.05) at treatments from 25 μM FB_1 and above (Fig. 3A). The Sa/So ratio showed a slight dose response and was significantly (Po0.05) higher at 25 mM FB_1, increased further significantly (Po0.05) at 25 mM and plateaued at the higher concentrations. The sphingoid base content in primary rat hepatocytes has been published previously [21] but is presented in Fig. 3B for comparative purposes. So content did not differ significantly from control treatment, while Sa and the Sa/So ratio increased significantly (Po0.05) from 1 μM FB_1 and above. The decrease at higher concentrations is likely to be related to an increased cytotoxic effect. The increase in Sa content in response to FB_1 was approximately 4 fold higher in Chang cells when compared to primary hepatocytes.

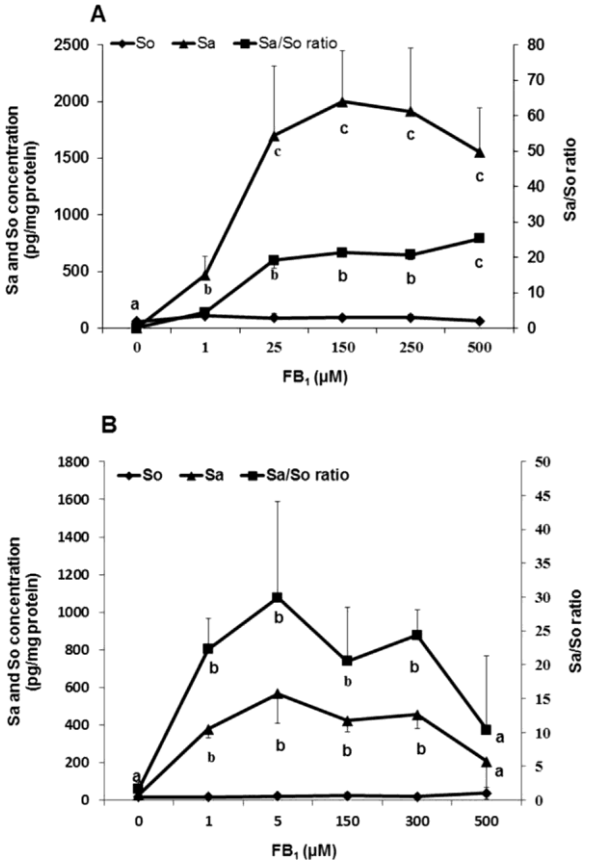

Fig. 3. Sphingosine, sphinganine content and sphinganine/sphingosine ratios in Chang cells (A) and primary hepatocytes (B) treated with FB_1 for 48 (Chang cells) and 44 h (primary hepatocytes). *The data in 3B were adapted from Gelderblom* et al. [21] *for comparative purposes.* Abbreviations: FB [1] – fumonisin B [1], Sa – sphinganine, So – sphingosine. Values are means of triplicate determinations 7 SD. If letters on error bars differ then Po0.05.

3.2.2. Effect on cholesterol and phosphoglyceride metabolism

Chol content in Chang cells was approximately 30% higher than in primary hepatocytes, while SM content was 68% higher (Table 1). The PC content in Chang cells, on the other hand, was only 69% of that in primary hepatocytes, leading to a PC/PE ratio that was 25% lower in Chang cells. The Chol/total phosphoglyceride (TPG) ratio was 74% higher in Chang cells compared to primary hepatocytes as a result of lower TPG and higher Chol content.

No significant difference in Chol content was recorded in FB_1-treated Chang cells although it was markedly decreased. The Chol content was significantly (Po0.05) decreased in the primary rat hepatocytes at all FB_1 concentrations used, lacking any dose-response effect. The SM levels significantly (Po0.05) decreased in both Chang cells and primary rat hepatocytes at all FB_1 concentrations lacking a dose-response effect. The PC content increased (Po0.05) significantly by approximately 14% in Chang cells treated with the 250 μM FB_1, whereas it decreased significantly (Po0.05) by 17% in primary rat hepatocytes exposed to 150 μM. In contrast, the PE content significantly (Po0.05) increased in both FB_1-treated Chang cells (approximately 70% at 25 μM) and primary hepatocytes (45% at 25 μM) although it tended to decrease again at 250 μM and 500 μM FB_1 in primary hepatocytes, presumably due to the cytotoxic effect. The membrane fluidity parameters, Chol/TPG and PC/PE ratios decreased significantly in Chang cells and in primary hepatocytes at all FB_1 concentrations although no dose response effects noticed. FB_1 treatment also resulted in a decreased PC/(PE+SM) ratio (Po0.05) in both cell lines. However, the ratio was higher in the primary hepatocytes than Chang cells.

3.2.3. Effect of FB_1 on fatty acid metabolism

3.2.3.1. Saturated fatty acids (16:0 and 18:0)

3.2.3.1.1. Absolute FA content (μg FA/mg protein). Total SFA content in PE and PC was approximately two fold lower in Chang cells compared to primary hepatocytes, mainly due to higher 18:0 content (Table 2). Although FB_1 significantly (Po0.05), increased 16:0 and total SFA in the PE fraction of the Chang cells, no dose-response effects were apparent. In primary rat hepatocytes, 16:0 increased significantly in PE at FB_1 concentrations up to 250 μM and 18:0 up to 150 mM (Table 3). The total SFA was increased significantly (Po0.05) at all FB_1 concentrations, except for the highest level where it was markedly increased in Chang cells with no effect in primary hepatocytes.

3.2.3.1.2. Relative FA content (% of the total FA). The relative levels of total SFA in PC is very similar in the Chang cells and primary hepatocytes, although the 18:0 level in Chang cells is about 6 fold lower and the 16:0 level 1.5 fold higher. A similar effect is noticed in PE where the 18:0 level is approximately 2 fold lower in the Chang cells. In Chang cells, FB_1 treatment significantly (Po0.05) further increased the 16:0 level at 25, 75, 500 μM and markedly at 150 and 250 μM in PC, resulting in a marked to significant increase of the total SFA. In PE, 18:0 decreased significantly at all FB_1 concentrations, except at 250 μM where only marked

Table 1
Phospholipid and cholesterol content with selected fluidity parameters of Chang cells and primary hepatocytes treated with FB_1.

