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Fumonisin B_1 protects against long-chained polyunsaturated fatty acid-induced cell death in HepG2 cells – implications for cancer promotion

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ABSTRACT

Fumonisin B_1 (FB_1), a food-borne mycotoxin, is a cancer promoter in rodent liver and augments proliferation of initiated cells while inhibiting the growth of normal hepatocytes by disrupting lipid biosynthesis at various levels. HepG2 cancer cells exhibited resistance to FB_1-induced toxic effects presumably due to their low content of polyunsaturated fatty acids (PUFA) even though FB_1-typical lipid changes were observed, e.g. significantly increased phosphatidylethanolamine (PE), decreased sphingomyelin and cholesterol content, increased sphinganine (Sa) and sphinganine/sphingosine ratio, increased C18:1ω-9, decreased C20:4ω-6 content in PE and decreased C20:4ω-6_PC/PE ratio. Increasing PUFA content of HepG2 cells with phosphatidylcholine (PC) vesicles containing C20:4ω-6 (SAPC) or C22:6ω-3 (SDPC) disrupted cell survival, cellular redox status and induced oxidative stress and apoptosis. A partially protective effect of FB_1 was evident in PUFA-enriched HepG2 cells which may be related to the FB_1-induced reduction in oxidative stress and the disruption of key cell membrane constituents indicative of a resistant lipid phenotype. Interactions between different ω-6 and ω-3 PUFA, membrane constituents including cholesterol, and the glycerophospho- and sphingolipids and FB_1 in this cell model provide further support for the resistant lipid phenotype and its role in the complex cellular effects underlying the cancer promoting potential of the fumonisins.

1. Introduction

The foodborne fumonisin B_1 (FB_1) mycotoxin is classified as a group 2B carcinogen based on rodent carcinogenesis studies, widespread exposure and association with different human diseases [1]. The modulation of lipid metabolism, including glycerophospholipids, sphingolipids and fatty acids, are key mechanistic determinants underlying the different toxic and carcinogenic properties of FB_1 [1–3]. Studies in cell culture indicated that FB_1 modulates cell proliferation and apoptosis in a variety of cell types [4–11]. It became apparent that FB_1 differentially alters the growth responses and seems to be more cytotoxic in the non-transformed or primary cell lines than in the transformed cells [12]. Such changes may be critical for facilitating the proliferation of resistant cell clones originating from initiated cells while inhibiting the growth of normal hepatocytes in vivo, which is typically observed for tumour promoters [13–16]. FB_1 creates such a growth differential in the liver by modulating the composition of cell membrane lipids and fatty acids thus facilitating the growth of pre-neoplastic liver lesions, while selectively inhibiting the proliferation of normal hepatocytes [17–19].

Cancer cells are known to contain high levels of antioxidants [20], while few studies have also reported low levels of peroxidation substrates, such as polyunsaturated fatty acids (PUFA) to prevail in cancer cells [21–25]. Low PUFA levels might be beneficial to cell survival, especially under stressful conditions, aiding in resistance towards lipid

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peroxidation associated oxidative stress and the induction of apoptosis [26]. In the liver, early preneoplastic lesions were associated with reduced levels of PUFA when compared to the surrounding and normal tissue [27,28].

FB_1-induced changes in lipid and PUFA metabolism in rodent liver have previously been associated with cancer promotion [18,29–31]. FB_1 lacked cytotoxic effects in rapidly proliferating Chang cells exhibiting a very low PUFA content, while primary hepatocytes with a high PUFA content were more susceptible [8]. To test whether FB_1 exerts similar resistance in HepG2 liver cancer cells, their cell survival and lipid composition in response to FB_1 treatment were investigated. To further assess the hypothesis that cellular lipids and specifically a lack of PUFA confers resistance towards FB_1-induced cell death, HepG2 cells were enriched with selected ω-6 and ω-3 PUFA and changes in lipid composition and redox status following treatment with FB_1 were determined. Synthetic PC vesicles containing C22:6ω-3, (SDPC) an important membrane ω-3 FA [32] and C20:4ω-6 (SAPC), a key FA of the ω-6 FA pathway modulating cell survival [33] were utilised for the supplementation. These FA classes play important but opposing roles in the regulation of cell growth parameters during cancer development [34]. The specific objectives of this study were to investigate i) the effects of different FB_1 concentrations on HepG2 cell survival, lipid and FA indices, and ii) the effect of enrichment of HepG2 liver cancer cells with PUFA on FB_1-induced modulation of cell survival, lipid and FA parameters related to cellular resistance.

2. Materials and method

2.1. Chemicals

Fumonisin B_1 was purified at the Applied Microbial and Health Biotechnology Institute, Cape Peninsula University of Technology, Bellville, South Africa, according to the method described previously [35] to a purity of >95 %. Synthetic phosphatidylcholine (PC) was used as long-chained PUFA (LCPUFA) delivery vehicle, which contained C18:0 in the sn-1 position and either C20:4ω-6 (SAPC) or C22:6ω-3 (SDPC) in the sn-2 position and were obtained from Avanti-Polar Lipids Inc. (Alabaster, AL, USA). HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA), catalogue number HB8065. Eagle's minimum essential medium (EMEM), foetal bovine serum (FBS) and L-glutamine were obtained from Invitrogen, Ltd. (Carlsbad, USA). Hank's balanced salts, trypsin-ethylenediamine tetra-acetic acid (EDTA), sodium pyruvate, penicillin-streptomycin-amphotericin B mixture and non-essential amino acid solutions were purchased from Lonza (Walkersville, USA). The CellTiter-Glo® commercial kit (Promega, Madison, WI, USA) was used to assess ATP content as a measure of metabolically active cells. The incorporation of BrdU into the cellular DNA was assessed using a chemiluminescence kit (Roche Applied Sciences, Randburg, RSA). Apoptosis was monitored using a Caspase 3/7 activity chemiluminescent assay kit (Caspase-Glo® 3/7, Promega Corp., Madison, WI, USA). Butylated hydroxytoluene (BHT), malachite green, C17:0 free FA, HPLC-grade methanol, chloroform and hexane were obtained from Sigma Chemical Corporation, Cape Town, RSA. The free FA standards were acquired from Nu-Chek-Prep Inc. (Elysian, MN, USA). C20-Sphinganine was a kind gift from Prof. A.H. Merrill Jr. All other chemicals were of analytical grade (Sigma Chemical Corporation, Cape Town, RSA).

2.2. Effect of FB_1 on HepG2 cell survival

For determining cell survival indices in the presence of FB_1 (compare Fig. 1A), cells were seeded at 2×10^4 cells per well in EMEM containing 10 % FBS, 2 mM L-glutamine, 0.2 mM non-essential amino acids and 2 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B as described for each assay below. After 24 h, the media was changed to 0.5 % FBS containing FB_1 (75, 150 and 250 μM) and cells incubated for a further 24 and 48 h and endpoints measured as described below.

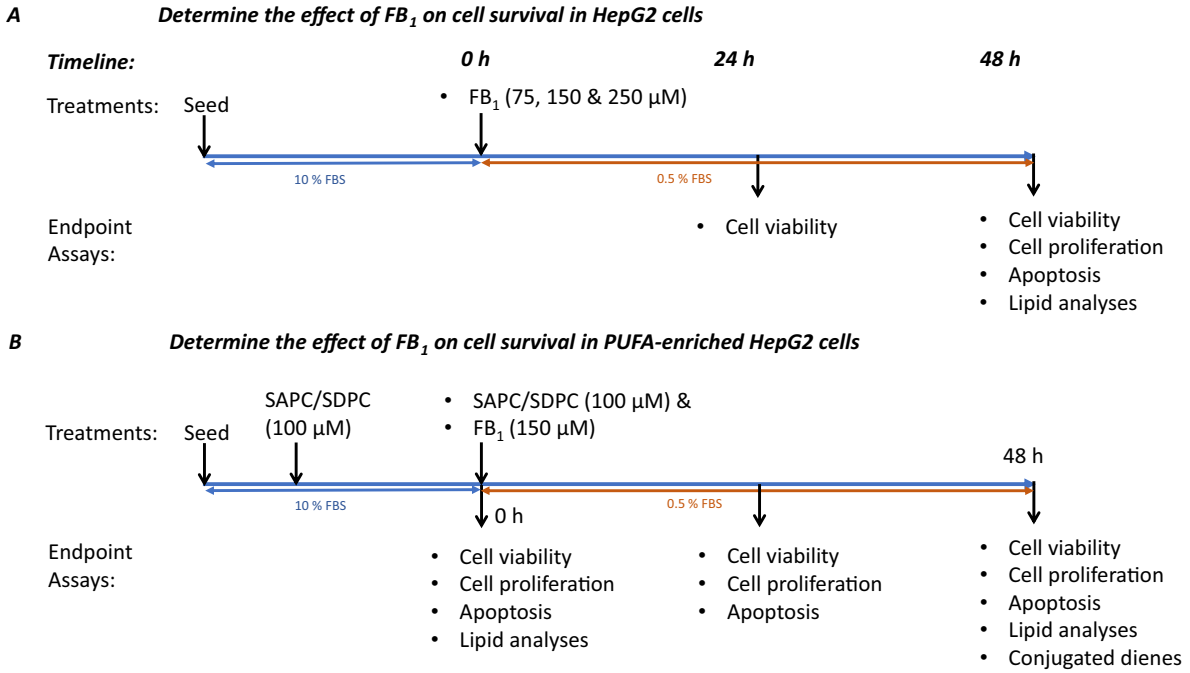

Fig. 1. Experimental outline and cell culture conditions (A) to determine the effect of FB_1 on cell survival in HepG2 cells and (B) to enrich HepG2 cells with selected polyunsaturated fatty acids (PUFA) and determine the effect of FB_1 on their cell survival. Cells were seeded in standard EMEM containing 10 % FBS. Cells were allowed to attach for 8 h, whereafter the vesicles containing arachidonic acid and docosahexaenoic acid were added to the medium for 16 h. The medium was replaced 24 h after seeding and new treatments added. Endpoint assays were completed at the indicated time points. Abbreviations: FB_1 – fumonisin B_1, FBS – foetal bovine serum, SAPC – 1-stearoyl-2-arachidonyl-sn-glycero-3-phosphocholine, SDPC – 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine.

