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► To cite this version:

Randa Sghaier, Thomas Nury, Valerio Leoni, Claudio Caccia, Jean-Paul Pais de Barros, et al.. Dimethyl fumarate and monomethyl fumarate attenuate oxidative stress and mitochondrial alterations leading to oxiaoptophagy in 158N murine oligodendrocytes treated with 7 β -hydroxycholesterol. *Journal of Steroid Biochemistry and Molecular Biology*, 2019, 194, pp.105432. 10.1016/j.jsbmb.2019.105432 . hal-03487954

HAL Id: hal-03487954

<https://hal.science/hal-03487954>

Submitted on 20 Jul 2022

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1 **Dimethyl fumarate and monomethyl fumarate attenuate oxidative stress and mitochondrial**
2 **alterations leading to oxiaoptophagy in 158N murine oligodendrocytes treated with 7 β -**
3 **hydroxycholesterol**

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26 the supervision of the study.

27

28 Abstract

29 Oxidative stress and mitochondrial dysfunction contribute to the pathogenesis of neurodegenerative
30 diseases and favor lipid peroxidation, leading to increased levels of 7 β -hydroxycholesterol (7 β -OHC)
31 which induces oxiaoptophagy (OXIdative stress, APOPTOsis, autoPHAGY). The cytoprotective
32 effects of dimethylfumarate (DMF), used in the treatment of relapsing remitting multiple sclerosis and
33 of monomethylfumarate (MMF), its main metabolite, were evaluated on murine oligodendrocytes
34 158N exposed to 7 β -OHC (50 μ M, 24 h) with or without DMF or MMF (25 μ M). The activity of 7 β -
35 OHC in the presence or absence DMF or MMF was evaluated on several parameters: cell adhesion;
36 plasma membrane integrity measured with propidium iodide (PI), trypan blue and fluoresceine
37 diacetate (FDA) assays; LDH activity; antioxidant enzyme activities (superoxide dismutase (SOD),
38 catalase (CAT) and glutathione peroxidase (GPx)); generation of lipid peroxidation products
39 (malondialdehyde (MDA), conjugated dienes (CDs)) and protein oxidation products (carbonylated
40 proteins (CPs)); reactive oxygen species (ROS) overproduction conducted with DHE and DHR123.
41 The effect on mitochondria was determined with complementary criteria: measurement of succinate
42 dehydrogenase activity, evaluation of mitochondrial potential ($\Delta\Psi_m$) and mitochondrial superoxide
43 anions ($O_2^{\bullet-}$) production using DiOC₆(3) and MitoSOX, respectively; quantification of mitochondrial
44 mass with Mitotracker Red, and of cardiolipins and organic acids. The effects on mitochondrial and
45 peroxisomal ultrastructure were determined by transmission electron microscopy. Intracellular sterol
46 and fatty acid profiles were determined. Apoptosis and autophagy were characterized by staining with
47 Hoechst 33342, Giemsa and acridine orange, and with antibodies raised against caspase-3 and LC3.
48 DMF and MMF attenuate 7 β -OHC-induced cytotoxicity: cell growth inhibition; decreased cell
49 viability; mitochondrial dysfunction (decrease of succinate dehydrogenase activity, loss of $\Delta\Psi_m$,
50 increase of mitochondrial $O_2^{\bullet-}$ production, alteration of the tricarboxylic acid (TCA) cycle, and
51 cardiolipin content); oxidative stress induction (ROS overproduction, alteration of GPx, CAT, and
52 SOD activities, increased levels of MDA, CDs, and CPs); changes in fatty acid and cholesterol
53 metabolism; and cell death induction (caspase-3 cleavage, activation of LC3-I in LC3-II).
54 Ultrastructural alterations of mitochondria and peroxisomes were prevented. These results demonstrate
55 that DMF and MMF prevent major dysfunctions associated with neurodegenerative diseases: oxidative
56 stress, mitochondrial dysfunction, apoptosis and autophagy.

57

58 **Keywords:** dimethyl fumarate, monomethyl fumarate, 7 β -hydroxycholesterol, 158N cells, oxidative
59 stress, mitochondria, peroxisome, oxiaoptophagy, apoptosis, autophagy, lipid profile.

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Introduction

Oxidative stress is characterized by an imbalance between excessive production of reactive oxygen species (ROS) and the capacity of antioxidant defenses to attenuate damage caused by free radicals [1]. Oxidative stress is amplified in a variety of neurodegenerative, chronic inflammatory, vascular and neurodegenerative diseases. Thus, several studies have carefully characterized the relationships between oxidative stress and demyelinating and non demyelinating neurodegenerative diseases [2, 3]. Oxidative injury can be involved in brain lesions via the induction of protein and lipid oxidation [4], or mitochondrial dysfunction [5]. In the presence of oxidative stress, the high content of cholesterol in the brain makes it vulnerable to oxidation, which favors the generation of deleterious compounds, named oxysterols (cholesterol oxide products), in the central nerve system (CNS) [6]. Some of these oxysterols (mainly those oxidized on C7, such as 7-ketocholesterol (7KC) and 7 β -hydroxycholesterol (7 β -OHC), mostly generated by the autoxidation of cholesterol [7]) are known to amplify oxidative stress and mitochondrial damage which contributes to cell death [8]. In humans, 7KC and 7 β -OHC levels were increased in the plasma, cerebrospinal fluid (CSF) or postmortem brain tissues of patients with neurodegenerative diseases, including multiple sclerosis (MS) [9], Parkinson's disease [10], Huntington's disease [11] or Alzheimer's disease (AD) [12]. A β peptide, a toxic peptide characteristic of AD, was shown to participate in the production of 7 β -OHC [13]. It is also known that 7KC can be converted to 7 β -OHC [14] by 11 β -hydroxyxteroid dehydrogenase type 1 (11 β -HSD1) [15]. In humans, the 11 β -HSD1 is also able to catalyse the conversion of 7 β -OHC into 7KC [14]. As 7 β -OHC can be formed from 7KC and vice versa, the deleterious effects of both are closely related. It is therefore important to consider the activities of 7KC and 7 β -OHC since these two cytotoxic oxysterols are involved in neurodegeneration [3]. Currently, it has been demonstrated in different cell types from various species [16-19], that 7 β -OHC induces a more or less pronounced apoptotic mode of cell death associated with oxidative stress. Later, a complex cell death process defined as oxiapoptophagy ((OXIdative stress, APOPTOsis, autoPHAGY)) was described on microglial cells and oligodendrocytes [8, 20]. This particular type of cell death involving oxidative stress, mitochondrial dysfunction, and induction of apoptosis and autophagy, which are hallmarks of neurodegenerative diseases, was observed in 7 β -OHC-treated murine oligodendrocytes 158N [8]. The cellular model used in the present study (murine oligodendrocytes 158N treated with 7 β -OHC) is therefore an interesting tool for identifying molecules targeting major cell alterations (oxidative stress and mitochondrial dysfunction leading to cell death) associated with demyelination, since oligodendrocytes are myelin synthesizing cells.