	Control	25 μM	75 μM	150 μM	250 μM	500 μM
Chang cells						
Chol	20.2±2.1	18.5±1.3	18.9±1.3	19.4±1.2	19.9±1.1	18.9±1.2
SM	11.75±2.11^{A}	5.70±1.28^{B}	5.60±1.62^{B}	5.34±1.54^{B}	5.08±1.09^{B}	4.98±1.26^{B}
PC	71.8±4.5^{A}	77.3±2.4^{A}	78.3±4.2^{A}	79.1±3.8^{A}	81.8±5.2^{B}	81.2±6.0^{B}
PE	30.3±3.1^{A}	50.4±3.2^{B}	50.8±3.4^{B}	50.0±2.0^{B}	52.5±4.8^{B}	51.2±3.0^{B}
PC/PE	2.25±0.11^{A}	1.47±0.07^{B}	1.47±0.10^{B}	1.50±0.06^{B}	1.47±0.08^{B}	1.49±0.06^{B}
Chol/TPL	0.40±0.05^{A}	0.29±0.01^{B}	0.29±0.02^{B}	0.30±0.02^{B}	0.29±0.02^{B}	0.28±0.01^{B}
PC/(PE+SM)	1.57±0.08^{A}	1.30±0.09^{B}	1.32±0.06^{B}	1.35±0.06^{B}	1.33±0.07^{B}	1.36±0.06^{B}
Primary hepatocytes						
Chol	15.6±1.4^{A}	12.5±1.1^{B}	12.6±0.8^{B}	12.4±0.5^{B}	11.9±0.9^{B}	12.6±2.0^{B}
SM	6.98±1.73^{A}	3.20±0.21^{B}	3.12±0.81^{B}	3.05±0.52^{B}	3.36±0.34^{B}	3.80±0.56^{B}
PC	104.7±5.1^{A}	100.3±6.2^{A}	96.1±5.8^{A}	93.4±8.1^{B}	86.8±4.4^{B}	85.5±6.2^{B}
PE	33.6±2.2^{A}	48.7±2.7^{C}	47.6±3.2^{C}	45.0±4.9^{C}	40.9±4.9^{B}	35.5±2.5^{B}
PC/PE	2.99±0.16^{A}	1.94±0.06^{Ba}	1.91±0.13^{Ba}	1.89±0.17^{Ba}	1.98±0.16^{B}	2.22±0.16^{Bb}
Chol/TPL	0.23±0.03^{A}	0.17±0.01^{B}	0.18±0.01^{B}	0.18±0.00^{B}	0.18±0.01^{B}	0.18±0.01^{B}
PC/(PE+SM)	3.16±0.16^{A}	2.05±0.06^{Ba}	2.02±0.14^{Ba}	2.00±0.18^{Ba}	2.09±0.17^{B}	2.35±0.17^{Bb}

Values represent means ± standard deviation of 4–5 replications. Differing letters indicate statistical significance ($P<0.05$). Upper case letters – comparison to control, lower case letters comparison between concentrations. Abbreviations: Chol – cholesterol, FB_1 – fumonisin B_1, PC – phosphatidylcholine, PE – phosphatidylethanolamine, SM – sphingomyelin, TPL – total phospholipids (PC+PE); PC/(PE+SM), PC/PE and Chol/TPL are molar ratios.

decrease was noticed. A similar response was noticed in primary hepatocytes at the lowest FB_1 concentration.

3.2.3.2. Monounsaturated fatty acids (MUFA, 16:1 and 18:1)

3.2.3.2.1. Absolute FA content (μg FA/mg protein). In Chang cells, total MUFA content in PE was 2 fold higher than in primary hepatocytes, due to higher levels of 18:1. Total MUFA in PC were also higher in Chang cells compared to primary hepatocytes (1.5 fold) due to a higher 16:1 content.

FB_1 significantly ($P<0.05$) increased the 16:1, 18:1 and total MUFA content in PE of Chang cells at all concentrations although the levels tended to decrease slightly at the two highest concentrations. In the primary hepatocytes, 18:1 in PE increased markedly at 25 and 75 mM and significantly at 150 μM FB_1, while it tended to decrease at the highest concentrations (250 and 500 μM FB_1). In PC, 16:1, 18:1 and total MUFA levels were significantly ($P<0.05$) decreased in primary hepatocytes at the highest concentration (500 μM FB_1).

3.2.3.2.2. Relative FA content (% of the total FA). In the Chang cells, MUFA constituted up to 40% and 60% of the total FA in PC and PE, respectively. In PC, 16:1 and 18:1 represent a 9 and 2 fold higher level, respectively resulting in a 3 fold higher MUFA level. In PE a similar effect is noticed although the levels are lower when compared to PC. The 16:1 and the total MUFA levels increased significantly ($P<0.05$) in PE at all FB_1 concentrations. In the PC fraction of primary hepatocytes, 18:1 and total MUFA decreased markedly and significantly in cells exposed to 250 and 500 μM respectively, while no effect was noticed in the Chang cells.

3.2.3.3. Polyunsaturated ω6 fatty acids (PUFA, 18:2ω6, 20:4ω6, 22:4ω6 and 22:5ω6)

3.2.3.3.1. Absolute FA content (μg FA/mg protein). In Chang cells the total ω6 FA content was approximately two fold and 16 fold lower in PE and PC, respectively, compared to primary hepatocytes. In PC, the levels of 18:2ω6 and 20:4ω6 was approximate 8 fold and 30 fold lower, respectively in the Chang cells.

FB_1 markedly to significantly ($P<0.05$) increased 18:2ω6 and 20:4ω6 in PE of the Chang cells, which led to significantly increase in the total ω6 fatty acids, although they tended to decrease at 500 μM. In PC no effect was noticed on the 22:4ω6 and 22:5ω6 and total ω6 FA, however, levels were very low. In primary hepatocytes, the levels of 18:2ω6, 20:4ω6, 22:5ω6 and total ω6 FA increased markedly to significantly ($P<0.05$) in PE at FB_1 concentrations up to 150 μM, but tended to decrease at higher concentrations. Similarly to the Chang cells, low levels of 22:4ω6 and 22:5ω6 were noticed in the primary hepatocytes which were not affected by FB_1.

3.2.3.3.2. Relative FA content (% of the total FA). In PC of the Chang cells the total ω6 FA are approximately 8 fold lower when compared to the primary hepatocytes while in PE a slight decrease was noticed. Upon FB_1 treatment, the 18:2ω6 content in PC of the Chang cells was significantly ($P<0.05$) lowered at 25 and 75 μM FB_1 and markedly at higher concentrations, while 20:4ω6 decreased markedly in PC and significantly ($P<0.05$) in PE in all FB_1 treated groups. Very low levels of 22:5ω6 were noticed in both PE and PC while no effect was noticed following FB_1 treatment. The decrease in the individual ω6 FA resulted in significant reduction of the total ω6 FA in PC at 25, 75 and 150 μM FB_1. Primary hepatocytes showed a marked to significant $P<0.05$ increase in 18:2ω6 in PE at 150 μM FB_1, whereas 20:4ω6 was decreased significantly ($P<0.05$) at the 150 μM treatment and markedly at all other FB_1 concentrations.

3.2.3.4. Polyunsaturated ω3 fatty acids (22:5ω3 and 22:6ω3)

3.2.3.4.1. Absolute FA content (μg FA/mg protein). Total ω3 FA content was approximately 3 fold lower in PE and 13 fold lower in PC in Chang cells compared to primary hepatocytes, mainly due to lower 22:6ω3 content.

In the Chang cells, the total and individual ω3 FA markedly increased in PE by FB_1. In the PC fraction, 20:5ω3 and 22:5ω3 decreased markedly to significantly ($P<0.05$) following FB_1 treatment resulting in an overall decreased in the total ω3 FA. In the PE phosphoglyceride fraction of the primary hepatocytes, levels of C22:5ω3 increased significantly at concentrations up to 250 μM and 22:6ω3 increased up to 150 μM FB_1, while it decreased at higher concentrations. The same trend was observed in the total ω3 FA in PE. In PC, the ω3 FA also tended to decrease at higher concentrations of FB_1, but changes were not significant.