2.2.1. Cell viability

Cell viability (ATP content) was assessed utilising a CellTiter-Glo^R commercial kit (Promega, Madison, WI, USA) as a measure of metabolically active cells by quantifying ATP. Cells (2×10^4 per well) were seeded in white, clear bottom tissue culture treated polystyrene 96-well microtiter plates (Krystal 2000, Labnet, Woodbrigde, NJ, USA). After 24 and 48 h treatment with FB_1 (75, 150 and 250 μM), plates were equilibrated to room temperature, the Celltiter-Glo reaction mixture was added and chemiluminescence measured using a Veritas Microplate Luminometer (Turner BioSystems, Sunnyvale, CA, USA). Results were expressed as percentage viable cells referring to the control group as 100 %.

Cytotoxicity, or necrotic cell death, was determined after the 48 h incubation period using the Cytotox96®–non-radioactive cytotoxicity kit (Promega, Madison, WI, USA). The cell supernatant was used for analysis of lactate dehydrogenase (LDH) levels leaked from the cells according to the manufacturer's instructions using a microplate reader (Opsys MR, Dynex Technologies, Chantilly, VA, USA). Total LDH release was also included for each treatment following complete cell lysis. Cytotoxicity was expressed as a percentage cell death (percentage of total LDH).

2.2.2. Cell proliferation

Incorporation of BrdU was assessed using a chemiluminescence cell proliferation ELISA kit (Roche Applied Sciences, Randburg, SA). Cells (2×10^4 per well) were seeded in solid black polystyrene 96-well microtiter plates (Labnet, Woodbrigde, NJ, USA) and treated with FB_1 (75, 150 and 250 μM) for 48 h. Labelling solution, diluted in EMEM, was added to each well 2 h prior to the end of the incubation time points. The manufacturer's instructions were followed during the assay procedure allowing 90 min for the incubation with the Anti-BrdU antibody and chemiluminescence was recorded with a Veritas Microplate Luminometer. Results were expressed as percentage of proliferating cells, referring to control treatment as 100 %.

2.2.3. Apoptosis

Apoptosis was determined utilising the Caspase 3/7-Glo kit. Cells (2×10^4 cells per well) were incubated in 96 well microtitre plates in the presence of FB_1 (75, 150 and 250 μM; 48 h). Plates were equilibrated to room temperature, the Caspase-Glo reaction mixture added and the chemiluminescence measured using a Veritas Microplate Luminometer. Apoptosis was expressed as fold increase referring to the control group as 1.

2.2.4. Modulation of lipid parameters by FB_1

Cells were seeded at a density of 1×10^6 cells per 60 mm petri dish in EMEM containing 10 % FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate. After 24 h, the medium was replaced with EMEM containing 0.5 % FBS, 2 mM L-glutamine, 0.2 mM non-essential amino acids and 2 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and the different FB_1 concentrations (75, 150 and 250 μM) for a further 48 h of treatment. For lipid analysis, cells were washed 3× with cold saline and cells of 4 dishes combined unless described otherwise and scraped into 0.5 ml cold saline. An aliquot (100 μl) was removed for protein analysis and cell suspensions were treated for the respective lipid extractions as described below. Each treatment comprised at least 3 biological repetitions.

2.2.4.1. Sphingolipid analysis. The cells from two petri dishes were scraped as described above, pelleted (1000 g, 4 °C, 10 min) and resuspended in 300 μl phosphate buffered saline (PBS; 50 mM, pH 7.0). The content of sphingoid bases (sphinganine, Sa and sphingosine, So) was determined as described previously [8,36]. Data were expressed as pmol Sa or So per mg protein.

2.2.4.2. Cholesterol, glycerophospholipid, sphingomyelin and fatty acid analysis. For lipid extractions, 4 Petri dishes (60 mm) were pooled and scraped as described above. Cell suspensions were extracted using the Folch extraction method [37] for FA, glycerophospholipid, sphingomyelin and cholesterol determinations as described previously [8]. Briefly, thin layer chromatography was used to separate the glycerophospholipid (PE and PC) and sphingomyelin (SM) fractions using chloroform/petroleum benzene/methanol/acetic acid/boric acid (40:30:20:10:1.8 *v*/v/v/v/w) as running solvent which contained 2,5-bis-(5′tertbutylbenzoxazolyl-[2′])-thiophene for visualisation under UV light at 366 nm [38]. Inorganic phosphate content of PC, PE and SM was determined spectrophotometrically [39] and expressed as μg inorganic phosphate (P_i) per mg protein. The FA content of PC and PE was determined following transmethylation of the separated lipid fractions with methanol-18 M sulfuric acid (95:5, *v*/v) at 70 °C for 2 h under nitrogen gas [40]. FA methylester analysis was conducted on a Varian model 3400 Gas Chromatograph (SGE Analytical Science Inc., Austin, Texas, USA) equipped with 30 m fused silica BPX-70 capillary columns. An internal standard (C17:0) was added to each sample for quantification and peaks were identified by comparison with a commercial standard mixture containing C14:0 to C24:1 (Nu-Chek-Prep, Elysian, MN, USA). Data were expressed as μg FA/mg protein and as % of total FA content.

2.2.4.3. Lipid peroxidation-conjugated dienes. Conjugated dienes (CD) were determined according to Hu et al. [41]. Cells were harvested as described above for lipid analysis (2.2.4), the cell suspensions centrifuged (1000 *g*, 4 °C, 10 min) and the cell pellet suspended in 1 ml 1.15 % (*w/v*) KCl/10 mM potassium phosphate buffer, pH 7.4. An aliquot (100 μl) was removed for protein analysis and the remainder was extracted three times with chloroform/methanol (CM 1:2, v/v). The combined organic phases were evaporated to dryness under nitrogen, the lipid residues dissolved in 1 ml of hexane and absorbency measured at 233 nm on a Uvikon 923 spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA). Conjugated dienes levels were calculated using the extinction coefficient of 27,000 M^{-1} cm^{-1} and expressed as nmol CD/mg protein.

2.3. Modulation of lipid parameters by PUFA PC vesicles in the presence or absence of FB_1

It is known that addition of PUFA to the cells in culture can cause cytotoxicity and oxidative damage in HepG2 cells [42–45]. Incubation of mammalian cells with PC vesicles can modulate cellular lipid content as vesicles are taken up via fusion with the plasma membrane [46,47]. In this study, synthetic PC vesicles containing C18:0 in sn-1 and either C20:4ω-6 or C22:6ω-3 in the sn-2 position were used as vehicles to alter the PUFA status of HepG2 cells.

2.3.1. Preparation of PUFA PC vesicles

An aliquot of SAPC and SDPC (between 5 and 10 mg, stored at −20 °C under nitrogen) was evaporated to dryness and dried in vacuo for 3 h in the dark. The lipid residue was suspended in DPBS (10 mg/ml), hydrated for 1 h on ice with intermittent vortexing and sonicated (1 h) on ice using a bath sonicator. The PC vesicle suspensions were added to the cells at a final concentration of 100 μM following filtration through a 0.2 μm syringe filter with reconstituted cellulose membrane (Corning, Lowell, MA, USA).

2.3.2. Equilibrium phase with PUFA PC vesicles

HepG2 cells were seeded as described above (Section 2.2), allowed to attach for 8 h and then pre-incubated with the respective SAPC (100 μM) and SDPC (100 μM) vesicles for 16 h in culture medium containing 10 % FBS (Fig. 1B). This incubation period was included to increase the PUFA content before treatment with FB_1. Cell viability parameters as well as

FA incorporation into cellular membrane phospholipids were assessed to monitor adequate uptake before treatment (0 h).

2.3.3. Incubation with SAPC, SDPC vesicles in the presence or absence of FB_1

Following the equilibrium of HepG2 cells with SAPC and SDPC for 16 h, the medium was removed and replaced with medium containing 0.5 % FBS, supplemented with 100 μM SAPC or SDPC (100 μM) in the absence or presence of FB_1 (150 μM). After the 48 h treatment period, cells were washed and collected for detailed lipid analysis as described above.

2.4. Protein determination

The protein content of the cell suspension was determined using the bicinchoninic acid reagent [48] as described previously [8].

2.5. Statistical analysis

Statistical analysis was performed using SAS's generalized linear modelling procedures (version 9.3, SAS Institute Inc., Cary, NC, USA). One-way ANOVAs were used to test for group differences for the concentration range of FB_1 and between control, SAPC and SAPC/FB_1 and separately for control, SDPC and SDPC/FB_1. The number of biological repetitions for each experiment was between three and five. Statistical significance was considered at $p < 0.05$, whereas a $0.05 < p < 0.1$ was considered marginally significant. Graphs were prepared using GraphPad Prism version 8.2.1.

3. Results

3.1. Modulation of HepG2 cell survival indices and lipid parameters by FB_1 (Table 1)

No loss in cell viability, as determined by cellular ATP content, was observed at FB_1 concentrations 75, 150 and 250 μM after 48 h of exposure. FB_1 did not affect cell proliferation or apoptosis at those concentrations. A significant decrease in SM as well as the total cholesterol (TC), free cholesterol (FC) and cholesteryl esters (CE) was induced by FB_1, while PE was increased significantly, lacking dose response effects. The PC/PE and FC/TPL ratios were significantly decreased. Sphinganine (Sa) and the Sa/So ratio were significantly increased at all FB_1 concentrations again lacking a dose-response effect, while the level of sphingosine (So) was noticeably but not significantly increased.