93 To prevent demyelination, different molecules have been tested for their neuroprotective capacities
94 such as dimethyl fumarate (DMF), the active component of Tecfidera (Biogen) also named BG-12
95 [21]. DMF is a fumaric acid ester which has immunomodulatory, anti-inflammatory, and antioxidant
96 properties [22-24]. This small drug has been approved as an oral therapeutic agent for the treatment of
97 patients with relapsing-remitting MS [25, 26]. Many studies have provided that the pharmacological
98 effect of DMF is due to its bioactive metabolite, monomethyl fumarate (MMF) [21, 27]. This
99 metabolite has beneficial effects on neuroinflammation, neurodegeneration and oxidative stress [23,
100 28]. It is supposed that DMF and MMF act by the activation of Nrf2 (nuclear factor E2-related factor2)
101 pathway, through the induction of antioxidant protein expression [22]. Under physiological conditions,
102 Nrf2 is sequestered in the cytoplasm through its interaction with the repressor protein 'kelch-like ECH-
103 associated protein 1' (Keap1). Treatment with compounds, like DMF, results in the conformational
104 modifications of cysteine residues on Keap1 [28], which allows activation and nuclear translocation of
105 Nrf2 to promote protective genes such as heme-oxygenase-1 [22] and NADPH-quinone
106 oxidoreductase-1 [28].

107 In the present study, we evaluated the cytoprotective effects of DMF and MMF on 7 β -OHC-treated
108 murine oligodendrocytes 158N. We asked whether DMF and MMF were able to prevent or reduce 7 β -
109 OHC-induced cytotoxicity (oxidative stress, mitochondrial dysfunction, modification of lipid profile,
110 apoptosis and autophagy) accompanying demyelination in MS. In addition, as 158N cells express
111 major myelin proteins, proteolipid protein (PLP) and myelin basic protein (MBP) [29], which are
112 major compounds of myelin (a proteolipidic complex which wraps around the axons and is essential
113 for the transmission of nerve impulse) [30], the effects of 7 β -OHC in the presence or absence DMF
114 and MMF on PLP and MBP expression were also evaluated.

115

116 2. Material and methods

117 2.1. Cell culture and treatments

118 Murine oligodendrocyte cells (158N) [29] were cultured in Dulbecco's Modified Eagle Medium
119 (DMEM) (Lonza, Amboise, France) containing 5% (v/v) heat-inactivated fetal bovine serum (FBS)
120 (Dutscher, Brumath, France) and 1% antibiotics (penicillin, streptomycin) (Dutscher). Cells were
121 incubated in a humidified atmosphere (5% CO₂, 95% air) at 37°C and passaged twice a week.

122 The stock solutions of 7 β -hydroxycholesterol (7 β -OHC) (Sigma-Aldrich, St Quentin Fallavier, France)
123 was prepared at 800 μ g/mL (2 mM) as previously described [19] and stored at 4°C. Dimethyl fumarate
124 (DMF) (ref: 242926, Sigma-Aldrich) and monomethyl fumarate (MMF) (ref: 651419; Sigma-Aldrich)
125 stock solutions, were prepared at 50 mM in DMSO (Sigma-Aldrich). α -Tocopherol (the major

126 component of Vitamin E; ref: T3251, Sigma-Aldrich) solution was prepared at 80 mM as previously
127 described [19].

128 In the different experimental conditions, murine oligodendrocytes 158N were seeded either in Petri
129 dishes (30,000 cells/cm², 100 mm diameter), or in six-well plates (2.4 x 10⁵ cells per well). After 24 h
130 of culture, and for an additional 24 h period of time, DMF (25 μM), MMF (25 μM) or α-tocopherol
131 (400 μM) (used as a positive control for cytoprotection) were introduced in the culture medium 2 h
132 before the addition of 7β-OHC (50 μM). The choice of the concentrations of 7β-OHC (50 μM) as well
133 as of DMF (25 μM) and MMF (25 μM) was based on a study by Ragot et al. [19] and on the dose-
134 effect relationship of 7β-OHC (1, 6.25, 12.5, 25, 50, 100 and 200 μM) (Supplementary Fig. 1) and of
135 DMF or MMF in the presence or absence of 7β-OHC (Supplementary Fig. 2). With 7β-OHC, the
136 concentration of 50 μM corresponds to the 50 % inhibiting concentration on 158N cells
137 (Supplementary Fig. 1). In vitro, it is important to underline that only a part of oxysterols (1-35%
138 depending on the cell type considered) accumulates in the cells [3, 31]. It has been established that the
139 median level of 7β-OHC was 1.11 μg/mL (2.8 μM) and 1.43 μg/mL (3.6 μM) in the cerebrospinal
140 fluid of normal subjects and of patients with multiple sclerosis, respectively [9]. Few data are available
141 on the intracellular level of oxysterols in nerve cells. However, when the oxysterols such as 7β-OHC
142 are used in vitro at μM concentrations (50 μM in the present study), the estimated intracellular levels
143 of this compound are in the range of those found in the frontal and occipital cortex of patients with
144 Alzheimer's disease [12]. Additional experiments were also performed to assess the effect of DMF and
145 MMF in the recovery of 7β-OHC (50 μM)-induced cell death. In these experiments, after 24 h of
146 culture, 158N cells were incubated with 7β-OHC for 6 and 10 h before the addition of DMF or MMF,
147 or α-tocopherol. The cells were further incubated with 7β-OHC in combination with DMF, MMF or α-
148 tocopherol for an additional 14 or 18 h.

149

150 2.2. Evaluation of cell morphology by phase contrast microscopy

151 After treatment of 158N cells with or without 7β-OHC (50 μM) in the presence or absence of DMF
152 (25 μM), MMF (25 μM) or α-tocopherol (400 μM), cell morphology, cell density and cell detachment
153 (loss of cell adhesion) were evaluated by phase contrast microscopy using an inverted-phase contrast
154 microscope (Axiovert 40 CFL, Zeiss, Jena, Germany). Images were obtained with a camera (AxioCam
155 ICm 1, Zeiss).