3.2.3.4.2. Relative FA content (% of the total FA). The total ω3 FA was approximately 8 fold and 4 fold lower in PC and PE, respectively in the Chang cells mainly due to a low level of 22:6ω3. In Chang cells, FB_1 significantly ($P<0.05$) decreased the 22:5ω3 level at all concentrations while the C20:5ω3 level was significantly to markedly decrease resulting in a corresponding decrease in the total amount of ω3 FA. In PE, C22:5ω3, C22:6ω3 and total ω3 FA levels were also lowered markedly to significantly ($P<0.05$) by

Table 2
Modulation of the absolute (mg/mg protein) and relative (% of the total FA) fatty acid profiles of Chang cells exposed to different FB_1 concentrations.

Treatment	Control		25 μM		75 μM		150 μM		250 μM		500 μM	
	PE	PC	PE	PC	PE	PC	PE	PC	PE	PC	PE	PC
μg/mg protein												
C16:0	1.75±0.38[A]	11.43±2.10	3.17±0.34[B]	12.74±1.44	3.11±0.91[B]	13.13±0.79	2.90±0.59[B]	12.00±0.84	3.25±0.56[B]	11.86±2.14	2.90±0.37[B]	11.64±1.14
C18:0	1.90±0.28	0.86±0.14	2.43±0.25	1.12±0.30	2.39±0.40	1.08±0.32	2.52±0.51	1.15±0.18	2.86±0.69	1.09±0.21	2.27±0.18	1.10±0.27
Total SFA	3.65±0.52[A]	12.30±2.21	5.60±0.46[B]	13.87±1.40	5.50±1.17[B]	14.22±0.84	5.42±1.07[B]	13.16±0.94	6.11±1.17[B]	12.95±2.10	5.17±0.48	12.74±1.35
C16:1	1.16±0.28[A]	6.58±1.23	3.17±0.34[B]	7.08±0.89	3.00±1.10[B]	7.25±0.69	3.05±0.55[B]	6.28±1.13	2.96±0.35[B]	6.51±1.92	2.97±0.39[B]	6.40±0.97
C18:1	5.69±1.04[A]	14.62±1.42	10.80±1.91[B]	13.45±1.67	10.48±2.46[B]	13.34±2.51	11.24±2.22[B]	14.07±3.13	11.34±2.09[B]	13.35±1.30	9.16±0.73	11.93±0.58
Total MUFA	6.84±0.82[A]	21.20±2.36	13.97±2.00[B]	20.53±2.11	13.48±3.42[B]	20.59±2.14	14.29±2.60[B]	20.35±2.46	14.30±2.09[B]	19.86±2.14	12.13±0.92[B]	18.33±1.26
C18:2	0.49±0.14[A]	0.70±0.18[A]	0.90±0.20[A]	0.46±0.10[A]	0.95±0.23[A]	0.42±0.10[B]	1.05±0.39[B]	0.47±0.07[A]	1.11±0.28[B]	0.50±0.14[A]	0.91±0.19[A]	0.51±0.16[A]
C20:3	0.10±0.06	0.09±0.02[A]	0.26±0.04	0.05±0.01[A]	0.25±0.13	0.04±0.02[B]	0.28±0.20	0.05±0.02[A]	0.33±0.23	0.04±0.02[B]	0.22±0.08	0.06±0.03[A]
C20:4	2.76±0.38[A]	0.43±0.10	3.31±0.30[A]	0.22±0.06	3.48±0.54[A]	0.16±0.09	3.50±0.33[B]	0.21±0.06	3.34±0.29[A]	0.35±0.29	3.31±0.23[A]	0.28±0.18
C22:4	0.21±0.12	0.06±0.03	0.24±0.05	n.d.	0.33±0.34	n.d.	0.28±0.08	0.02±0.03	0.10±0.13	0.05±0.05	0.21±0.03	0.04±0.05
C22:5	0.01±0.01	0.01±0.01	0.01±0.02	n.d.	0.01±0.02	n.d.	0.02±0.04	n.d.	n.d.	0.01±0.03	0.02±0.02	0.02±0.02
Total ω6 PUFA	3.57±0.66[A]	1.30±0.19	4.72±0.47[B]	0.73±0.16	5.02±0.79[B]	0.63±0.20	5.13±0.60[B]	0.74±0.16	4.88±0.29[B]	0.94±0.35	4.67±0.51[A]	0.92±0.35
C20:5	0.55±0.16	0.11±0.02[A]	0.59±0.37	0.06±0.01[A]	0.84±0.28	0.04±0.03[B]	0.79±0.10	0.04±0.03[B]	0.65±0.39	0.03±0.03[B]	0.73±0.15	0.05±0.04[B]
C22:5	0.41±0.04	0.11±0.02[A]	0.38±0.06	0.03±0.03[B]	0.37±0.05	0.03±0.02[B]	0.38±0.06	0.02±0.03[B]	0.44±0.21	0.04±0.04[B]	0.32±0.04	0.03±0.03[B]
C22:6	0.57±0.04	0.14±0.03	0.73±0.25	0.07±0.07	0.67±0.17	0.09±0.07	0.71±0.18	0.06±0.07	0.63±0.10	0.13±0.07	0.57±0.05	0.04±0.06
Total ω3 PUFA	1.57±0.17	0.40±0.08[A]	1.76±0.64	0.17±0.10[B]	1.91±0.41	0.20±0.14[A]	1.92±0.34	0.13±0.11[B]	1.74±0.62	0.21±0.14	1.65±0.19	0.12±0.09[B]
% of total FA												
C16:0	11.16±2.05	32.31±1.94[A]	12.26±1.57	36.10±1.38	11.90±2.25	36.93±1.43[B]	10.82±1.29	35.02±2.40[A]	12.05±1.61	34.78±3.50[A]	12.25±0.99	36.23±0.73
C18:0	12.12±0.91[A]	2.46±0.24	9.39±0.92[B]	3.19±0.89	9.27±1.04[B]	3.05±0.96	9.36±0.75[B]	3.35±0.22	10.47±1.38	3.25±0.71	9.62±0.58[B]	3.40±0.58
Total SFA	23.28±1.80	34.76±1.99[A]	21.65±2.13	39.29±0.54[B]	21.17±1.98	39.98±1.65[B]	20.18±1.81	38.36±2.22[A]	22.52±2.38	38.03±2.85[A]	21.86±1.01	39.62±0.84[B]
C16:1	7.43±1.79[A]	18.60±1.75	12.22±1.18[B]	20.08±1.76	11.43±2.99[B]	20.49±2.70	11.39±1.33[B]	18.41±3.66	11.04±1.76	18.90±3.97	12.56±1.23[B]	19.84±1.52
C18:1	36.38±5.91	41.76±3.32	41.35±3.49	38.08±2.20	40.23±2.56	37.23±3.97	41.83±3.41	40.71±6.01	41.73±3.74	39.68±5.51	38.79±2.39	37.35±3.14
Total MUFA	43.81±4.24[A]	60.36±1.64	53.57±2.55[B]	58.16±0.52	51.67±4.67[B]	57.72±1.32	53.22±2.79[B]	59.12±2.37	52.77±2.60[B]	58.59±1.58	51.34±2.33[B]	57.19±1.66
C18:2	3.14±0.82	2.00±0.45[A]	3.47±0.75	1.30±0.25[B]	3.71±0.88	1.18±0.25[B]	3.89±1.11	1.37±0.19[A]	4.09±0.87	1.44±0.25[A]	3.84±0.78	1.58±0.41[A]
C20:3	0.62±0.37	0.26±0.04[A]	0.98±0.07	0.15±0.04	0.98±0.50	0.10±0.06[B]	0.99±0.61	0.13±0.05	1.19±0.75	0.10±0.06[B]	0.93±0.33	0.19±0.09[A]
C20:4	17.66±1.80[A]	1.27±0.43	12.75±0.74[B]	0.63±0.18	13.61±2.19[B]	0.45±0.25	13.32±2.24[B]	0.60±0.14	12.51±1.98[B]	1.05±0.98	14.04±1.12[B]	0.85±0.47
C22:4	1.33±0.75	0.17±0.10	0.92±0.20	n.d.	1.38±1.45	0.01±0.02	1.04±0.23	0.04±0.09	0.39±0.53	0.14±0.15	0.91±0.17	0.13±0.15
C22:5	0.04±0.09	0.02±0.05	0.03±0.07	n.d.	0.03±0.08	n.d.	0.05±0.13	n.d.	n.d.	0.05±0.10	0.06±0.09	0.05±0.07
Total ω6 PUFA	22.82±3.58	3.75±0.73[A]	18.18±1.43	2.08±0.44[B]	19.72±4.01	1.76±0.50[B]	19.31±2.11	2.14±0.40[B]	18.18±1.77	2.78±1.13[A]	19.79±2.20	2.80±0.88[A]
C20:5	3.52±0.94	0.31±0.09[A]	2.16±1.27	0.18±0.03[A]	3.30±1.25	0.10±0.07[B]	3.01±0.50	0.11±0.08[B]	2.47±1.42	0.09±0.11[B]	3.07±0.58	0.16±0.11[A]
C22:5	2.62±0.27[A]	0.32±0.09[A]	1.47±0.16[B]	0.09±0.09[B]	1.44±0.21[B]	0.08±0.05[B]	1.46±0.31[B]	0.07±0.08[B]	1.62±0.70[B]	0.12±0.13[B]	1.38±0.21[B]	0.09±0.09[B]
C22:6	3.65±0.22[A]	0.39±0.08	2.77±0.61[B]	0.20±0.20	2.56±0.24[B]	0.25±0.18	2.69±0.61[B]	0.19±0.23	2.35±0.48[B]	0.37±0.22	2.44±0.31[B]	0.12±0.17
Total ω3 PUFA	10.09±0.89[A]	1.13±0.24[A]	6.60±1.63[(B)]	0.47±0.25[B]	7.44±1.59	0.53±0.38[A]	7.29±1.37	0.38±0.34[B]	6.53±2.32[B]	0.61±0.43[A]	7.01±0.92[B]	0.38±0.26[B]