3.2. FB_1 modulation of fatty acid profiles in HepG2 cells (Table 2)

Major FA changes were noticed in the PE phospholipid fraction with no statistically significant changes observable in PC (Table 2). Trends in the PC fraction included the marked reduction in C20:4ω-6 and C22:6ω-3 levels at all concentrations of FB_1 which resulted in concomitant marked reduction in the LCPUFA (defined as FA containing 20 and more carbons) in PC.

In PE, FB_1 significantly increased C16:0, C18:0 and the total saturated fatty acids (SFA), while the C18:0/C16:0 ratio decreased significantly at FB_1 concentrations of 75 and 150 μM. The levels of C18:1ω-7, C18:1ω-9 and total MUFA increased significantly, resulting in a significant increase in the MUFA/SFA ratio. The C18:0/C18:1ω-9 ratio, reflecting enzymatic activity of the stearoyl coenzyme A desaturase (SCD) was significantly reduced at all concentrations tested.

FB_1 significantly increased C18:2ω-6 but not C20:4ω-6 levels resulting in a marginal increase ($p < 0.1$) in the total ω-6 PUFA with the 75 and 250 μM FB_1 treatments. The C20:5ω-3 concentrations were increased significantly at all FB_1 concentrations, while C22:6ω-3 increased markedly but not significantly. FB_1 significantly decreased the C20:4ω-6/C20:5ω-3 ratio, while the P/S ratio was markedly but not significantly decreased across all concentrations. A significant reduction in the C20:4ω-6_PC/PE ratio was observed. For the PUFA and ω-6LCPUFA levels a marked but not significant increase was noticed.

Table 1
Cell growth parameters, lipid content and lipid ratios in HepG2 cells in the presence of FB_1

	Control	75 μM	150 μM	250 μM
Cytotoxicity (% cell death)				
48 h	6.8 ± 1.8	6.8 ± 1.8	7.1 ± 1.9	7.3 ± 1.9
Cell viability (% of Control)				
24 h	100.0 ± 6.9	101.7 ± 8.8	110.3 ± 13.8	107.0 ± 13.6
48 h	100.0 ± 2.8	89.0 ± 15.3	96.2 ± 13.9	101.7 ± 11.8
Cell proliferation (% of Control)				
48 h	100.0 ± 4.5	**109.4 ± 15.6a**	**93.2 ± 15.2b**	**91.8 ± 16.0b**
Apoptosis (Fold change)				
48 h	1.00 ± 0.04	1.12 ± 0.09	1.11 ± 0.07	1.13 ± 0.06
Membrane lipids (48 h)				
Cholesterol (μg/mg protein)				
TC	**22.20 ± 1.63a**	**17.72 ± 1.13b**	**17.69 ± 0.97b**	**17.87 ± 0.76b**
FC	**18.74 ± 0.44a**	**16.52 ± 0.68b**	**16.31 ± 0.48b**	**16.67 ± 0.48b**
CE	**3.46 ± 1.30a**	**1.21 ± 0.84b**	**1.31 ± 0.66b**	**1.20 ± 0.94b**
Glycerophospholipids (μg P_i/mg protein)				
SM	**8.16 ± 0.44a**	**2.76 ± 0.35b**	**2.83 ± 0.48b**	**2.74 ± 0.42b**
PC	83.79 ± 10.09	89.16 ± 6.43	87.10 ± 4.74	88.64 ± 3.92
PE	**25.62 ± 4.06a**	**38.36 ± 7.65b**	**38.42 ± 5.81b**	**39.46 ± 5.68b**
Lipid ratios				
PC/PE	**3.23 ± 0.21a**	**2.28 ± 0.34b**	**2.31 ± 0.40b**	**2.28 ± 0.32b**
FC/TPL	**0.33 ± 0.03a**	**0.26 ± 0.02b**	**0.25 ± 0.02b**	**0.25 ± 0.02b**
Sphingolipids (pmol/mg protein)				
So	64.7 ± 14.3	75.1 ± 17.5	71.4 ± 17.5	76.0 ± 15.7
Sa	**12.5 ± 3.2a**	**1672.5 ± 450.6b**	**1661.0 ± 413.4b**	**1843.0 ± 393.9b**
Sa/So	**0.19 ± 0.04a**	**22.30 ± 2.16b**	**23.24 ± 0.97b**	**24.22 ± 0.58b**

Values are expressed as mean ± standard deviation of at least 3 repetitions. Data were tested for group differences using One-way ANOVA, with significance ($p < 0.05$) indicated by differing letters and in bold. Abbreviations: CE - cholesteryl esters, FB_1 – fumonisin B_1, FC – free cholesterol, FC/TPL – free cholesterol/total phospholipid (PC + PE) ratio, PC - phosphatidylcholine, PE – phosphatidyl-ethanolamine, Sa – sphinganine, SM - sphingomyelin, So - sphingosine, TC – total cholesterol, TPL – total phospholipids (PC + PE).

Table 2
Modulation of FA levels (μg/mg protein), total PUFAs and selected FA ratios in the PC and PE glycerophospholipids of HepG2 cells in the presence of different FB_1 concentrations.

	PC (μg/mg protein)				PE (μg/mg protein)			
Fatty Acids	Control	75 μM	150 μM	250 μM	Control	75 μM	150 μM	250 μM
C16:0	12.74 ± 0.91	13.54 ± 0.81	13.69 ± 0.86	13.81 ± 0.75	**1.51 ± 0.08a**	**2.47 ± 0.32b**	**2.42 ± 0.34b**	**2.26 ± 0.32b**
C18:0	1.23 ± 0.11	1.28 ± 0.07	1.31 ± 0.09	1.34 ± 0.19	**1.75 ± 0.27a**	**2.15 ± 0.06b**	**2.11 ± 0.10b**	**2.19 ± 0.10b**
Total SFA	15.04 ± 0.71	15.88 ± 1.12	16.21 ± 0.99	16.32 ± 0.94	**3.36 ± 0.32a**	**4.75 ± 0.35b**	**4.64 ± 0.48b**	**4.55 ± 0.42b**
C18:0/C16:0 ratio	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	**1.04 ± 0.12a**	**0.80 ± 0.13b**	**0.80 ± 0.10b**	0.89 ± 0.10
C16:1ω-7	3.34 ± 0.30	2.93 ± 0.19	2.99 ± 0.13	3.01 ± 0.25	**0.54 ± 0.16(a)**	**1.10 ± 0.36(b)**	**1.07 ± 0.28(b)**	0.96 ± 0.26
C18:1ω-7	6.45 ± 1.03	6.21 ± 0.07	6.32 ± 0.26	6.27 ± 0.18	**1.59 ± 0.21a**	**3.29 ± 0.51b**	**3.20 ± 0.49b**	**3.24 ± 0.55b**
C18:1ω-9	9.39 ± 1.23	9.62 ± 0.54	9.82 ± 0.78	9.99 ± 0.70	**2.97 ± 0.64a**	**5.87 ± 0.73b**	**5.75 ± 0.86b**	**5.85 ± 1.00b**
Total MUFA	19.62 ± 2.51	19.29 ± 0.52	19.64 ± 1.13	19.80 ± 0.78	**5.31 ± 0.82a**	**10.55 ± 1.60b**	**10.30 ± 1.63b**	**10.34 ± 1.82b**
C18:0/C18:1 ratio (SCD)	0.13 ± 0.03	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	**0.60 ± 0.10a**	**0.37 ± 0.06b**	**0.37 ± 0.05b**	**0.38 ± 0.06b**
MUFA/SFA ratio	1.21 ± 0.10	1.12 ± 0.06	1.12 ± 0.02	1.12 ± 0.07	**1.53 ± 0.27a**	**2.11 ± 0.18b**	**2.12 ± 0.14b**	**2.17 ± 0.19b**
C18:2ω-6	1.07 ± 0.35	0.87 ± 0.25	0.87 ± 0.21	0.87 ± 0.21	**0.53 ± 0.14a**	**0.80 ± 0.05b**	**0.73 ± 0.06b**	**0.74 ± 0.06b**
C20:4ω-6	0.69 ± 0.14	0.44 ± 0.13	0.49 ± 0.14	0.47 ± 0.17	1.87 ± 0.23	2.06 ± 0.14	2.05 ± 0.16	2.13 ± 0.22
Total ω-6 PUFA	2.30 ± 0.60	1.80 ± 0.46	2.00 ± 0.44	1.90 ± 0.50	**2.79 ± 0.44(a)**	**3.35 ± 0.18(b)**	3.25 ± 0.18	**3.34 ± 0.27(b)**
C20:5ω-3	0.12 ± 0.03	0.08 ± 0.03	0.09 ± 0.03	0.13 ± 0.04	**0.16 ± 0.02a**	**0.20 ± 0.01b**	**0.22 ± 0.01b**	**0.21 ± 0.01b**
C22:6ω-3	0.55 ± 0.13	0.37 ± 0.13	0.38 ± 0.12	0.40 ± 0.18	1.61 ± 0.27	1.82 ± 0.22	1.81 ± 0.20	1.93 ± 0.22
Total ω-3 PUFA	0.75 ± 0.17	0.49 ± 0.19	0.56 ± 0.22	0.58 ± 0.18	1.94 ± 0.30	2.18 ± 0.28	2.18 ± 0.28	2.29 ± 0.26
C20:4ω-6/C20:5ω-3 ratio	5.86 ± 1.46	5.96 ± 1.79	5.56 ± 1.28	3.89 ± 0.92	**11.80 ± 0.53a**	**10.12 ± 0.81b**	**9.48 ± 0.46b**	**10.03 ± 1.15b**
C20:4ω-6/C22:6ω-3 ratio	1.37 ± 0.16	1.29 ± 0.20	1.42 ± 0.19	1.29 ± 0.09	1.26 ± 0.06	1.23 ± 0.08	1.23 ± 0.07	1.19 ± 0.05
ω-6/ω-3 ratio	3.35 ± 0.32	4.37 ± 1.12	4.20 ± 1.24	3.74 ± 0.52	1.56 ± 0.05	1.68 ± 0.13	1.64 ± 0.15	1.60 ± 0.12
P/S ratio	0.17 ± 0.04	0.13 ± 0.03	0.13 ± 0.03	0.13 ± 0.04	1.23 ± 0.11	1.02 ± 0.17	1.03 ± 0.18	1.09 ± 0.16
C20:4ω-6_PC/PE ratio	**0.37 ± 0.06a**	**0.21 ± 0.05b**	**0.24 ± 0.06b**	**0.22 ± 0.07b**				
C22:6ω-3_PC/PE ratio	**0.34 ± 0.03a**	**0.23 ± 0.08b**	**0.21 ± 0.05b**	**0.22 ± 0.07b**				
PUFA	3.06 ± 0.76	2.30 ± 0.62	2.55 ± 0.63	2.48 ± 0.67	4.73 ± 0.73	5.53 ± 0.46	5.43 ± 0.43	5.63 ± 0.50
ω-6LCPUFA	1.09 ± 0.19	0.75 ± 0.17	0.86 ± 0.21	0.83 ± 0.22	2.16 ± 0.24	2.41 ± 0.14	2.39 ± 0.16	2.49 ± 0.25
ω-3LCPUFA	0.75 ± 0.17	0.49 ± 0.19	0.56 ± 0.22	0.58 ± 0.18	1.94 ± 0.30	2.18 ± 0.27	2.18 ± 0.28	2.29 ± 0.26
LCPUFA	1.84 ± 0.36	1.24 ± 0.34	1.42 ± 0.42	1.40 ± 0.39	4.09 ± 0.54	4.58 ± 0.39	4.57 ± 0.42	4.77 ± 0.49