156

157 2.3. Quantification of adherent cells with crystal violet staining

158 Adherent cells were estimated by staining with crystal violet (Sigma-Aldrich). After treatment of 158N
159 cells, adherent cells were stained with crystal violet for 5 min, rinsed three times with water. Crystal

160 violet was then dissolved with 0.1 % sodium citrate (Sigma-Aldrich) in 50% ethanol (Carlo-Erba, Val
161 de Reuil, France) and the optical density per well was read at 570 nm with a microplate reader (Tecan
162 Sunrise, Tecan, Lyon, France).

163

164 **2.4. Evaluation of cell viability with the fluorescein diacetate (FDA) assay**

165 The FDA assay evaluates the ability of living cells to transform the FDA to fluorescein after cleavage
166 by plasma membrane esterases [32]. After 24 h of treatment with or without 7 β -OHC (50 μ M) in the
167 presence or absence of DMF or MMF (25 μ M), 158N cells were incubated for 5 min at 37°C with 50
168 μ M FDA (Sigma-Aldrich), and then lysed with 10 mM of Tris-HCl solution containing 1% sodium
169 dodecyl sulfate (SDS, Sigma-Aldrich). Fluorescence intensity of fluorescein (λ_{Ex} Max: 485 nm, λ_{Em}
170 Max: 528 nm) was measured with a TECAN fluorescence microplate reader (Tecan Infinite M 200 Pro,
171 Lyon, France).

172

173 **2.5. Cell counting of viable cells with trypan blue**

174 Adherent and non-adherent cells were collected by trypsinization and resuspended in culture medium.
175 The total number of viable cells was determined with a Mallasez counting chamber under a light
176 microscope in the presence of trypan blue (v/v), which stains dead cells in blue.

177

178 **2.6. Evaluation of plasma membrane permeability and cell death by staining with propidium** 179 **iodide**

180 Propidium iodide (PI) was used to quantify plasma membrane permeability and cell death [33]. At the
181 end of treatment with or without 7 β -OHC (50 μ M) in the presence or absence of DMF (25 μ M), MMF
182 (25 μ M) or α -tocopherol (400 μ M). 158N cells (adherent and non-adherent cells) collected by
183 trypsinization, were stained with 1 μ g/mL of PI (5 min at 37°C) and then immediately analyzed with a
184 Galaxy flow cytometer (Partec, Münster, Germany). Fluorescence was collected through a 590/10
185 bandpass filter on a Galaxy flow cytometer (Partec): 10,000 cells were acquired. Data were analyzed
186 with Flomax (Partec) or FlowJo (Tree Star Inc.) software.

187

188 **2.7. Mitochondrial analysis**

189 **2.7.1. Measurement of mitochondrial succinate dehydrogenase activity with the MTT assay**

190 Mitochondrial activity and/or cell proliferation were evaluated with an MTT based assay. Only
191 metabolically active cells can transform MTT into formazan by mitochondrial succinate
192 dehydrogenase. MTT data was interpreted by comparison with results obtained by cell counting in the

193 presence of trypan blue. The MTT assay was carried as described previously [34]. The absorbance was
194 read at 570 nm with a microplate reader (TECAN Sunrise, Tecan, Lyon, France).

195

196 **2.7.2. Measurement of mitochondrial transmembrane potential with DiOC₆(3)**

197 The mitochondrial transmembrane potential ($\Delta\Psi_m$) was assessed by flow cytometry with the cationic
198 lipophilic dye 3,3'-dihexyloxacarbocyanine iodide DiOC₆(3) (Thermo Fisher Scientific, Courtaboeuf,
199 France). At the end of treatment, adherent and non-adherent cells were pooled, incubated with
200 DiOC₆(3) (40 nM, 15 min, 37°C) and then analyzed with a Galaxy flow cytometer (Partec) [19, 35].

201 The loss of transmembrane mitochondrial potential ($\Delta\Psi_m$) is indicated by a decreased of the green
202 fluorescence collected through a 520 ± 10 nm band pass filter. For each sample, 10,000 cells were
203 acquired and data were analyzed with Flomax (Partec) or FlowJo (Tree Star Inc.) software.

204

205 **2.7.3. Measurement of mitochondrial mass with Mitotracker Red**

206 Mitotracker Red (Thermo Fisher Scientific) was used to evaluate mitochondrial mass [36]. Mitotracker
207 Red stock solution was prepared at 1 mM in DMSO. Adherent and non-adherent cells were pooled,
208 washed with DMEM without FBS and then incubated for 30 min at 37°C in a Mitotracker Red solution
209 (100 nM), which was prepared in DMEM without FBS. The fluorescent signals were collected through
210 a 580 ± 20 nm band pass filter on a Galaxy flow cytometer (Partec). Data were analyzed with Flomax
211 software (Partec).

212

213 **2.7.4. Measurement of mitochondrial reactive oxygen species production with MitoSOX-Red**

214 Mitochondrial production of superoxide anion ($O_2^{\bullet-}$) was quantified by flow cytometry after
215 staining with MitoSOX Red (Thermo Fisher Scientific). This positively charged probe accumulates in
216 the mitochondria [36] and exhibits an orange/red fluorescence ($\lambda_{Ex} Max = 510$; $\lambda_{Em} Max = 580$ nm).
217 MitoSOX Red stock solution was prepared at 5 mM in PBS and used at 5 μ M in the cells. After cell
218 treatments, adherent and non-adherent cells were pooled by trypsinization and incubated with
219 MitoSOX Red for 15 min at 37°C. The fluorescent signals were collected through a 580 ± 20 nm band
220 pass filter on a Galaxy flow cytometer (Partec). For each sample, 10,000 cells were acquired. Data
221 were analyzed with Flomax software (Partec).

222

223 **2.7.5. Measurement of mitochondrial cardiolipin levels**

224 The characterisation and quantification of cardiolipins was conducted by liquid chromatography
225 tandem-mass spectrometry (LC-MS/MS) on an Agilent 6460 QqQ (Agilent Technologies, Santa Clara,
226 CA, USA). Total lipid was extracted from cell pellet as described previously by Folch et al. [37], then

227 dissolved in 100 μ L of chloroform/methanol/water mixture. About 1 to 8 μ L of extracted lipid were
228 used for lipidomic analysis. Lipid standards and chemicals of the highest grade available were from
229 Avanti Polar Lipids (Coger SAS, Paris, France) and Sigma-Aldrich, respectively. LC-MS/MS quality
230 grade solvents were from Thermo Fischer Scientific. Extracted lipids for cardiolipin analysis were
231 determined as described previously by Vial et al. [38].