Values represent means ± standard deviation of 4–5 replications and expressed as μg per mg protein or % of total FA. Statistical analysis: One-way ANOVA, differing letters/combination of letters indicate statistical significance ($P \leq 0.05$). Upper case letters – comparison to control, lower case letters comparison between concentrations. Abbreviations: FB1 – fumonisin B1, PUFA – polyunsaturated fatty acids, SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, n.d. = not detectable.

Table 3
Modulation of absolute (mg/mg protein) and relative (% of the total FA) fatty acid profiles of primary hepatocytes exposed to different FB_1 concentrations.

Treatment	Control		25 μM		75 μM		150 μM		250 μM		500 μM	
	PE	PC	PE	PC	PE	PC	PE	PC	PE	PC	PE	PC
μg/mg protein												
C16:0	3.09 ± 0.85^{A}	12.87 ± 2.46	5.13 ± 0.95^{B}	12.19 ± 2.97	5.02 ± 0.50^{B}	13.45 ± 1.99	5.49 ± 1.33^{B}	11.96 ± 2.20	4.81 ± 0.64^{B}	12.04 ± 3.32	3.94 ± 0.76^{A}	13.28 ± 1.19
C18:0	6.61 ± 0.98^{A}	12.41 ± 1.00^{A}	8.62 ± 0.82^{B}	11.45 ± 2.47^{A}	8.53 ± 0.96^{B}	10.55 ± 1.09^{A}	8.87 ± 0.80^{B}	10.66 ± 1.53^{A}	7.87 ± 1.33^{A}	10.78 ± 1.57^{A}	6.55 ± 1.52^{A}	9.94 ± 1.04^{B}
Total SFA	9.70 ± 1.63^{A}	25.29 ± 3.11	13.76 ± 0.45^{B}	23.64 ± 1.82	13.55 ± 1.07^{B}	24.00 ± 2.13	14.36 ± 2.06^{B}	22.62 ± 2.30	12.68 ± 1.63^{B}	22.81 ± 2.11	10.49 ± 1.86^{A}	23.22 ± 1.22
C16:1	0.46 ± 0.21	1.77 ± 0.85^{A}	0.60 ± 0.22	1.89 ± 0.88^{A}	0.41 ± 0.27	1.72 ± 0.73^{A}	0.63 ± 0.12	1.42 ± 0.88^{A}	0.53 ± 0.14	1.05 ± 0.69^{AB}	0.59 ± 0.34	0.80 ± 0.09^{B}
C18:1	2.81 ± 0.94^{A}	12.82 ± 2.06^{A}	4.13 ± 0.64^{A}	12.75 ± 3.06^{A}	3.71 ± 0.63^{A}	11.81 ± 1.88^{A}	4.74 ± 2.16^{B}	11.12 ± 1.16^{AB}	3.45 ± 0.76^{A}	10.25 ± 1.63^{AB}	2.56 ± 0.45^{A}	8.45 ± 1.42^{B}
Total MUFA	3.27 ± 1.04^{A}	14.59 ± 2.74^{A}	4.73 ± 0.78^{A}	14.64 ± 2.91^{A}	4.12 ± 0.81^{A}	13.53 ± 2.19^{A}	5.37 ± 2.27^{B}	12.54 ± 1.20	3.98 ± 0.80^{A}	11.29 ± 2.00	3.14 ± 0.67^{A}	9.25 ± 1.41^{B}
C18:2	0.73 ± 0.18^{A}	5.82 ± 0.94	1.40 ± 0.21^{A}	5.75 ± 1.05	1.32 ± 0.23^{A}	5.52 ± 0.83	1.90 ± 0.98^{B}	5.13 ± 0.80	1.26 ± 0.22^{A}	5.44 ± 1.34	1.19 ± 0.16^{A}	5.23 ± 0.49
C20:3	0.06 ± 0.07	0.84 ± 0.31	0.07 ± 0.08	0.90 ± 0.49	0.13 ± 0.09	0.65 ± 0.35	0.09 ± 0.08	0.65 ± 0.33	0.12 ± 0.11	0.61 ± 0.29	0.07 ± 0.11	0.61 ± 0.32
C20:4	7.05 ± 0.74^{A}	13.57 ± 1.72	9.95 ± 1.19^{B}	12.79 ± 1.89	8.82 ± 1.18^{A}	12.40 ± 2.69	8.99 ± 0.84^{A}	12.07 ± 3.18	8.04 ± 1.21^{A}	13.08 ± 3.66	6.92 ± 1.63^{A}	12.03 ± 0.98
C22:4	0.06 ± 0.03	0.09 ± 0.03	0.12 ± 0.04	0.07 ± 0.03	0.11 ± 0.06	0.05 ± 0.03	0.12 ± 0.13	0.05 ± 0.04	0.06 ± 0.04	0.07 ± 0.04	0.09 ± 0.06	0.05 ± 0.02
C22:5	0.02 ± 0.02	0.05 ± 0.01	0.02 ± 0.02	0.05 ± 0.05	0.04 ± 0.04	0.03 ± 0.01	0.01 ± 0.02	0.02 ± 0.01	0.01 ± 0.02	0.03 ± 0.03	n.d.	0.01 ± 0.01
Total ω 6 PUFA	7.95 ± 0.92^{A}	20.40 ± 2.18	11.59 ± 1.39^{B}	19.58 ± 2.95	10.44 ± 1.52^{A}	18.67 ± 2.86	11.13 ± 1.82^{B}	17.94 ± 3.46	9.53 ± 1.44^{A}	19.23 ± 3.98	8.29 ± 1.83^{A}	17.94 ± 1.52
C20:5	0.38 ± 0.16	0.64 ± 0.24	0.33 ± 0.20	0.57 ± 0.41	0.53 ± 0.28	0.49 ± 0.20	0.46 ± 0.20	0.46 ± 0.16	0.49 ± 0.25	0.41 ± 0.25	0.26 ± 0.15	0.39 ± 0.13