Values are expressed as mean ± standard deviation of at least 3 repetitions and expressed as μg per mg protein. Data were tested for group differences using One-way ANOVA and significance ($p < 0.05$) is indicated by differing letters and in bold. Letters in parenthesis represent marginal differences ($0.05 < p < 0.1$). Abbreviations: C20:4ω-6-PC/PE - ratio of C20:4ω-6 content in PC to PE, FB_1 – fumonisin B_1, LCPUFA – long-chained PUFA, i.e. FA with C20 and above, MUFA – total monounsaturated fatty acids, PC – phosphatidylcholine; PE – phosphatidylethanolamine, P/S ratio – ratio of polyunsaturated to saturated fatty acids, PUFA – polyunsaturated fatty acids, SCD – stearoyl coenzyme A desaturase, SFA –saturated fatty acids, ω-6/ω-3ratio - total omega-6/omega-3 polyunsaturated fatty acid ratio.

3.3. Effects of FB_1 on PUFA-enriched HepG2 cells

3.3.1. Cell viability and modulation of lipid parameters by PC vesicles during 16-h equilibrium phase

Cell viability was slightly increased for cells treated with both PUFA-enriched PC vesicles (SAPC and SDPC) for 16 h (Table 3). Cell proliferation was significantly decreased by both PC vesicle treatments, while apoptosis was not altered. No significant differences were detected in the phospholipid levels. The TC content was significantly decreased by both SAPC and SDPC due to a marked reduction in CE and FC. The level of C16:0 marginally decreased ($p < 0.1$) with the SDPC compared to the control, while C18:0 was significantly increased by both PUFA-enriched PC vesicle treatments resulting in a significant increase in the C18:0/C16:0 ratio. No changes were noticed in the total SFA and MUFA levels and the MUFA/SFA ratio. SAPC significantly increased C20:4ω-6, while SDPC significantly increased the level of C22:6ω-3 resulting in increase of the ω-6/ω-3 ratio with the SAPC and decrease with the SDPC treatment. The C20:4ω-6/C22:6ω-3 ratio was increased significantly by the SAPC and decreased by the SDPC. The C20:4ω-6/C20:5ω-3 and the C20:4ω-6_PC/PE ratios were significantly increased by SAPC. The PUFA content was significantly increased by the treatment with SAPC vesicles due to a significant increase in the ω-6LCPUFA. The ω-3LCPUFA increased due to the SDPC treatment.

3.3.2. Modulation of cell survival parameters and cellular redox status of PUFA-enriched PC vesicles by FB_1

A non-cytotoxic dosage of FB_1 (150 μM) and pre-determined cytotoxic SAPC and SDPC concentrations (100 μM) were selected to investigate the interactive responses between PUFA loading in HepG2 cells and FB_1 treatment. Data on dose selection is summarised in Supplementary materials S2 and S3. After the 48-h incubation with both SAPC and SDPC treatments, a significant increase in cytotoxicity and decrease in cell viability was observed (Fig. 2A and B).

The increase in cytotoxicity was significantly attenuated, but not completely abolished in the presence of FB_1. Cell proliferation (Fig. 2C) was significantly inhibited with SAPC and SDPC treatments to a far larger extent when compared to the 16-h equilibrium treatment (Table 3), while FB_1 countered this response, although not completely. There were no significant differences in the responses between the two different PC vesicle treatments. Apoptosis was significantly increased with SAPC and SDPC treatments (Fig. 2D), while the FB_1 treatment countered the effect in cells treated with SAPC. FB_1 did not reduce the SDPC-induced increase in apoptosis. For conjugated dienes (CD, Fig. 2E), the SAPC and SDPC treatments significantly increased CD content when compared to control, while FB_1 significantly reduced conjugated dienes in SAPC and SDPC treated cells to similar levels as the control (Fig. 2E). FB_1 treatment alone did not change CD levels (FB_1 (9.79 ± 1.70 nmol/mg protein) versus Control (10.0 ± 2.05 nmol/mg protein)).

3.3.3. Modulation of lipid parameters

3.3.3.1. Cholesterol, glycerophospholipids and sphingolipids. Group differences indicated that SAPC increased the TC and CE, while SDPC did not induce a similar increase (Table 4). FB_1 significantly lowered TC and FC as well as CE significantly in SAPC and SDPC treated cells (main effect). Both PUFA-enriched PC vesicles significantly increased SM and PC content. These effects were countered by FB_1 with SM significantly reduced to below control levels for both SAPC and SDPC, while PC remained still above the control with the SAPC/FB_1 treatment but not

Table 3
Effect of equilibration (16 h) with SAPC and SDPC on cell growth parameters of HepG2 cells and selected FA profiles and ratios in PC.

Parameters	Ctrl	SAPC	SDPC
Cell viability (% of control)	**100.0 ± 1.9a**	**105.0 ± 3.4b**	**107.2 ± 2.4c**
Cell proliferation (% of control)	**100.0 ± 6.2a**	**85.4 ± 9.4b**	**79.5 ± 9.3b**
Apoptosis (fold change)	1.00 ± 0.03	1.03 ± 0.07	1.09 ± 0.08
PC (μg P_i/mg protein)	81.70 ± 5.8	84.0 ± 1.3	83.8 ± 6.4
PE (μg P_i/mg protein)	27.2 ± 3.5	27.4 ± 3.9	28.0 ± 3.8
SM (μg P_i/mg protein)	8.1 ± 0.7	7.8 ± 0.8	6.9 ± 0.7
TC (μg/mg protein)	**22.8 ± 1.3a**	**20.4 ± 0.9b**	**20.5 ± 1.6b**
FC (μg/mg protein)	17.1 ± 1.3	15.8 ± 0.7	15.7 ± 1.2
CE (μg/mg protein)	5.8 ± 0.9	4.6 ± 0.8	4.7 ± 0.6
Fatty acids (μg/mg protein)			
C16:0	**12.69 ± 0.88(a)**	11.85 ± 1.08	**10.44 ± 2.20(b)**
C18:0	**1.57 ± 0.12a**	**2.55 ± 0.27b**	**2.46 ± 0.31b**
Total SFA	15.23 ± 0.85	15.46 ± 0.93	13.73 ± 2.52
C18:0/C16:0 ratio	**0.11 ± 0.01a**	**0.20 ± 0.04b**	**0.22 ± 0.03b**
C18:1ω-9	8.22 ± 0.68	8.07 ± 0.32	7.73 ± 1.51
Total MUFA	16.37 ± 1.39	15.83 ± 0.53	15.01 ± 3.03
MUFA/SFA ratio	0.99 ± 0.06	0.96 ± 0.05	1.02 ± 0.05
C18:2ω-6	0.90 ± 0.02	0.88 ± 0.09	0.78 ± 0.13
C20:4ω-6	**1.18 ± 0.07a**	**1.92 ± 0.24b**	**1.02 ± 0.19a**
Total ω-6	**2.82 ± 0.10a**	**3.53 ± 0.24b**	**2.52 ± 0.45a**
C20:5ω-3	**0.32 ± 0.01a**	**0.27 ± 0.04b**	**0.32 ± 0.04(a)**
C22:6ω-3	**1.13 ± 0.09a**	**0.97 ± 0.16a**	**1.55 ± 0.31b**
total ω-3	1.82 ± 0.12	**1.56 ± 0.28a**	**2.16 ± 0.40b**
C20:4ω-6/C20:5ω3- ratio	**3.64 ± 0.31a**	**7.20 ± 0.87b**	**3.16 ± 0.42a**
C20:4ω-6/C22:6ω-3 ratio	**1.13 ± 0.03a**	**2.16 ± 0.22b**	**0.72 ± 0.02c**
ω-6/ω-3 ratio	**1.55 ± 0.08a**	**2.32 ± 0.35b**	**1.16 ± 0.05c**
C20:4ω-6_PC/PE ratio	**0.59 ± 0.08a**	**0.82 ± 0.23b**	**0.57 ± 0.06a**
PUFA	**4.64 ± 0.20a**	**5.08 ± 0.52b**	4.68 ± 0.84
LCPUFA	3.55 ± 0.19	3.96 ± 0.58	3.66 ± 0.68
ω-6LCPUFA	**1.73 ± 0.09a**	**2.41 ± 0.30b**	**1.49 ± 0.29a**
ω-3LCPUFA	1.82 ± 0.12	**1.56 ± 0.28a**	**2.16 ± 0.40b**