232

233 **2.8. Mass spectrometry analysis of sterols, fatty acids phospholipids and organic acids**

234 Total lipid was extracted by the Folch's method [37], and fatty acids, sterols and organic acids were
235 quantified as described previously [31, 39, 40]. Gas chromatography-mass spectrometry (GC-MS)
236 analysis was performed on a Clarus 600D (Perkin Elmer, USA). The GC was equipped with an Elite
237 column (30 m x 0.32 mm id x 0.25 mm film; Perkin Elmer) and injection was performed in splitless
238 mode using helium (1 mL/min) as carrier gas. The temperature program was as follows: initial
239 temperature of 80°C was held for 1 min, followed by a linear ramp of 10°C/min to 220°C, 20°C/min to
240 of 280°C and 5°C/min up to 290°C, which was held for 10 min. The mass spectrometer operates in full
241 mass scan or selected ion-monitoring mode. Peak integration was performed manually, and
242 metabolites were quantified from selected-ion monitoring analyses against internal standards using
243 standard curves for all the compounds [39, 40]. For phospholipid analysis, total lipid was also
244 extracted by the Folch's method [37]; phosphatidylcholine and sphingomyelin were quantified as
245 described previously [19].

246

247 **2.9. Evaluation of oxidative stress**

248 **2.9.1. Analysis of antioxidant enzymes activities: catalase, glutathione peroxidase (GPx) and** 249 **superoxide dismutase (SOD)**

250 Catalase (CAT) activity was determined by photometric measurement of H₂O₂ consumption at 240 nm.
251 158N cells were trypsinized, washed with PBS, and lysed in RIPA buffer (10 mM Tris-HCl, pH 7.2,
252 150 mM NaCl, 0.5% Nonidet NP40, 0.5% Na deoxycholate, 0.1% SDS, 2 mM EDTA and 50 mM
253 NaF) in the presence of 1/25 complete protease inhibitor cocktail tablets (Roche Diagnostics,
254 Indianapolis, IN, USA) for 30 min on ice. Cell lysates were collected after centrifugation at 12,000 g
255 for 20 min at 4°C. The reaction was initiated by the addition of Tris-HCL (1 M, pH 7.4), and H₂O₂
256 (400 mM), and the amount of H₂O₂ remaining after 2 min was measured spectrophotometrically (240
257 nm), using a microplate reader (Tecan Infinite M 200 Pro).

258 Glutathione peroxidase (GPx) activity was quantified as previously described [41]. 158N cells were
259 lysed by sonication and centrifuged at 20,000 g for 30 min at 4°C. Cell lysates were incubated for 5
260 min at 25°C with 0.1 mM reduced glutathione (GSH) and phosphate buffer saline (50 mM, pH 7.8).

261 The reaction mixture was initiated by addition of H₂O₂ and stopped by the incubation with 1%
262 trichloric acid, for 30 min at 4°C, and then centrifuged at 1,000g for 10 min. The absorbance was
263 measured at 412 nm (Lambda 25 Spectrophotometer, Perkin Elmer, Villebon sur Yvette, France).

264 Superoxide dismutase (SOD) activity was quantified using the method of Misra and Fridovich [42].
265 This colorimetric assay, evaluates the ability of SOD to inhibit the Cytochrome c-reduction, induced
266 by superoxide anions produced by xanthine oxidase enzyme [34]. To this end, cell lysates were added
267 to potassium phosphate buffer (0.05 M, pH 7), and 50 µM xanthine (ref 69-89-6, Sigma-Aldrich).
268 Then 0.01U of xanthine oxidase (ref 9002-17-9, Sigma–Aldrich) and 5 µM of cytochrome c were
269 added. Absorbance was measured at 550 nm at 20 min intervals for at least five reading. Antioxidant
270 enzyme activities were expressed relative to the protein content determined with the Bradford method.

271

272 **2.9.2. Measurement of thiol (SH group) levels**

273 Measurement of thiols (SH group) was conducted according to the method of Flaure & Lafon [43].
274 Cellular lysates were mixed with 5-5’DiThiobs-(2-NitroBenzoic acid) (DTNB), and incubated in dark
275 for 15 min. The absorbance was measured at 415 nm on a Lambda 25 Spectrophotometer (Perkin Elmer).

276

277 **2.9.3. Measurement of reactive oxygen species (ROS) overproduction with dihydroethidium and** 278 **dihydrorhodamine 123**

279 ROS overproduction, including superoxide anion (O₂^{•-}), was quantified by flow cytometry after
280 staining with dihydroethidium (DHE; Thermo Fisher Scientific) used at 2 µM. Once in the cell, DHE
281 is rapidly oxidized and exhibits an orange fluorescence ($\lambda_{\text{Ex Max}} = 488 \text{ nm}$; $\lambda_{\text{Em Max}} = 575 \text{ nm}$) [44].

282 ROS overproduction, including hydrogen peroxide (H₂O₂), was also detected with dihydrorhodamine
283 123 (DHR 123, Cliniscience, Nanterre, France) used at 6 µM, which is oxidized to Rhodamine 123
284 (RH123) ($\lambda_{\text{Ex Max}} = 505 \text{ nm}$; $\lambda_{\text{Em Max}} = 529 \text{ nm}$) [45]. After 24 h of treatment, adherent and non-
285 adherent cells were stained with DHE or DHR123 and incubated at 37°C for 15 min. Fluorescent
286 signals were collected with 590 ± 20 nm (DHE) and 520 ± 20 nm (DHR 123) band pass filters,
287 respectively. Ten thousand cells were acquired from each sample. Data were analyzed with Flomax
288 (Partec) or FlowJo (Tree Star Inc.) software.

289

290 **2.9.4. Measurement of lipid peroxidation products: conjugated dienes (CDs) and** 291 **malondialdehyde (MDA)**

292 Conjugated dienes (CDs) are the products of polyunsaturated fatty acid peroxidation. The level of CDs
293 was determined with the method of Esterbauer et al. [46]. Malondialdehyde (MDA) is a lipid
294 peroxidation product. It is an alkylating agent capable of reacting with biologic macromolecules. The

295 level of MDA was evaluated as previously described [47]. The experimental procedures on 158N cells
296 were performed as described previously [31].

297

298 **2.9.5. Measurement of carbonylated proteins (CPs) level**

299 Similarly than lipids, proteins can also be oxidized. The measurement of carbonylated proteins (CPs)
300 level was conducted as described by Oliver et al. [48]. This method, which is based on the reaction of
301 2, 4-dinitrophenylhydrazine (DNPH) with CP groups to form protein hydrazones, was realized as
302 previously described [31].