C22:5	0.44 ± 0.13^{A}	0.67 ± 0.18	0.84 ± 0.14^{B}	0.81 ± 0.20	0.76 ± 0.09^{B}	0.54 ± 0.25	0.75 ± 0.16^{B}	0.61 ± 0.24	0.67 ± 0.05^{B}	0.66 ± 0.29	0.56 ± 0.13^{A}	0.62 ± 0.27
C22:6	3.12 ± 0.29^{A}	3.69 ± 0.81	5.73 ± 0.85^{B}	3.60 ± 0.54	5.17 ± 0.74^{B}	3.05 ± 0.45	4.66 ± 1.03^{B}	3.06 ± 0.94	4.34 ± 0.69^{A}	3.23 ± 1.25	3.70 ± 0.80^{A}	2.78 ± 0.16
Total ω 3 PUFA	4.02 ± 0.34^{A}	5.16 ± 0.93	7.01 ± 1.02^{B}	5.12 ± 0.93	6.55 ± 0.83^{B}	4.24 ± 0.54	5.93 ± 1.08^{B}	4.29 ± 1.13	5.60 ± 0.77^{B}	4.45 ± 1.52	4.62 ± 0.83^{C}	3.89 ± 0.51
% of total FA												
C16:0	12.28 ± 2.74	19.59 ± 2.67	13.91 ± 2.83	19.70 ± 5.72	14.62 ± 2.14	22.35 ± 3.55	14.77 ± 1.90	20.98 ± 4.05	15.28 ± 2.52	21.17 ± 6.39	15.11 ± 3.61	24.57 ± 2.70
C18:0	26.50 ± 1.72^{A}	19.01 ± 0.92	23.27 ± 1.93^{B}	18.04 ± 2.17	24.59 ± 0.82	17.43 ± 0.60	24.22 ± 1.76	18.52 ± 1.50	24.65 ± 1.25	18.59 ± 1.43	24.52 ± 1.53	18.28 ± 0.90
Total SFA	38.78 ± 2.89	38.60 ± 2.31	37.19 ± 2.24	37.74 ± 3.62	39.21 ± 2.32	39.78 ± 3.08	38.99 ± 2.22	39.49 ± 3.29	39.93 ± 1.93	39.77 ± 5.08	39.63 ± 3.25	42.85 ± 1.95
C16:1	1.83 ± 0.79	2.65 ± 1.07	1.63 ± 0.59	3.04 ± 1.49	1.17 ± 0.76	2.87 ± 1.19	1.72 ± 0.22	2.56 ± 1.69	1.66 ± 0.35	1.83 ± 1.11	2.19 ± 1.39	1.47 ± 0.18
C18:1	11.03 ± 2.25	19.63 ± 2.66^{A}	11.12 ± 1.46	20.06 ± 2.75^{A}	10.66 ± 1.11	19.47 ± 2.05^{A}	12.57 ± 4.25	19.46 ± 2.21^{A}	10.79 ± 1.49	17.69 ± 2.10	9.67 ± 1.01	15.51 ± 1.70^{B}
Total MUFA	12.86 ± 2.50	22.28 ± 3.13^{A}	12.75 ± 1.86	23.10 ± 2.49^{A}	11.83 ± 1.61	22.34 ± 2.74^{A}	14.28 ± 4.38	22.02 ± 3.10^{A}	12.46 ± 1.27	19.52 ± 2.79	11.86 ± 1.98	16.98 ± 1.60^{B}
C18:2	2.90 ± 0.49^{A}	8.89 ± 0.99	3.77 ± 0.43	9.08 ± 0.90	3.79 ± 0.37	9.13 ± 1.11	5.08 ± 2.17^{B}	8.94 ± 1.19	3.97 ± 0.54	9.38 ± 2.02	4.51 ± 0.16	9.62 ± 0.38
C20:3	0.23 ± 0.24	1.27 ± 0.40	0.18 ± 0.22	1.38 ± 0.58	0.37 ± 0.23	1.07 ± 0.55	0.26 ± 0.24	1.14 ± 0.56	0.39 ± 0.36	1.03 ± 0.43	0.24 ± 0.33	1.12 ± 0.56
C20:4	28.49 ± 3.09^{A}	20.78 ± 2.20	26.74 ± 1.70	20.36 ± 2.82	25.38 ± 1.18	20.47 ± 3.61	24.59 ± 2.45^{B}	20.85 ± 4.24	25.27 ± 1.25	22.49 ± 4.83	25.85 ± 2.09	22.15 ± 0.89
C22:4	0.24 ± 0.11	0.13 ± 0.04	0.32 ± 0.11	0.11 ± 0.04	0.31 ± 0.14	0.09 ± 0.05	0.31 ± 0.31	0.09 ± 0.06	0.19 ± 0.12	0.12 ± 0.05	0.37 ± 0.26	0.09 ± 0.05
C22:5	0.06 ± 0.07	0.08 ± 0.02	0.06 ± 0.06	0.07 ± 0.06	0.12 ± 0.09^{A}	0.05 ± 0.02	0.02 ± 0.05	0.04 ± 0.02	0.04 ± 0.05	0.05 ± 0.05	n.d.	0.02 ± 0.02
Total ω 6 PUFA	32.06 ± 3.04	31.20 ± 2.10	31.17 ± 2.07	31.05 ± 3.02	30.02 ± 1.77	30.83 ± 3.37	30.35 ± 3.89	31.09 ± 4.02	29.94 ± 1.76	33.08 ± 4.51	31.05 ± 1.83	33.02 ± 0.97
C20:5	1.53 ± 0.55	0.97 ± 0.31	0.89 ± 0.54	0.86 ± 0.50	1.48 ± 0.64	0.80 ± 0.30	1.30 ± 0.70	0.80 ± 0.29	1.48 ± 0.61	0.71 ± 0.41	1.07 ± 0.69	0.72 ± 0.20
C22:5	1.76 ± 0.44	1.03 ± 0.26	2.28 ± 0.37	1.30 ± 0.32	2.22 ± 0.35	0.90 ± 0.41	2.07 ± 0.49	1.04 ± 0.35	2.14 ± 0.28	1.14 ± 0.41	2.09 ± 0.23	1.13 ± 0.42
C22:6	12.69 ± 1.86	5.67 ± 1.31	15.44 ± 1.96	5.73 ± 0.73	14.99 ± 2.11	5.07 ± 0.76	12.84 ± 3.43	5.28 ± 1.29	13.72 ± 1.82	5.56 ± 1.81	13.95 ± 2.02	5.13 ± 0.37
Total ω 3 PUFA	16.30 ± 1.97	7.92 ± 1.50	18.89 ± 2.38	8.11 ± 1.03	18.95 ± 2.02	7.05 ± 0.90	16.37 ± 3.94	7.40 ± 1.49	17.68 ± 1.57	7.64 ± 2.10	17.46 ± 2.26	7.15 ± 0.52