Values are expressed as mean ± standard deviations of 5 repetitions. The concentration of SAPC and SDPC used was 100 μM. Statistical significance ($p < 0.05$) between groups, determined by One-way ANOVA, is indicated by differing lowercase letters and in bold. Letters in parenthesis indicate marginal differences ($0.05 < p < 0.1$). Abbreviations: C20:4ω-6_PC/PE - ratio of C20:4ω-6 content in PC to PE, CE – cholesteryl esters, FA - fatty acids, FC – free cholesterol, LCPUFA – long-chained polyunsaturated fatty acids, MUFA – monounsaturated fatty acids, SAPC – 1-stearoyl-2-arachidonyl-sn-glycero-3-phosphocholine, SDPC – 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine, PC - phosphatidylcholine, PE - phosphatidylethanolamine, PUFA – polyunsaturated fatty acids, SFA – saturated fatty acids, SM - sphingomyelin, TC – total cholesterol

SDPC/FB_1. Overall FB_1 significantly decreased PC and increased and PE levels (main effect). The So, Sa and Sa/So ratio were not significantly altered by the SAPC and SDPC, while FB_1 significantly increased Sa levels as well as the Sa/So ratio.

3.3.3.2. Lipid ratios as indicators of membrane fluidity. The PC/PE and free cholesterol/TPL (PC + PE) ratios were not affected by the PUFA-enriched PC vesicles, while FB_1 significantly decreased both ratios (main effect, Table 4).

3.4. Effects of FB_1 and PUFA-enriched PC vesicles on FA content and ratios

The complete set of quantitative FA data in PC and PE can be found in Supplementary Table S3.

3.4.1. Saturated fatty acids (SFA, Table S3 and Fig. 3A–D)

SAPC significantly increased C16:0, C18:0 and the total SFA (Fig. 3A) whilst SDPC increased C18:0 and total SFA in PC. FB_1 significantly reduced the increased levels of C18:0 with the SAPC treatment. In the presence of SDPC, FB_1 significantly increased C16:0 and total SFA but countered the increase in C18:0. In both cases the levels of the FA did not return to the level observed in the control cells. In PE, SAPC vesicles increased C18:0, while C16:0 and total SFA levels (Fig. 3B) were not affected. SDPC increased C18:0 significantly and tended to increase total SFA ($0.05 < p < 0.1$). FB_1 increased C16:0, C18:0 and total SFA levels significantly, except for C16:0 where only a trend towards an increase with the SAPC/FB_1 treatment ($0.05 < p < 0.1$) was observed. The C18:0/C16:0 ratio was significantly increased in PC and PE (Fig. 3C and D) by both SAPC and SDPC, which was significantly countered by FB_1, except for the SDPC/FB_1 treatment in PE.

3.4.2. Monounsaturated fatty acids (MUFA, Table S3 and Fig. 3E–H)

SAPC increased the levels C18:1ω-7, C18:1ω-9 (Fig. 3E), and the total MUFA while SDPC increased the C18:1ω-9 and total MUFA significantly in PC. FB_1 countered the SAPC-induced increase, while no effect was noticed in the presence of SDPC. In PE, no effect was noticed for SAPC and SDPC, while FB_1 increased the levels C18:1ω-7, C18:1ω-9 (Fig. 3F), and the total MUFA in the presence of both PUFA-enriched PC vesicles. The C18:0/C18:1ω-9 ratio, reflecting the SCD activity, was increased by both PUFA-enriched PC vesicles in PC and PE (Fig. 3G and H), while FB_1 significantly countered the effects in PE. The MUFA/SFA ratio was decreased by FB_1 in PC with the PUFA-enriched PC vesicles lacking any response. In PE, on the other hand, both PUFA-enriched PC vesicles significantly decreased the MUFA/SFA ratio, whilst FB_1 increased the values to reach levels similar to the control.

3.4.3. ω−6 polyunsaturated fatty acids (ω-6 PUFA, Table S3 and Fig. 3I–K)

Both PUFA-enriched PC vesicles increased the content of the total ω-6 PUFA by increasing the levels of C18:2ω-6 and C20:4ω-6 in PC (Fig. 3I and J), while FB_1 reduced the levels with both SAPC and SDPC

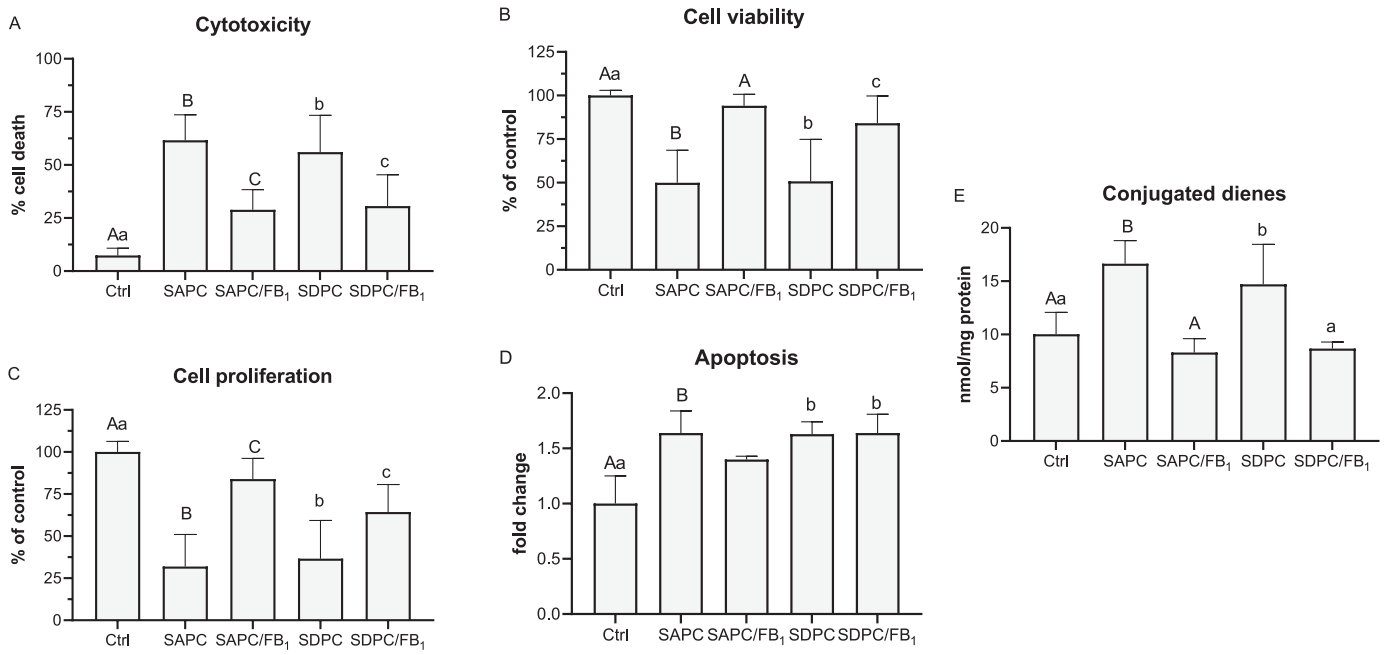

Fig. 2. Modulation of cytotoxicity (A), cell viability (B), cell proliferation (C) and apoptosis (D) in HepG2 cells exposed to SAPC (100 μM) and SDPC (100 μM) in the absence and presence of FB_1 (150 μM) for 48 h. Levels of conjugated diene (E) in HepG2 cells treated with the PC vesicles in the absence and presence of FB_1. Values are expressed as mean ± standard deviation of 4 to 5 repetitions. Significant differences, determined by One-way ANOVA are indicated by differing upper case (comparing Ctrl, SAPC, SAPC/FB_1) and lower case (comparing Ctrl, SDPC, SDPC/FB_1) letters. Abbreviations: Ctrl – control, SAPC – 1-stearoyl-2-arachidonyl-sn-glycero-3-phosphocholine, SDPC – 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine, FB_1 – fumonisin B_1.

treatments. In PE, SAPC increased the levels of C20:4ω-6 (Fig. 3K) and total ω-6 PUFA.

3.4.4. ω−3 polyunsaturated fatty acids (ω-3 PUFA, Table S3 and Fig. 3L and M)

Both SAPC and SDPC significantly increased levels of C20:5ω-3, C22:6ω-3 (Fig. 3L) and total ω-3 PUFA, while FB_1 significantly reduced C22:6ω-3 and total ω-3 PUFA. In PE, C22:6ω-3 (Fig. 3M) and the total ω-3 PUFA increased with the SDPC treatment, while FB_1 significantly increased C20:5ω-3 when compared to the control and C22:6ω-3 and total ω-3 PUFA when compared to the SDPC treatment and the control.

3.4.5. Fatty acid ratios (Table S3 and Fig. 3N–P)

In PC, SAPC significantly increased the C20:4ω-6/C20:5ω-3, C20:4ω-6/C22:6ω-3, ω-6/ω-3 and P/S (Fig. 3N) ratios while FB_1 significantly countered the effect except for the ω-6/ω-3 ratio. SDPC significantly decreased C20:4ω-6/C20:5ω-3, C20:4ω-6/C22:6ω-3 and ω-6/ω-3 ratios while FB_1 lacked any effect. When considering the P/S ratio, SDPC increased the ratio while it was countered by FB_1. In PE, the SAPC significantly increased the C20:4ω-6/C20:5ω-3, C20:4ω-6/C22:6ω-3 and ω-6/ω-3 ratios, while FB_1 decreased the SAPC-induced increase in the C20:4ω-6/C22:6ω-3 ratio. The SDPC significantly decreased the C20:4ω-6/C20:5ω-3, C20:4ω-6/C22:6ω-3 and ω-6/ω-3 ratio's while FB_1 further reduced the C20:4ω-6/C20:5ω-3 ratio.