303

304 **2.10. Cell death characterization**

305 **2.10.1. Evaluation of cell death by the measurement of lactate dehydrogenase activity**

306 The culture medium of 158N cells treated with or without 7 β -OHC (50 μ M) in combination with with
307 DMF (25 μ M), MMF (25 μ M) or α -tocopherol (400 μ M) were collected, and centrifuged to eliminate
308 non adherent cells. Lactate dehydrogenase (LDH) activity was quantified using a commercial kit
309 (#ab102526, Abcam, Paris, France). A microplate reader (TECAN Infinite M 200 Pro) was used to
310 measure LDH activity at a wavelength of 450 nm in a kinetic mode at 25°C for 30-60 min.

311

312 **2.10.2 Characterization of cell death by the evaluation of nuclear morphology after staining with** 313 **Giemsa or H \ddot{o} chst 33342**

314 Normal and apoptotic versus necrotic cells can be identified by nuclear morphology evaluated after
315 staining with Giemsa and/or H \ddot{o} chst 33342 as described previously [33]. Apoptotic cells were
316 characterized by condensed and/or fragmented nuclei whereas normal cells have regular and round
317 nuclei. A total of 300 cells per sample were counted to determine the percentage of apoptotic cells.

318

319 **2.10.3. Measurement of lysosomal destabilization with Acridine Orange**

320 Acridine orange (AO) was used to evaluate lysosomal integrity [49] and or to quantify autophagic
321 vesicular (acidic vesicular) formation [50-52]. AO stock solution was prepared at 1 mg/mL in DMSO
322 and used at 1 μ g/mL. After 15 min of incubation at 37°C, cells were washed, and resuspended in PBS
323 for flow cytometry analysis. The fluorescence of AO was collected using a 630 nm longpass filter and
324 measured on a logarithmic scale. Ten thousand cells were acquired on a Galaxy flow cytometer
325 (Partec). Data were analyzed with Flomax (Partec) or FlowJo (Tree Star Inc.) software.

326

327 **2.10.4. Polyacrylamide gel electrophoresis and Western blotting**

328 Adherent and floating cells were harvested and lysed for 30 min on ice in a RIPA buffer (10 mM Tris-
329 HCl, pH 7.2, 150 mM NaCl, 0.5% Nonidet NP40, 0.5% Na deoxycholate, 0.1% SDS, 2 mM EDTA
330 and 50 mM NaF) containing a complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis,
331 IN, USA). The cell debris were eliminated by centrifugation (20 min, 10,000 g), and the supernatant
332 was collected. The protein concentrations were measured using bicinchoninic acid reagent (Sigma
333 Aldrich). Seventy micrograms of protein were diluted in a loading buffer (125 mM Tris-HCl, pH 6.8,
334 10% β -mercaptoethanol, 4.6% SDS, 20% glycerol, and 0.003% bromophenol blue), separated on a
335 SDS-PAGE gel, and transferred onto a nitrocellulose membrane (Thermo Fischer Scientific).
336 Nonspecific binding sites were blocked by incubation with 5% milk powder in PBST (PBS, 0.1%
337 Tween 20, pH 7.2) and the membranes were incubated overnight at 4°C with the primary antibody
338 diluted in 5% milk in PBST. For apoptosis and autophagy analysis, the antibodies used were directed
339 against caspase-3 (Ozyme / Cell Signaling (# 9662) (for detection of the endogenous level of full
340 length caspase-3 (35 kDa) and its large cleavage product (17 kDa)), and LC3-I/II (#L8918, Sigma
341 Aldrich) (detecting LC3-I (18 kDa) and LC3-II (16 kDa)), respectively; all antibodies were rabbit
342 polyclonal antibodies; they were used at a final dilution of 1/1000. For myelin protein analysis
343 (proteolipid protein (PLP); myelin basic protein (MBP)), PLP expression was determined with a rabbit
344 polyclonal antibody (PLP; Novus, NB10074503) and MBP expression with a mouse monoclonal
345 antibody (MBP; Millipore, MAB381). An antibody directed against β -actin (mouse monoclonal
346 antibody (#A2228, Sigma Aldrich)) was used at a final concentration of 1/10,000. Membranes were
347 washed three times for 5 min, with PBST and incubated for 1 h at room temperature with horseradish
348 peroxidase-conjugated goat anti-rabbit (for caspase-3, LC3-I/-II, and PLP) diluted at 1/5,000 in 1%
349 milk powder in PBST or anti-mouse antibody against MBP or β -actin, diluted at 1/5,000 in 1% milk
350 powder in PBST. The membranes were then washed and antibody binding revealed using an enhanced
351 chemiluminescence detection kit (Supersignal West Femto Maximum Sensitivity Substrate, Thermo
352 Fisher Scientific) and Chemidoc XRS+ (Bio-Rad, Marnes la Coquette, France). Band intensity was
353 determined with Image Lab software (Bio-Rad). The expression of caspase-3 (uncleaved and cleaved)
354 and the ratio LC3-II / LC3-I were calculated with Image Lab software (Bio-Rad). The expression of
355 caspase-3 and the level of cleaved caspase-3 were normalized versus β -actin, and expressed as
356 normalized expression versus control (untreated cells). The ratio LC3-II / LC3-I was calculated for
357 each assay.

358

359 **2.10.5. Evaluation of the activation of PKA, PKC and MEK / ERK signalling pathways in 7 β -** 360 **hydroxycholesterol-treated 158N cells**

361 To evaluate the signalling pathways activated during 7 β -OHC-induced cell death, different inhibitors
 362 were used. To this end, 158N cells were seeded in 24 wells at 60,000 cells / well. After 24 h of culture,
 363 158N cells were cultured in the presence or absence of 7 β -OHC (50 μ M) with or without different
 364 inhibitors such as H89 (20 μ M, Sigma-Aldrich), U73122 (1 μ M, Sigma-Aldrich), chelerythrine (1 μ M,
 365 Sigma-Aldrich), and U0126 (20 μ M, Calbiochem, San Diego, CA, USA), which inhibit protein kinase
 366 A (PKA), phospholipase C (PLC), protein kinase C (PKC) and MEK, respectively. **These inhibitors**
 367 **were chosen for the following reasons: cAMP level leading to PKA activation is increased during**
 368 **during 7 β -OHC-induced cell death; a cytosolic Ca²⁺ increase contributing to PLC activation is**
 369 **observed during 7 β -OHC-induced cell death; and 7 β -OHC-induced cell death is associated with**
 370 **inflammatory cytokines overproduction as well as enhanced expression of integrins via the activation**
 371 **of the nuclear factor κ B (NF- κ B) involving PKC and MEK-ERK signalling pathways [16, 18].** These
 372 compounds were introduced in culture medium 30 min before the 7 β -OHC. Stock solutions of these
 373 inhibitors were prepared as follow: the H89 stock solution was prepared in distilled water at 1 mM,
 374 while U0126 (0.1 mM), U73122 (0.1 mM) and chelerythrine (1 mM) stock solutions were prepared in
 375 DMSO. At the end of the treatments, an FDA assay was carried out as described previously.