Values represent means ± standard deviation of 4–5 replications and expressed as μg per mg protein or % of total FA. Statistical analysis: One-way ANOVA, differing letters/combination of letters indicate statistical significance ($p \leq 0.05$). Upper case letters – comparison to control, lower case letters comparison between concentrations. Abbreviations: FB_1 – fumonisin B_1, PUFA – polyunsaturated fatty acids, SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, n.d.=not detectable.

Table 4
Total FA and selected molar FA ratios of Chang cells and primary hepatocytes treated with FB_1.

	Control	25 μM	75 μM	150 μM	250 μM	500 μM
Chang cells						
PC						
tPUFA (μg/mg)	1.69±0.25^{A}	0.90±0.22^{B}	0.83±0.34^{B}	0.87±0.25^{B}	1.14±0.43	1.04±0.37^{B}
tPUFA (% of total)	4.88±0.92^{A}	2.55±0.55	2.29±0.88^{B}	2.52±0.72^{B}	3.39±1.41	3.18±0.93^{A}
ω6/ω3 ratio	3.63±0.64	4.88±2.37	3.63±1.57	6.95±4.99	3.57±1.08	6.59±2.37
P/S ratio	0.12±0.03^{A}	0.06±0.01^{B}	0.05±0.02^{B}	0.06±0.02^{B}	0.08±0.04	0.07±0.02^{B}
C16:0/C16:1 (SCD)	1.73±0.10	1.79±0.11	1.81±0.21	1.93±0.31	1.87±0.29	1.82±0.12
C18:0/C18:1 (SCD)	0.06±0.01	0.08±0.02	0.08±0.03	0.08±0.01	0.08±0.01	0.09±0.02
PE						
tPUFA (μg/mg)	5.14±0.62^{A}	6.48±1.01^{A}	6.93±1.17^{B}	7.05±0.58^{B}	6.61±0.81^{A}	6.32±0.65^{A}
tPUFA (% of total)	32.91±2.78^{A}	24.78±1.64^{B}	27.16±5.51	26.60±2.53	24.71±3.75^{B}	26.79±2.95
ω6/ω3 ratio	2.42±0.54	3.08±0.85	2.79±0.25	2.90±0.73	2.71±0.48	2.98±0.28
P/S ratio	1.25±0.07	1.01±0.13	1.02±0.15	1.17±0.15	0.99±0.21	1.08±0.17
C16:0/C16:1(SCD)	1.52±0.17^{A}	0.99±0.07^{B}	1.07±0.21^{B}	0.95±0.11^{B}	1.09±0.15^{B}	0.97±0.07^{B}
C18:0/C18:1 (SCD)	0.34±0.04^{A}	0.23±0.03^{B}	0.23±0.04^{B}	0.22±0.03^{B}	0.25±0.03^{B}	0.25±0.01^{B}
C20:4ω6-PC/PE	0.16±0.05^{A}	0.07±0.03^{B}	0.06±0.00^{B}	0.06±0.01^{B}	0.07±0.04^{B}	0.08±0.05^{B}
Primary hepatocytes						
PC						
tPUFA (μg/mg)	25.56±2.52	24.70±3.81	22.91±3.19	22.23±4.56	23.68±5.37	21.83±1.96
tPUFA (% of total)	39.12±2.68	39.16±3.89	37.88±3.80	38.48±5.44	40.72±6.38	40.17±1.14
ω6/ω3 ratio	4.41±0.81	4.20±0.35	4.81±0.64	4.72±0.39	5.05±0.68	5.06±0.37
P/S ratio	0.91±0.09	0.94±0.19	0.85±0.14	0.80±0.07	0.82±0.06	0.83±0.06
C16:0/C16:1 (SCD)	8.45±3.54^{A}	6.05±0.98^{Aa}	8.85±3.32^{A}	8.06±4.88^{A}	12.54±4.63^{b}	16.59±0.84^{B}
C18:0/C18:1 (SCD)	0.97±0.12^{A}	0.90±0.08^{A}	0.90±0.08^{A}	0.93±0.10^{A}	1.02±0.11^{AB}	1.18±0.09^{B}
PE						
tPUFA (μg/mg)	11.97±1.04^{A}	18.60±2.02^{B}	16.99±2.04^{B}	17.06±2.06^{B}	15.13±1.97^{A}	12.91±2.54^{A}
tPUFA (% of total)	48.35±4.30	50.06±3.02	48.96±2.55	46.72±6.11	47.61±1.76	48.51±6.45
ω6/ω3 ratio	2.13±0.27	1.81±0.28	1.73±0.24	2.09±0.50	1.84±0.25	1.95±0.24
P/S ratio	1.12±0.18	1.19±0.14	1.11±0.12	1.07±0.19	1.06±0.09	1.09±0.17
C16:0/C16:1 (SCD)	7.92±3.67	9.42±3.32	12.71±6.06	8.58±1.00	9.32±1.83	6.61±2.09
C18:0/C18:1 (SCD)	2.46±0.48	2.12±0.43	2.31±0.27	2.08±0.64	2.31±0.41	2.54±0.34
C20:4ω6-PC/PE	1.94±0.34^{A}	1.27±0.18^{Bb}	1.33±0.21^{B}	1.29±0.14^{Bb}	1.48±0.28	1.83±0.38^{a}

Values represent means ± standard deviation of 4–5 replications. Differing letters/combination of letters indicate statistical significance ($p<0.05$). Upper case letters – comparison to control, lower case letters comparison between concentrations. Abbreviations: SCD – stearoyl coenzyme A desaturase, FA – fatty acids, FB_1 – fumonisin B_1, PC – phosphatidylcholine, PE – phosphatidylethanolamine, P/S ratio – ratio of polyunsaturated to saturated fatty acids, tPUFA – total polyunsaturated fatty acids, C20:4ω6-PC/PE – ratio of C20:4ω6 content in PC to PE.

FB_1 in the Chang cells. No significant changes were observed in both phosphoglyceride fractions of the primary hepatocytes.