Group effects for the 20:4ω-6_PC/PE (Fig. 3O) and 22:6ω-3_PC/PE (Fig. 3P) ratios indicated an increase by SAPC and SDPC, which was countered in the presence of FB_1.

3.4.6. Cumulative PUFA parameters (Table S3)

When considering the cumulative FA parameters, SAPC and SDPC increased PUFA, LCPUFA, ω-6LCPUFA and ω-3LCPUFA in PC, which was significantly countered by FB_1 for LCPUFA, ω-6LCPUFA and ω-3LCPUFA with SAPC treatment and for PUFA, LCPUFA, ω-6LCPUFA and ω-3LCPUFA with the SDPC treatment. In PE, SAPC significantly increased the ω-6LCPUFA, while SDPC increased ω-3LCPUFA. FB_1 significantly increased the PUFA, LCPUFA and ω-3LCPUFA only in the presence of SDPC.

4. Discussion

Cellular resistance of pre-cancerous and cancerous cells towards toxic substances including drugs is well documented [49–51]. These adaptive changes seem to aid pre-cancerous cells in developing strategies to evade immediate toxic environments and the potential loss of normal cellular control mechanisms is thought to be the underlying process resulting in malignant transformation and cancerous growth [52,53]. Membrane lipids are known to play a vital role in maintaining membrane integrity and function and the induction of a pro-carcinogenic altered lipid phenotype with differential changes in cholesterol, SM, PE, membrane FA's including SFA, MUFA and PUFA were associated with neoplastic development previously [28,31,54,55]. Changes in FA metabolism related to C16:0, C18:1ω-9 and C20:4ω-6 were suggested to play a key role in the selective growth kinetics of cancer cells compared to normal cells [28,31,56–58]. A previous study indicated that rapidly proliferating Chang cells were resistant to the cytotoxic effects of FB_1, which linked altered membrane lipid composition to cell survival in rapidly proliferating cells in contrast to the cytotoxicity observed in primary hepatocytes [8]. As the modulation of cancer growth by the cytotoxic and antiproliferative properties of ω-6 and ω-3 PUFAs and their metabolites via changes in redox status and cell signalling pathways has been well established [27,33,59–61], we hypothesized that increasing PUFA content using SAPC and SDPC could heighten the susceptibility of HepG2 cells to FB_1-induced cytotoxic effects. Although FB_1 protected against PUFA-induced cytotoxicity, PUFA-loaded HepG2 cells also appeared to be sensitized towards FB_1 effects with respect to levels of cytotoxicity (Fig. 2A) and cell proliferation (Fig. 2B). That these effects occurred after 48 h of incubation may be related to the method of PUFA delivery in this study. The PC vesicles were used to match naturally occurring PC for the cells to internalise and metabolise similar to an endogenous phospholipid. That would imply that uptake, remodelling of intracellular phospholipids and metabolism, such as enzymatic and non-enzymatic oxidation, may not happen

Table 4
Lipid parameters of HepG2 cells exposed to SAPC and SDPC in the absence and presence of FB_1.

Lipid parameters	Control	SAPC	SAPC/FB_1	SDPC	SDPC/FB_1	Main Effects
Cholesterol (μg/mg protein)						
TC	20.9±0.6 Aa	25.7±1.3 B	16.7±1.1 C	22.1±2.6 a	16.3±1.5 b	FB_1 ↓
FC	18.1±0.4 Aa	22.8±1.1 B	16.0±0.9 C	19.8±2.5 a	15.0±2.0 b	FB_1 ↓
CE	2.8±0.4 Aa	3.1±0.9 A	0.7±0.4 B	2.4±0.4 a	0.8±0.5 b	FB_1 ↓
Phospho - and sphingolipids (μg P_i/mg protein)						
SM	7.05±0.62 Aa	10.50±1.73 B	2.62±0.54 C	8.69±0.81 b	3.20±0.91 c	FB_1 ↓
PC	78.88±3.52 Aa	104.82±6.24 B	90.20±7.63 C	90.01±3.06 b	87.62±6.23 b	FB_1 ↓
PE	24.21±5.63	26.05±4.76	33.20±7.26	26.08±5.37	33.77±8.33	FB_1 ↑
Sphingolipids (pmol/mg protein)						
So	64.5±11.8 (A)	54.3±15.0	41.1±7.6 (B)	55.4±14.2	46.2±11.5	(FB_1 ↓)
Sa	9.2±2.5 Aa	4.9±2.2 A	2015.5±471.5 B	5.9±2.6 a	2032.8±522.7 b	FB_1 ↑
Sa/So ratio	0.14±0.01 a	0.09±0.04 A	42.68±11.94 B	0.10±0.03 a	44.0±2.03 b	FB_1 ↑
Lipid ratios						
PC/PE ratio	3.46±0.71	3.95±0.73 A	2.79±0.42 B	3.75±0.46 (a)	2.83±0.41 (b)	FB_1 ↓
FC/TPL ratio	0.35±0.02 Aa	0.36±0.02 A	0.26±0.02 B	0.34±0.03 a	0.26±0.02 b	FB_1 ↓

Values are expressed as mean ± standard deviation of at least 3 repetitions. The data set was divided into an SAPC subset (Ctrl, SAPC and SAPC/FB_1) and a SDPC subset (Ctrl, SDPC, SDPC/FB_1) and these were analysed separately for group differences by One-way ANOVA. Means followed by differing letters indicate significant ($p < 0.05$) differences: using uppercase for SAPC (blue) and lowercase for SDPC (red). Two-Way ANOVA was performed for the SAPC, SAPC/FB_1, SDPC and SDPC/FB_1 treatments to determine main effects of FB_1. ↑ and↓ arrows indicate increased or decreased main effects. Abbreviations: CE - cholesteryl ester, FB_1 - fumonisin B_1, FC – free cholesterol, FC/TPL - free cholesterol to total phospholipid (PC + PE) ratio, PC - phosphatidylcholine, PE - phosphatidylethanolamine, SM – sphingomyelin, Sa – sphinganine, SAPC - 1-stearoyl-2-arachidonyl-sn-glycero-3-phosphocholine, SDPC – 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine, So – sphingosine, TC – total cholesterol.

immediately [62]. The data presented (Fig. 3 and Table S3) show that both SAPC and SDPC vesicles were able to deliver C20:4ω-6 and C22:6ω-3, respectively, into HepG2 cell as indicated by increased C18:0, C20:4ω-6 and C22:6ω-3 content in the PC phospholipid fraction. The data further suggested that these FA were also incorporated into other lipid fractions, such as PE, which is consistent with literature showing that external FA can enter the cellular pool and are metabolised similar to intracellular FA [22].

Since PUFA are easy targets of lipid peroxidation [63,64], it is possible that modulation of oxidative stress in addition to altered lipid environment accounted for the observed effects. To delineate how FB_1 was able to decrease PUFA-induced cytotoxicity and why this was not completely reversed, lipid peroxidation as well as lipid profiles were determined in these cells after the 48-h incubation period.

4.1. FB_1-induced cell survival and lipid changes

The FB_1-induced attenuation of the cytotoxic effects was evident at various levels as demonstrated by changes in the membrane cholesterol, glycerophospholipids, sphingolipid and different FA parameters that varied between the PC and PE glycerophospholipids, which is summarised below (Fig. 4). The loss in membrane integrity due to changes and/or redistribution of the major membranal lipid constituents were previously associated with adverse toxic effects [65]. However, changes to membrane lipid symmetry and/or the loss thereof is a common feature in cancer cells and was implicated in their resistance to anti-cancer drugs [66,67]. FB_1 countered the membranal changes induced by PUFA supplementation in HepG2 cancer cells, specifically the increase in cholesterol, PC and SM whilst increasing the level of PE. As a result, FB_1 decreased the major membrane fluidity parameters (PC/PE and Chol/TPL) suggesting a more rigid membrane structure, a common feature of cancer cells [68]. PUFA supplementation increased the P/S ratio in PC and reduced it in PE, while FB_1 selectively attenuated these changes due to alteration in the SFA and PUFA, specifically C20:4ω-6, C20:5ω-3 and C22:6ω-3. These FA are known to play a key role in membrane fluidity due to the high degree of conformational flexibility resulting from multiple double bonds [69,70]. The interaction of C22:6ω-3 with membrane lipids, particularly cholesterol and sphingomyelin, has also been suggested to play a prominent role in lipid raft formation and integrity [71–73]. Since SM and PC constitute >50 % of membrane sphingo- and glycerophospholipids, specific interactions with cholesterol may have significant impact on cell viability [74]. Cholesterol in lipid raft domains is known to activate both pro- as well as anti-apoptotic signalling pathways supporting the survival of cancer cells [66,75–77]. A reduction in cholesterol by FB_1 could therefore interfere with apoptosis signalling while reducing the number of sphingolipid micro domains in the plasma membrane [78]. The depletion of SM content might be responsible for the low capacity of plasma membrane to solubilise cholesterol or for "activation" of the plasma membrane cholesterol pool [79,80]. These changes are likely associated with the resistance of HepG2 cells to undergo cell death or apoptosis via the ceramide and/or mitochondrial pathways [71].