376

377 **2.11. Quantification of 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) and 11 β -** 378 **hydroxysteroid dehydrogenase type 2 (11 β -HSD2) by RT-qPCR**

379 11 β -HSD1 is an intracellular enzyme that catalyzes interconversion of cortisone in cortisol [53]. 11 β -
 380 HSD1 also catalyses the conversion of 7KC to 7 β -OHC and reciprocally [54, 55]. However, the
 381 conversion of 7 β -OHC to 7KC can also be catalyzed by 11 β -HSD2 [56]. Total mRNA from 158N
 382 cells (and microglial BV-2 cells used as positive control [20]) were extracted and purified using the
 383 RNeasy Mini Kit (Qiagen, Courtaboeuf, France). Total mRNA concentration was measured with
 384 TrayCell (Hellma, Paris, France). The purity of nucleic acids was controlled by the ratio of absorbance
 385 at 260 nm and 280 nm (ratios of 1.8 - 2.2 were considered satisfactory). One microgram of total
 386 mRNA was used for reverse transcription with the iScript cDNA Synthesis Kit (Bio-Rad) according to
 387 the following protocol: 5 min at 25°C, 20 min at 46°C, 5 min at 95°C. cDNA was amplified using the
 388 FG Power SYBR Green (Thermo Fischer Scientific). All PCR reactions were performed on an Applied
 389 Biosystem Step One plus QPCR machine (Life Science Technologies). The primer sequence of 11 β -
 390 *HSD1* was:

391 ❖ 11 β -HSD1: forward 5'-actcagacctcgctgtctct-3' and reverse 5'-gcttgacagtagggagcaa-3'

392 ❖ 11 β -HSD2: forward 5'-ggttgtgacactggtttggc-3' and reverse 5'-agaacacggctgatgcctct-3'

393 Thermal cycling conditions were as follows: activation of DNA polymerase at 95°C for 10 min,
 394 followed by 40 cycles of amplification at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s, followed by

395 a melting curve analysis to test for the absence of non-specific products. Gene expression was
396 quantified using cycle to threshold (Ct) values and normalized by the *36B4* reference gene (*forward 5'-*
397 *gcgacctggaagtccaacta-3'* and *reverse 5'-atctgcttgagcccacat-3'*). The quantitative expressions of *11β-*
398 *HSD1* and *11β-HSD2* were determined as fold induction above the control.

399

400 **2.12. Statistical analysis**

401 Statistical analyses were performed using the Statview Software (SAS Institut Inc, NC, USA) using a
402 one way or a two way analysis of variance (ANOVA) followed by Student's t-test. Data shown are the
403 mean ± standard deviation (SD) of two or three independent experiments, usually conducted in
404 triplicate. Data were considered statistically significant at a P-value of 0.05 or less.

405

406 **3. Results**

407 **3.1. Effects of dimethyl fumarate and monomethyl fumarate on the adhesion and morphology of** 408 **158N cells**

409 158N cells treated with 7β-OHC are a relevant model to evaluate the relationship between oxidative
410 stress, apoptosis and autophagy (oxiaptophagy); to specify the role played by mitochondria, in this
411 process; and to identify natural or synthetic molecules capable of preventing the toxic effects of 7β-
412 OHC associated with major age-related and neurodegenerative diseases. To evaluate the cytoprotective
413 activity of DMF and MMF on 7β-OHC-treated 158N cells, these cells were cultured for 24 h with or
414 without 7β-OHC (50 μM, 24 h) in the presence or absence of DMF and MMF, used at 25 μM. α-
415 tocopherol (400 μM) was chosen as positive cytoprotective control. The effects of DMF and MMF
416 were evaluated compared to untreated cells (control) and vehicle-treated cells (ethanol 0.6% or DMSO
417 0.05%).

418 Based on the observations performed by phase contrast microscopy, morphological changes were
419 identified under treatment with 7β-OHC: a pronounced increase of the number of round and floating
420 cells, indicating of loss of cell adhesion and an induction of cell death, was observed; whereas only
421 few non adherent cells were present in control and vehicle-treated cells (**Fig 1A**).Of note, when 7β-
422 OHC was associated with DMF, MMF or α-tocopherol, the loss of cell adhesion was prevented and an
423 important decreases of the number of non adherent cells was observed. (**Fig 1A**).

424 The impact of DMF and MMF on cell adhesion was confirmed by the crystal violet assay. Under
425 treatment with 7β-OHC and compared to control and vehicle-treated cells, a significant decrease of
426 adherent cells was revealed (**Fig. 1B**). When 7β-OHC was associated with DMF and MMF the
427 percentage of adherent cells was significantly increased: DMF and MMF increased by 30-32% the

428 percentage of adherent cells, respectively. This effect is comparable to those observed with α -
429 tocopherol (**Fig. 1B**).

430 431 **3.2. Effects of dimethyl fumarate and monomethyl fumarate on plasma membrane integrity and** 432 **cell viability**

433 The impact of DMF and MMF on 7β -OHC-induced loss of plasma membrane integrity and on cell
434 death induction was determined on 158N cells. To this end, 158N cells were stained either with
435 fluoresceine diacetate (FDA), propidium iodide (PI) or trypan blue, and in addition LDH was
436 measured in the culture medium.

437 After staining with FDA, a significant decrease of FDA positive cells (FDA+ cells) was observed in
438 7β -OHC-treated cells compared to the corresponding vehicle and to the control (**Fig. 2A**). With the
439 trypan blue assay, a significant decrease of the total number of living cells was simultaneously
440 observed with 7β -OHC. Compared to 7β -OHC, co-treatment with DMF or MMF induced a significant
441 increase of almost 40% of (FDA+ cells) as well as an increase of the total number of living cells
442 evaluated after staining with trypan blue. More pronounced cytoprotective effects were seen with α -
443 tocopherol (**Fig. 2A-B**).

444 After staining with PI, in the presence of 7β -OHC, more than 70% of propidium iodide positive cells
445 (PI +) were observed, indicating an increased permeability of the plasma membrane to PI which stains
446 cells with damaged plasma membranes, often corresponding to dead cells (**Fig. 2C**). The increase of
447 permeability to PI was strongly attenuated by DMF and MMF (**Fig. 2C**).