3.2.3.5. FA parameters and ratios

3.2.3.5.1. Absolute FA content (mg FA/mg protein). The PUFA content in Chang cells was approximately 15 fold lower in PC and 2 fold lower in PE than in primary hepatocytes (Table 4). The ω6/ω3 FA ratios were similar in PC and PE in both cell cultures, while the PUFA/saturated (P/S) ratio was 8 fold lower in the PC fraction of the Chang cells. The 16:0/16:1 ratio was 5 fold lower in PC and PE and the 18:0/18:1 ratio was approximately 16 fold lower in PC and 7 fold lower in PE in Chang cells.

The total ω6 and ω3 PUFA in the Chang cells was significantly ($P<0.05$) lowered in PC at all FB_1 concentrations which resulted in a decrease of P/S ratio. In PE, the total PUFA increased significantly ($P<0.05$) in cells exposed up to 150 μM FB_1, while it tended the decrease at higher concentrations. A similar observation was noticed in PC, but changes were not significant. The 16:0/16:1 and 18:0/18:1 ratio in PE and the 20:4ω6-PC/PE ratio decreased significantly ($P<0.05$) at all FB_1 concentrations.

In the primary hepatocytes, the 16:0/16:1 and 18:0/18:1 ratios in PC increased significantly ($P<0.05$) at 500 μM FB_1 when compared to control. Similar to the Chang cells, the total PUFA increased significantly ($P<0.05$) in PE of cells exposed up to 150 μM FB_1, while it tended the decrease at higher concentrations. The ω6/ω3 and P/S ratios were not altered in PE or PC of the primary hepatocytes, while the 20:4ω6 PC/PE ratio decreased significantly ($P<0.05$) up to 150 μM FB_1.

3.2.3.5.2. Relative FA content (% of the total FA). The relative PUFA content in Chang cells was also lower in both PC and PE as described for the quantitative levels. Total PUFA content was markedly to significantly ($P<0.05$) reduced by FB_1 in both PE and PC fractions of the Chang cells, while no changes were observed in the primary hepatocytes.

4. Discussion

Altered lipid metabolism and its modulating role in carcinogenesis is becoming more prominent in cancer research since it presents an attractive target for chemoprevention [53,54]. Modulation of FA, phosphoglyceride and sphingolipid metabolism has been implicated in the toxic and carcinogenic properties of fumonisins in the liver [20,22,24,25], but their exact role still needs to be resolved. The present study investigated the changes in lipid profiles in sensitive and resistant cell models toward FB_1-induced cell cytotoxicity, antiproliferative and apoptotic effects in order to provide information regarding cell death and/or cell survival indices. Differential changes in lipid metabolism were defined and related to the underlying mechanism of FB_1-induced cancer promotion in rat liver.

Proliferating Chang cells were far more resistant to the cytotoxic, antiprolferative and pro-apoptotic effects of FB_1 compared to primary rat hepatocytes. The cytotoxicity of FB_1 was significantly increased in a dose dependent manner in primary hepatocytes, which was associated with an increased apoptosis. Previous

studies have shown that FB_1 induces necrosis and apoptosis in primary rat hepatocytes and different secondary mammalian cell lines [21,27,28,36,37], whereas some cell lines displayed resistance [29,38,55]. A similar lack of FB_1-induced cytotoxicity was observed in Hep3B liver cancer cells, although the incubation time was only 8 h using a low dose of 20 μM [56]. HT29 colon cancer cells also displayed resistance as there was no effect on cell viability or cell proliferation, despite the fact that FB_1-induced lipid peroxidation at a low concentration [57]. The differential cytotoxicity observed in the present study provides further support for the underlying mechanism associated with cancer promotion of FB_1 in the liver of rats [22], especially regarding the resistance of initiated hepatocytes to toxicity based on the resistant hepatocyte model [17,18]. A recent study indicated that lipid changes affected by cancer promoters, FB_1 and 2-acetylaminofluorene/partial hepatectomy (AAF/pH), in the liver of rats play an important role during cancer promotion and contribute to the differential responses of normal and initiated hepatocytes to apoptotic and cell proliferation signals [16].

Chang cells provided a model for pre-malignant and/or initiated cells, since distinct differences in lipid and FA content of untreated cells were evident in comparison to primary hepatocytes. These differences include higher Chol, SM and lower PC levels resulting in a reduction in the Chol/TPG, PC/PE and PC/PE þ SM ratios, which imply a more rigid membrane structure [58]. Chang cells contained approximately two fold higher SM levels and displayed lower PC levels than the primary hepatocytes. The low PUFA content in the PC fraction and the high level of SFA will further contribute to a more rigid membrane structure in Chang cells, which is likely to modulate membrane function known to alter the responsiveness of cells to toxins [59,60]. Increased lipid saturation in cancer cells is associated with an enhanced protection against toxic insults, such as free radicals [61]. In this study, 16:0 makes up 30% of the total FA pool in PC in Chang cells, compared to the only 20% primary hepatocytes. Even though other FA pools, such as the triglycerides, were not characterized in this study, it would appear that fatty acid synthase (FAS) is more active in Chang cells in order to satisfy the demand for membrane FA in proliferating cells. The overexpression of FAS, catalysing the rate limiting step in the *de novo* FA synthesis producing mainly 16:0, has been linked to aggressiveness of tumors and poor prognosis in human cancer patients [62]. It was also suggested that normal and pre-malignant cells display differences in FAS expression and therefore changes in SFA content [53].

The 18:0 content was low in Chang cells, despite that cells can produce it through elongation of 16:0. Both these FA, especially 16:0, induce apoptosis in different cell types by stimulating ceramide generation [63,64]. However, the low level of 18:0 could be due to the high levels of 18:1ω9, which contributes up to 36% of the total FA in PE and 41% in PC, indicating that desaturation of 18:0 is prominent in Chang cells. This is supported by the low 18:0/18:1ω9 fatty acid ratio in PE and PC, a surrogate marker for stearoyl co-enzyme A desaturase (SCD) activity [65]. Increased activity of SCD has been implicated in cancer development via modulation of lipid biosynthesis and survival signaling pathways that contribute to the development and progression of cancer [66]. The high levels of MUFA in Chang cells, constituting up to 40% and 60% of the total FA pool in PE and PC, respectively, compared to the 10% and 20% in primary hepatocytes, further support such a hypothesis. High levels of 18:1ω9, known to exhibited antioxidant properties [8,10], have been associated with protection against toxic insults in vitro [67], which is likely to contribute to the resistance of Chang cells to FB_1-induced cytotoxicity. In this regard FB_1-induced cytotoxicity in primary hepatocytes was associated with an increased oxidative stress which was counteracted by α-tocopherol [28]. In primary rat astrocytes, FB_1 inhibits mitochondrial respiration resulting depolarisation of the mitochondrial membrane with the induction of reactive oxygen species [68] suggesting a determining role of oxidative stress in the cytotoxic effects. The relative levels of 18:1 could therefore play an important role in the protection against oxidative stress in the Chang cells. A similar effect was noticed in rat hepatocyte nodules and human liver cancer biopsies that exhibited higher C18:1 level presumably contributing to a selective growth advantage during cancer promotion and liver carcinogenesis [9,11].