4.2. FB_1/PC vesicles lipid interactions

As reduced SM and increased PE levels were reported to be associated with continuous cell proliferation [55] it would appear that the FB_1-induced changes support cell survival. For instance, FB_1 decreased PC whilst increasing PE, thus restoring the PC/PE ratio and counteracting the effect of PUFA supplementation, thereby potentially contributing to the reduction of PUFA-induced cytotoxicity in HepG2 cells. Of interest was that SAPC and SDPC seemed to modulate sphingolipid metabolism, as Sa tended to decrease, although not significantly, while SM was increased (Table 4). This could be related to the stimulation of sphingomyelin synthase associated with the increased PC level due to convergence of glycerophospho- and sphingolipid metabolism [81]. The inhibition of ceramide synthase by FB_1 and accumulation of the sphingoid bases with a reduction of sphingomyelin levels [82]

countered the effect of the PUFA supplementation thus likely promoting cell survival. This was also evident in highly proliferating Chang cells, where the FB_1-induced disruption of sphingolipid biosynthesis was associated with or occurred despite of cellular resistance [8].

Of interest was that differential responses on the lipid parameters were induced by SAPC compared to SDPC. SAPC preferentially affected the increase in free cholesterol and SM, the decrease in Sa while both PC vesicles increased the PC levels, presumably as it was the vehicle for the PUFA enrichment. However, the role of C20:4ω-6 and C22:6ω-3 in determining membrane functions differ slightly [83–85]. Similarly, the modulating response of FB_1 on these membrane parameters also differed, as, for instance, the reduction in PC was only statistically

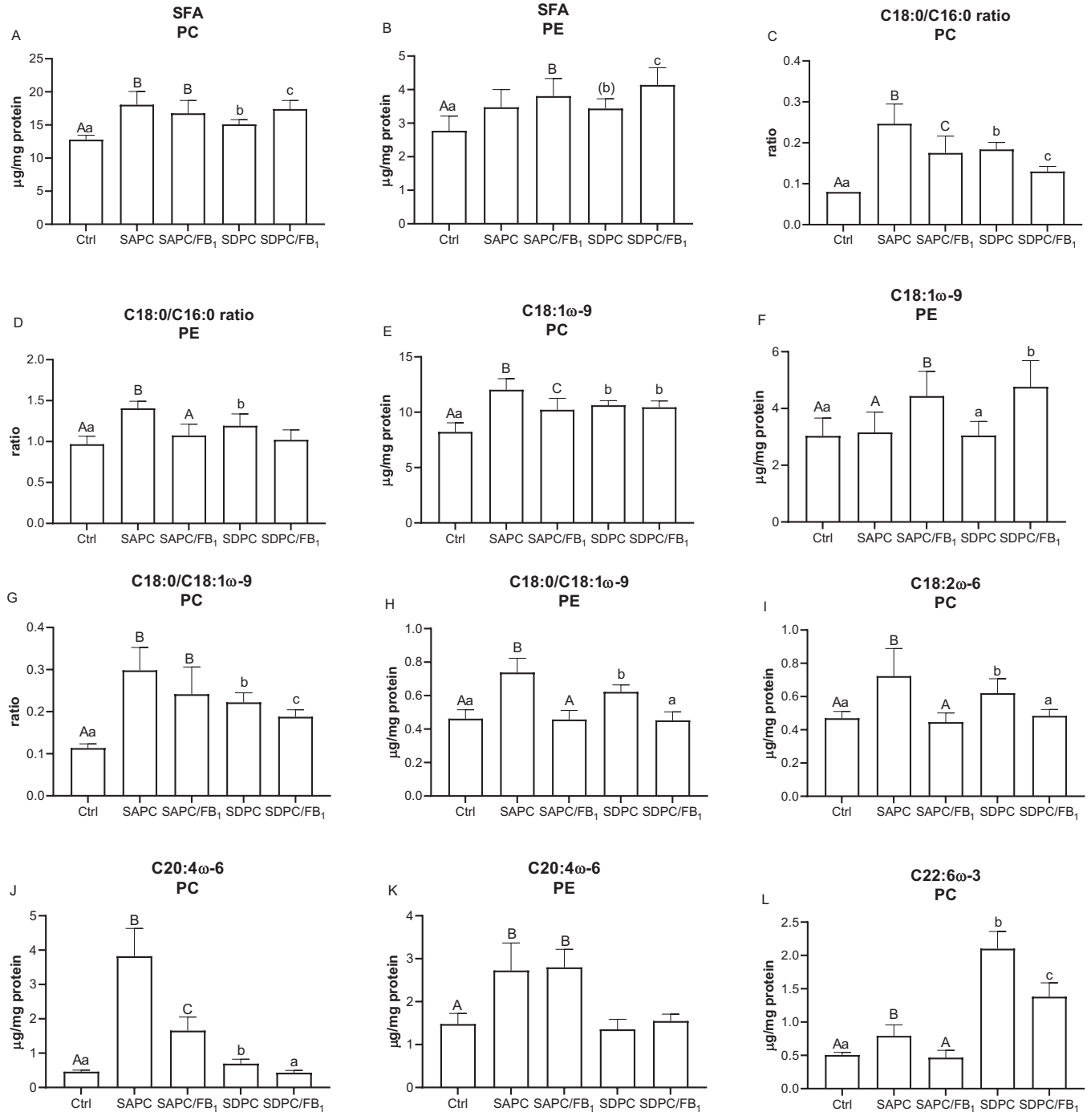

Fig. 3. Modulation of selected fatty acids and fatty acid ratios in PC and PE phospholipid fractions of HepG2 cells treated with SAPC or SDPC (100 µM) in the absence and presence of FB_1 (150 µM) for 48 h. Values are expressed as mean ± standard deviation of 4 to 5 repetitions. Significant differences, determined by One-way ANOVA, are indicated by differing upper case (comparing Ctrl, SAPC, SAPC/FB_1) and lower case (comparing Ctrl, SDPC, SDPC/FB_1) letters. Abbreviations: C20:4ω-6_PC/PE - ratio of C20:4ω-6 content in PC to PE, Ctrl – control, FB_1 – fumonisin B_1, PC - phosphatidylcholine, PE – phosphatidylethanolamine, P/S ratio – ratio of polyunsaturated to saturated fatty acids, SAPC – 1-stearoyl-2-arachidonyl-sn-glycero-3-phosphocholine, SDPC – 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine, SFA – saturated fatty acids.

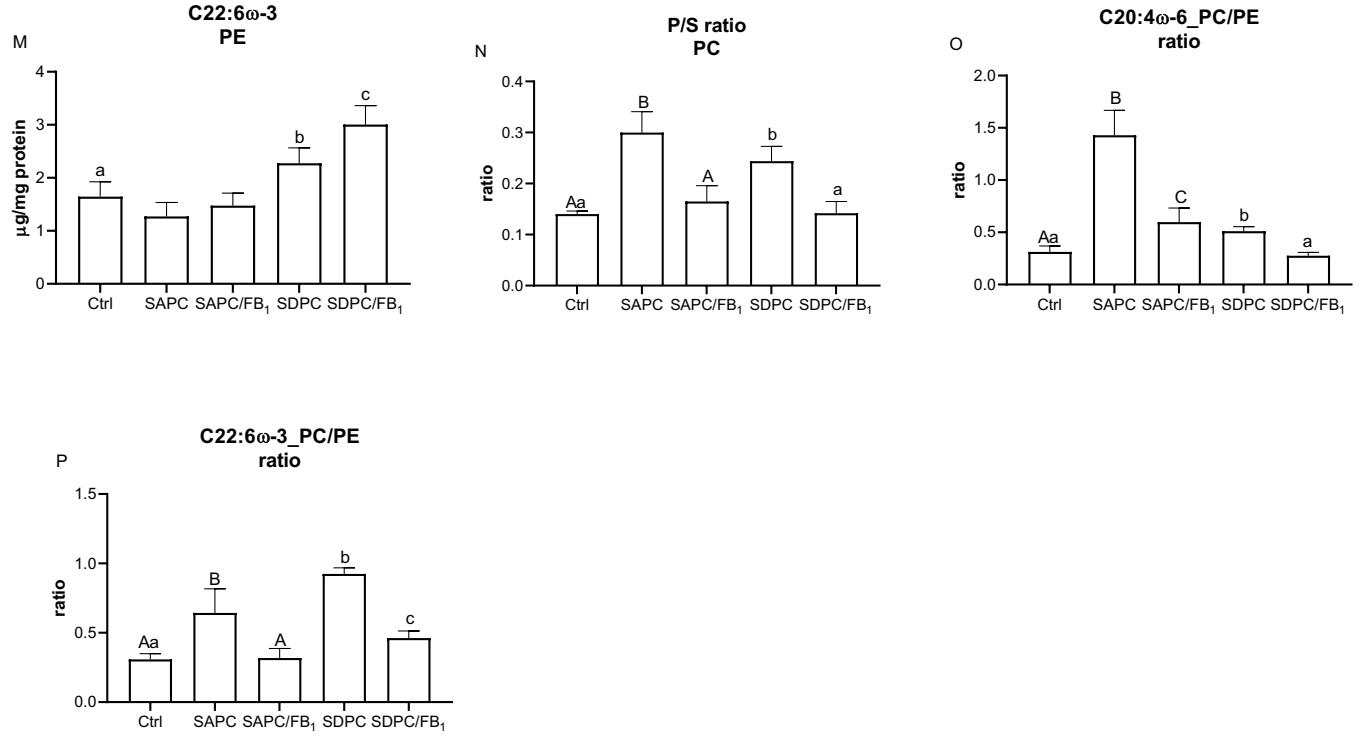

Fig. 3. (*continued*).

significant in the presence of SAPC.