448 In addition, the quantification of LDH activity in the culture medium, which is a criteria of cell death,
449 showed an increased LDH activity in 7β -OHC-treated cells (**Fig. 2D**). This increase was strongly
450 attenuated by DMF and MMF; in the culture medium of (7β -OHC + DMF) or (7β -OHC + MMF), the
451 LDH activity was only slightly higher than in control and vehicle-treated cells (**Fig. 2D**).

452 Of note, the ability of DMF and MMF to prevent the decrease of FDA+ cells and of living cells, as
453 well as the increase of PI+ cells was similar to that observed with α -tocopherol (**Fig. 2**).

454 455 **3.3. Effects of dimethyl fumarate and monomethyl fumarate on 7β -hydroxycholesterol-induced** 456 **oxidative stress**

457 To determine the ability of DMF and MMF to prevent 7β -OHC-induced oxidative stress, we studied
458 the impact of these fumarate esters on the production of reactive oxygen species (ROS: $O_2^{\bullet-}$, H_2O_2),
459 antioxidant enzyme (SOD, CAT, GPx) activities, and macromolecule oxidation products (MDA,
460 carbonylated proteins) as well as on the level of SH-groups and conjugated dienes (CDs).

461 The measurement of ROS production was evaluated by flow cytometry after staining with DHE and
462 DHR123. The highest percentage of DHE and DHR123 positive cells was observed when 158N cells
463 were exposed to 7 β -OHC compared to control and vehicle-treated cells. This increase was significantly
464 inhibited when 7 β -OHC was associated with DMF and MMF (**Fig. 3**). The most important attenuation
465 of 7 β -OHC-induced ROS overproduction was observed with α -tocopherol used as positive control
466 anti-oxidant molecule (**Fig. 3**).

467 Under treatment with 7 β -OHC, a significant increase of GPx, SOD, and CAT activities was observed
468 compared to control and vehicle-treated cells (**Fig. 4A-C**). Compared to 7 β -OHC-treated cells, GPx,
469 SOD and CAT activities were significantly increased when 7 β -OHC was associated with DMF or
470 MMF, and the most important increase was observed with DMF (**Fig. 4A-C**). In the presence of α -
471 tocopherol, the effects were similar to those of DMF and MMF. On the otherhand, a significant
472 decrease in the level of SH-groups was observed in 7 β -OHC-treated cells compared to control and
473 vehicle-treated cells (**Fig. 4D**). Of note, the level of SH-groups was almost normalized, and in the range
474 of that measured in control and vehicle-treated cells, when 7 β -OHC was associated with DMF, MMF
475 and α -tocopherol (**Fig. 4D**). With biochemical colorimetric methods of analysis, allowing evaluation of
476 lipid and protein oxidation products, a significant increase of CDs, MDA, and carbonylated proteins
477 levels (CPs) which are biomarkers of lipid and protein oxidation, was observed when 158N cells were
478 exposed to 7 β -OHC compared to control and vehicle-treated cells (**Fig. 5A-C**). Interestingly, this
479 increase was significantly attenuated when 7 β -OHC was associated with DMF and MMF (**Fig. 5A-C**).
480 Similar effects than DMF and MMF were found in the presence of α -tocopherol (**Fig. 5**).

481

482 **3.4. Effects of dimethyl fumarate and monomethyl fumarate on 7 β -hydroxycholesterol-induced**

483 **mitochondrial damage and peroxisomal changes**

484 As mitochondrial damage is induced by 7 β -OHC in 158N cells, the impact of DMF and MMF at the
485 mitochondrial level was studied. To this end, succinate dehydrogenase activity, transmembrane
486 mitochondrial potential ($\Delta\Psi_m$), mitochondrial mass, superoxide anions ($O_2^{\bullet-}$) production, and
487 cardiolipin levels were evaluated.

488 The MTT assay, used to measure succinate dehydrogenase activity, showed that the ratio [(% MTT
489 positive cells in the assay) / (% MTT positive cells in the control)] was significantly reduced with 7 β -
490 OHC compared to control and ethanol-treated cells (**Fig. 6A**). When 7 β -OHC was associated with
491 DMF and MMF this ratio was significantly increased. A similar effect was observed with α -
492 tocopherol. These data showed that DMF and its main metabolite MMF prevent the decrease of
493 succinate dehydrogenase activity which participates in both the TCA cycle and the electron transport
494 chain at the mitochondrial level. These data lead us to examine the effect of DMF and MMF on 7 β -

495 OHC-induced loss of transmembrane mitochondrial potential ($\Delta\Psi_m$) on 158N cells by staining with
496 DiOC₆(3). A high value (33%) of DiOC₆(3) negative cells, indicating a decrease in $\Delta\Psi_m$ (cells with
497 depolarized mitochondria) was detected under treatment with 7 β -OHC (**Fig. 6B**). The percentage of
498 DiOC₆(3) negative cells was strongly reduced when 158N cells were co-treated with DMF, MMF or α -
499 tocopherol (**Fig. 6B**). Another fluorochrome (MitoTracker Red) was used to evaluate the
500 mitochondrial mass. Upon staining with Mitotracker Red, an increase in the mean fluorescence
501 intensity (MFI) of this dye was observed when the cells were treated with 7 β -OHC compared to
502 control and vehicle-treated cells (**Fig. 6C**). This increase was significantly inhibited by DMF and
503 MMF, as well as by α -tocopherol (**Fig 6C**).

504 Due to the important role played by cardiolipins, which are essential mitochondrial phospholipids for
505 electron transport, oxidative phosphorylation and energy production [57, 58], it was important to
506 determine the effect of 7 β -OHC on the cardiolipin content. No difference in cardiolipin content was
507 observed between untreated cells (control) and vehicle-treated cells (**Fig. 6D**). The highest levels of
508 cardiolipins was found with DMF and MMF (**Fig. 6D**). Total cardiolipin content was significantly
509 decreased in 7 β -OHC-treated cells compared to control and vehicles-treated cells (**Fig. 6D**), When 7 β -
510 OHC was associated with DMF and MMF, this decrease was counteracted and the cardiolipin values
511 observed were in the range of that observed in control and vehicle-treated cells (**Fig. 6D**). The same
512 observation was made with α -tocopherol (**Fig. 6D**).