Primary hepatocytes contained considerably higher levels of ω6 and ω3 PUFA when compared to the Chang cells. Low PUFA levels in Chang cells closely mimicked the FA profile prevailing in cancer cells which, together with the high 18:1ω9 content, is associated with resistance to oxidative stress [7]. Differences in the 20:4ω6 content in the relative distribution in PE and PC in Chang cells are also of interest. Although the overall content of 20:4ω6 was very low, the relative contribution to the cellular FA pool in PE is higher in the Chang cells, while it is similar in both phosphoglyceride fractions in primary hepatocytes. This resulted in a nearly 10 fold lower 20:4ω6-PC/PE ratio in Chang cells compared to primary hepatocytes. The higher membrane distribution of 20:4ω6 in PE has been proposed as an indicator for cell proliferation in rat hepatocyte nodules and liver cancer in humans [9,11]. In addition to the other lipid associated changes in the Chang cells, differences in the FA profiles are likely responsible for the differential response to FB_1-induced cytotoxicity when compared to primary hepatocytes.

The typical FB_1-induced alterations in membrane lipids, reported previously [15,69,70], were observed in both cell types. These included increases ($P \leq 0.05$) in SFA (16:0), MUFA (18:1) and PUFA (18:2ω6; 20:4ω6) in PE, a decrease in SM, while Chol decreased in primary hepatocytes and only markedly in Chang cells, lacking a dose response effect. The high PE content was suggested to decrease the barrier function of cellular membranes due to the conical shape of this phosphoglyceride, therefore membrane permeability may increase [71]. However, when compared to primary hepatocytes, Chang cells seemed well adapted to this increase considering the difference in cytotoxicity to FB_1. The 18:0/18:1ω9 FA ratio in PE of the Chang cells was significantly further decreased by FB_1 due to an approximately two fold increase in 18:1ω9. The FB_1-induced increase of the SFA percentage in PC is mitigated in Chang cells by desaturation to yield MUFA, specifically in PE, which is a mechanism to decrease lipotoxicity and pro-inflammatory effects of SFA [72,73]. In primary hepatocytes, however, the opposite effect was noticed with a significant increase in the 16:0/16:1ω7 and 18:0/18:1ω9 FA ratios due to decreases in the levels of 16:1 and 18:1ω9, which might sensitise primary hepatocytes to the cytotoxicity of FB_1. The predominant FA in Chang cells (especially in PC) was 18:1ω9, which was shown to exhibit anti-oxidant properties as mentioned above [8,10]. Since 18:1ω9 was further increased by FB_1 treatment, it could therefore also contribute towards resistance against oxidative damage. In primary hepatocytes, 18:1ω9 decreased in both PC and PE at cytotoxic concentrations of FB_1, emphasizing that 18:1ω9 could protect during increased oxidative stress. The decrease in PUFA as a percentage of total FA in PC and, to some extent, in PE (especially 20:4ω6) in Chang cells, may also further limit the availability of oxidisable substrates contributing to the resistance towards the cytotoxic effects of FB_1. In primary hepatocytes, PUFA tended to decrease at cytotoxic FB_1 concentrations in both PE and PC, presumably due to an FB_1-induced lipid peroxidation [28]. The low content of 20:4ω6 in Chang cells may also compromise the availability of this key FA towards the 20:4ω6 cascade, thereby preventing the induction of apoptosis through the regulation of ceramide levels via the sphingomyelinase pathway [74,75]. The resultant shift of the cellular 20:4ω6 towards PE, as indicated by

the decrease in the 20:4ω6-PC/PE ratio, probably signals cell proliferation, presumably via the cyclooxygenase pathway, and possibly contributes to the selective outgrowth of initiated hepatocytes into hepatocyte nodules during cancer promotion [22,55,76]. The reduction in primary hepatocytes was far less prominent and hence C20:4ω6 presumably remained available for pro-apoptotic signaling via ceramide. Therefore, the relative distribution of 20:4ω6 in the membrane phosphoglycerides may play a critical role in governing cell survival indices to FB_1-induced cytotoxicity.

Apart from changes in the phosphoglyceride and FA profiles, FB_1 alters the sphingolipid content in a variety of tissues and cells [23,24]. The decrease in SM and the increase in Sa content were observed in both cell culture models. The increase in Sa did not follow a typical dose response, as it reached a maximum at 5 μM FB_1 in primary hepatocytes, which was well below cytotoxic concentrations [21]. However, the increase in Sa was more prominent in Chang cells, reaching levels approximately 4.7 fold higher compared to primary hepatocytes at 150 μM FB_1, implying that Chang cells were resistant to suggested cytotoxic and/or antiproliferative effects of Sa [77]. Although So was not significantly altered in both cells culture systems, So-1P is well established as a cancer promoting agent that induces cell proliferation [78,79], however, neither Sa- nor So-1P levels were assessed in the current study. In this regard sphingosine kinase, the enzyme responsible for the phosphorylation of both Sa and So, has also been implicated in the development of cancer [80]. Although, less information is available on Sa-1P, a metabolomic study on oesophageal cancer patients indicated an increased level of this metabolite together with phosphoglycerides such as PE and PC [81]. If the excess Sa in Chang cells is converted to Sa-1P, it is likely to contribute to the observed resistance, providing a selective growth advantage as was hypothesized for So-1P in FB_1-induced hepatocyte nodules *in vivo* [45]. Since increased levels of Sa have been associated with cytotoxicity of FB_1 in the liver and kidneys of rats [82,83], the cytotoxic effects observed in primary hepatocytes in this study might also be attributed to the increased Sa levels. The reduction of SM also indicated that complex sphingolipids were depleted which, together with the reduction of ceramide, could have important implications on modulating cell survival and apoptosis. Since the respective roles of Sa, So and their phosphate derivatives in the selective resistant of the Chang cells towards FB_1-induced cytotoxicity have not been clarified, their underlying role in cancer promotion of FB_1 in the liver needs further investigation.

Changes involving Chol, phosphoglycerides, sphingolipids, SFA, MUFA and PUFA are likely to be key role players associated with the differential response of the Chang cells and primary hepatocytes to FB_1-induced cytotoxic and/or growth regulatory effects. The current investigation indicated that cellular resistance to FB_1 is associated with:

- A more rigid membrane structure due to changes in the major membrane lipid constituents, including Chol, phosphoglycerides and sphingolipids as well as decreasing PC/PE, PC/PE▷SM and Chol/TPG membrane fluidity ratios.
- Increased 16:0 and 18:1ω9 and low 18:0 and PUFA levels, mimicking FA patterns in cancer cells [7,10,11,84] which may be indicative of resistance to apoptosis and oxidative damage.
- Decreased 20:4ω6-PC/PE ratio due to a relative higher level in PE and the disruption in the Sa/So ratio are important signaling events determining key growth regulatory responses following FB_1 exposure.

The current study supports previous findings where a FB_1-induced cancer promoting "lipid signal" was proposed [16] and it was suggested that cells with divergent lipid profiles may react differently to specific adverse toxicological conditions and that these distinct differences could facilitate the growth of pre-neoplastic lesions during cancer promotion in the liver of FB_1 treated rats, whereas similar changes will sensitise normal cells, such as primary hepatocytes, to undergo apoptosis, thereby creating a growth differential associated with cancer promotion in the liver.

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