4.3. FB_1/PC vesicles FA interactions

Apart from the major membrane constituents, the role of specific FA changes related to the effect of PUFA supplementation on cell viability and the interactive responses with FB_1 were complex, as suggested by the numerous significant effects highlighted in Fig. 3 and Supplementary Table S3. These differences could be related to altered FA incorporation kinetics and distribution between the different phospholipid fractions. PE is generally distributed towards the inner leaflet of cellular membranes [86,87] and C22:6ω-3 is preferentially incorporated into PE in

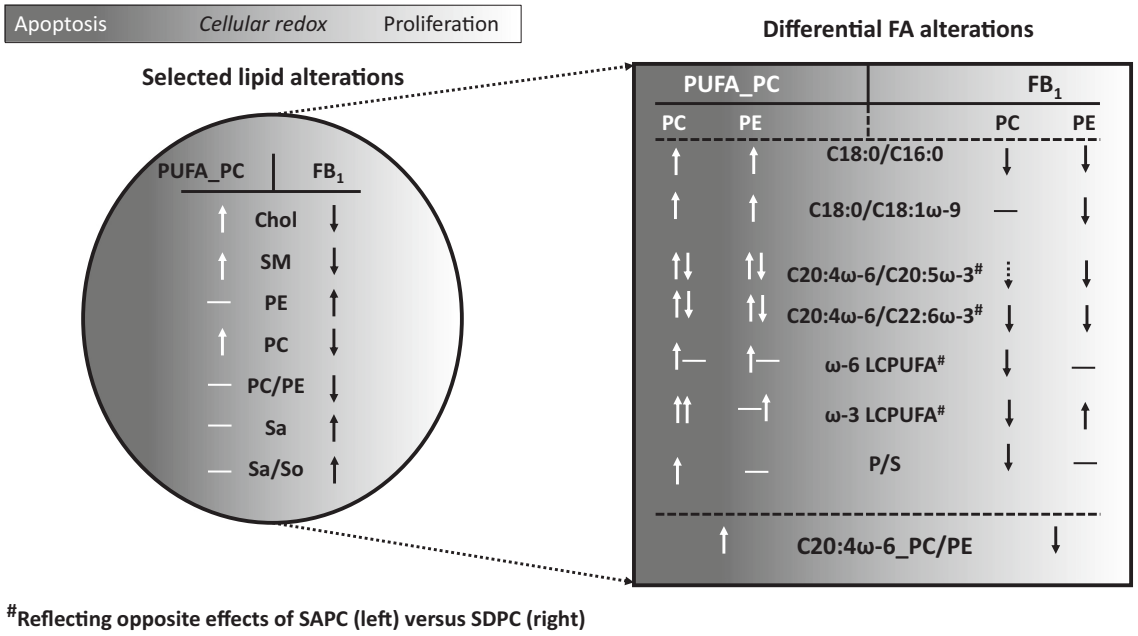

Fig. 4. Schematic diagram portraying interactions between FB_1 and PUFA-enriched PC vesicles modulating selected lipid and FA parameters associated with differential cell survival indices effected in HepG2 cancer cells. Interplay between apoptosis (dark shaded) and cell proliferation (light shaded) as a function of the role of cellular redox (intermediate shaded), depicted as the resultant oxidative stress, are illustrated as a function of the different lipid and FA parameters. PUFA-enriched PC vesicle-induced changes, associated with increase in oxidative stress, reduction in cell viability and induction of apoptosis, are illustrated and FB_1 attenuation thereof. Arrows ↑ or ↓ depict either an increase or decrease of the PUFA-enriched PC vesicle response, respectively, and the subsequent FB_1 attenuation thereof. "-" indicates an absence of the response, dashed arrows indicate a trend ($0.05 < p < 0.1$).

contrast to C20:4ω-6 which tends to be incorporated into PC in the liver and other tissues [83–85]. In the current study, C20:4ω-6 was also preferentially incorporated into PC (Fig. 3O). In contrast, C22:6ω-3 was equally distributed between PC and PE (Fig. 3P) suggesting a higher degree of flux from PC to PE. The increased LCPUFA levels and unequal distribution of C20:4ω-6 and C22:6ω-3 between PC and PE together with the other membrane fluidity markers, may contribute to the adverse toxic effect of the PUFA-enriched PC vesicles. These changes coincided with an increase in oxidative stress (Fig. 2E) and changes in cellular redox status as reported previously in HepG2 cells [42,43,45]. This also concurred with the findings by Di Nunzio et al. [88] who showed that PUFA alter the survival of HepG2 cells by changes in the redox status resulting in cytotoxic effects. As low PUFA levels contribute to the resistance phenotype of cancer cells, enhanced susceptibility of oxidative stress resulting from increased levels of LCPUFA was suggested to reduce cell viability [21,31,89].

FB_1 treatment reduced LCPUFA levels mainly in PC, which was likely a result of impairment of Δ6-desaturase activity [30] and an increase in oxidative stress as reported in different cell culture systems [90–92]. In the current study the induction of oxidative stress by FB_1, measured as conjugated dienes, could not be confirmed. However, FB_1 was previously shown to enhance the activities of glutathione peroxidase and glutathione reductase [93,94] and these enzymes rapidly remove lipid hydroperoxides and regenerate oxidised glutathione [95,96]. These protective mechanisms could help to maintain cellular homeostasis in cancer cells and contribute to resistance to redox changes [97], especially when considering that FB_1 also reduced conjugated diene levels in cells treated with SAPC and SDPC (Fig. 2E).

FB_1-induced interactions with SFA, MUFA and sphingolipids and modulation of downstream cell survival signalling events may also be influenced by the very low level of C18:0 in PC of HepG2 cells, which was almost 10-fold lower than the level of C16:0. As PC vesicles contained C18:0 in the sn-1 position, supplementation of HepG2 cells increased the level of this FA resulting in an increase in the C18:0/C16:0 ratio (Fig. 3C). Although the exact roles each of these SFA play are not fully understood, studies indicated that both C16:0 and C18:0 exhibit toxic effects in cells and tissues [98–101]. Several studies showed that SFA induced apoptosis via ceramide dependent and independent pathways [99,100,102]. An increased C18:0/C16:0 ratio was associated with changes in cell growth parameters [103] and the induction of apoptosis, presumably via the oxidative stress associated with ceramide synthesis [102]. The increase in SM and decrease in Sa indicated that the PUFA supplementation in HepG2 cells also disrupted sphingolipid metabolism and likely ceramide synthesis, which is associated with increased oxidative stress. A reduction of oxidative stress and the observed protection against cytotoxicity and inhibition of cell proliferation by FB_1 through the disruption SFA and sphingolipid metabolism [104], tends to support the notion that the modulation of these interactive pathways contributed to the resistance of HepG2 cancer cells.

When considering the MUFA content, PUFA supplementation increased C18:1ω-7, C18:1ω-9 and total MUFA content only in PC (compare Fig. 3E and F). However, the C18:0/C18:1ω-9 ratio was increased in both PC and PE (Fig. 3G and H) while the MUFA/SFA ratio was decreased only in PE. These changes could indicate decreased stearoyl coenzyme A desaturase (SCD) activity, which has been associated with reduced cell survival and induction of apoptosis [57]. The considerably lower levels of C18:0 relative to the C18:1ω-7 and C18:1ω-9 metabolites in both PC and PE in the presence of FB_1 suggested a higher activity of SCD in FB_1-treated HepG2 cells. The FB_1-induced changes to MUFA levels could therefore be related to the restoration of SCD activity and the relative distribution between the two glycerophospholipids. High activity of SCD can predict aggressiveness in certain cancers [105,106], while it has been well documented that high MUFA levels confer resistance towards cytotoxicity and apoptosis of cancer cells [57,105,107–110]. Several studies also indicated that MUFA and certain PUFA, such as C18:2ω-6 and C20:4ω-6, may protect against the induction of apoptosis by SFA [100,111]. While the role of excessive levels of C18:1ω-9 is not yet fully understood, provision of cellular building blocks for sustaining rapid cell replication, antioxidant properties and the removal of cytotoxic SFA, such as C18:0, were hypothesized to be part of the underlying mechanisms [57]. The FB_1-induced modulation of distribution of MUFA in PC and PE in HepG2 cells supplemented with PUFA is therefore likely a key contributor in restoring cell survival indices in HepG2 cells.

4.4. Lipid phenotype associated with cellular resistance

Apart from the modulation of the major membrane lipids including cholesterol, PE and SM, the disruption of sphingolipid and FA metabolism by FB_1 also seems to play a key role in determining cell survival suggestive of resistance of pre-neoplastic cells [18,31,112]. This became relevant as the low PUFA levels prevailing in cancer cells may play a protective role against cell cytotoxicity associated with a low redox status [22,113]. The low levels of C20:4ω-6 [30] and ceramide [82], known to be key regulators of apoptosis [33,114], is likely to contribute to cellular resistance of HepG2 cells towards FB_1-induced programmed cell death. This study highlighted that the resistance of HepG2 cells towards FB_1 was associated with changes to major membrane constituents including cholesterol, glycerophospho- and sphingolipid and FA, which were similar to changes previously observed in highly proliferating Chang cells [8]. However, in primary hepatocytes, the disruption of these lipid parameters, specifically in lipid rafts, was associated with the induction of a cytotoxic effects [8,112]. Increasing PUFA content in HepG2 cells sensitized these cells towards FB_1 while FB_1 also conferred a level of protection against PUFA-induced cytotoxicity. These counteractive responses of FB_1 against cytotoxicity in PUFA-enriched HepG2 cancer cells provided further evidence regarding potential mechanisms of cancer promotion by FB_1. It could be argued that FB_1, through modulation of oxidative stress and lipid metabolism, may assist cells to acquire cellular resistance which may then no longer respond to the inhibitory effects of FB_1 in the same manner as normal cells would [8,18,31]. This further supported the hypothesis of differential responses between normal and cancer cells, which have been proposed to underlie the cancer promoting activity of FB_1 in the liver [18,31,112].

CRediT authorship contribution statement

Sylvia Riedel: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Stefan Abel:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing. **Hester-Mari Burger:** Writing – review & editing. **Sonja Swanevelder:** Formal analysis, Writing – review & editing. **Wentzel C.A. Gelderblom:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & 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

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