513 In addition, flow cytometric analyses were performed with MitoSOX allowing the measurement of
514 O₂^{•-} production at the mitochondrial level (MitoSOX positive cells). In cells incubated with 7 β -OHC
515 compared control and to vehicle-treated cells an overproduction of O₂^{•-} was observed. This
516 overproduction of O₂^{•-} was counteracted by DMF, MMF and α -tocopherol (**Fig. 6E**).

517 As mitochondria and peroxisome are tightly connected organelles [59], the effects of 7 β -OHC on the
518 peroxisome was evaluated by transmission electron microscopy (**Fig. 7**). Whereas round peroxisomes
519 were mainly observed in control cells, several peroxisomes with irregular shapes, which were often
520 larger or smaller than in the control, were detected in 7 β -OHC-treated cells (**Fig. 7**). Several
521 mitochondria, which were smaller than in control cells, were also frequently observed in 7 β -OHC-
522 treated cells (**Fig. 7**). Of note, these modifications of the peroxisomal and mitochondrial morphologies
523 were prevented when 7 β -OHC was associated with DMF (**Fig. 7**).

524 Altogether, our data demonstrate that 7 β -OHC induced several types of mitochondrial damage, and
525 that DMF and MMF have strong cytoprotective effects at the mitochondrial level.

526

527 **3.5. Impact of 7 β -hydroxycholesterol, dimethyl fumarate, and monomethyl fumarate on cell**
528 **metabolism**

529 **3.5.1. Effects of 7 β -hydroxycholesterol, dimethyl fumarate, and monomethyl fumarate on**
530 **glycolysis and TCA cycle**

531 TCA cycle, also known as Krebs cycle, is the metabolic pathway used to release stored energy through
532 the oxidation of acetyl-CoA, to provide the reducing agent NADH and precursors of certain amino
533 acids. This cycle takes place in the matrix of the mitochondria. Thus, we considered pertinent to
534 understand the impact of 7 β -OHC, DMF, MMF and 7 β -OHC associated with DMF or MMF on the
535 TCA cycle. Lactate dehydrogenase (LDH) converts two pyruvates into 2 lactate molecules, using
536 NADH as a coenzyme so that the cell can continue to perform glycolysis by regenerating NAD⁺. The
537 effect of 7 β -OHC, DMF, MMF and 7 β -OHC associated with DMF or MMF on these metabolic
538 pathways was also evaluated. When exposed to 7 β -OHC, a significant increase in lactic acid (lactate)
539 levels was observed (220.59 ± 2.50 ng/million cells) compared to vehicle - treated cells (EtOH 0.6%:
540 122.64 ± 3.53 ng/million cells); this increase in lactic acid was significantly reduced, under treatment
541 with DMF and MMF in the presence of 7 β -OHC (**Table 1**). However, a marked and significant
542 decreases in pyruvic acid (pyruvate), succinic acid (succinate), fumaric acid (fumarate), malic acid
543 (malate) and citric acid (citrate) levels were observed in 7 β -OHC-treated 158N cells compared to
544 vehicle; this decrease was attenuated when the cells were simultaneously treated with 7 β -OHC and
545 DMF or MMF (**Table 1**).

546
547 **3.5.2. Effects of 7 β -hydroxycholesterol, dimethyl fumarate, and monomethyl fumarate on**
548 **cholesterol metabolism**

549 In the presence of 7 β -OHC, the cholesterol level was significantly decreased compared to vehicle-
550 treated cells (**Table 2**). This reduction of the cholesterol level was still observed when the cells were
551 simultaneously treated with 7 β -OHC and MMF (**Table 2**). Compared to 7 β -OHC-treated cells, the
552 cholesterol level was significantly increased, when the cells were co-treated with 7 β -OHC and DMF
553 (**Table 2**). When cholesterol precursors were measured after treatment with 7 β -OHC, significant
554 reduced levels of lathosterol, desmosterol, and lanosterol were observed compared to control and
555 vehicle (EtOH 0.6%)-treated cells (**Table 2**). While lanosterol level was normalized with DMF and
556 MMF, a most important decrease of other cholesterol precursors levels was detected (**Table 2**). In
557 addition, cholesterol auto-oxidation products (triol, ketocholesterol (7KC) and 7 β -hydroxycholesterol
558 (7 β -OHC)) were measured in the same conditions. In 158N cells treated with 7 β -OHC, important
559 levels of this oxysterol were found supporting an accumulation and/or an interaction of this
560 compounds with the cells (**Table 2**). After 24 h of incubation with 7 β -OHC, the levels of this oxysterol
561 present in the cells (when 158N cells were only cultured in the presence of 7 β -OHC) represents 1% of
562 the quantity of 7 β -OHC introduced in the culture medium (**Table 2**). This accumulation is lower than

563 those reported with 7KC and 27-hydroxycholesterol which represents 10-20% of the quantity of
564 oxysterols initially introduced in the culture medium [3]. It is noteworthy that in the plasma of X-ALD
565 patients, the concentration of 7 β -OHC can reach 2.5 μ M [20]. In the presence of 7 β -OHC, in
566 agreement with the ability of 7 β -OHC to stimulate the oxidative stress, higher levels of triol and 7KC
567 were also observed compared to control cells and vehicle-treated cells; this increase was significantly
568 reduced when 7 β -OHC was associated with DMF or MMF (Table 2). Despite the levels of triol and
569 7KC being significantly reduced, when 7 β -OHC was cultured in the presence of DMF or MMF, the
570 levels of 7 β -OHC remained increased, and were even higher than in cells only treated with 7 β -OHC
571 (Table 2). Therefore, DMF and MMF inhibit the signaling pathways leading to 7 β -OHC-induced
572 cytotoxicity without preventing the cellular accumulation of this oxysterol. In 158N cells, the Ct value
573 of the gene HSD11B1 encoding for the enzyme 11 β -HSD1 ($Ct_{(HSD11B1)} = 36.5 \pm 1.0$) was high
574 whereas the Ct value of the reference gene 36B4 was low ($Ct_{(36B4)} = 18.4 \pm 0.1$) supporting a good
575 expression of 36B4 in 158N cells (Supplementary Fig. 3). In 158N cells, the gene HSD11B1
576 encoding for the enzyme 11 β -HSD1 can be considered as non expressed since multiple peaks were
577 observed on the melt curve demonstrating a non specific amplification (Supplementary Fig. 3).
578 However, on microglial BV-2 cells, used as positive control, the Ct value of the gene HSD11B1
579 encoding for 11 β -HSD1 was higher ($Ct_{(HSD11B1)} = 30.2 \pm 0.6$) than in 158N cells ($Ct_{(HSD11B1)} = 36.5 \pm$
580 1.0); as a melt curve with homogeneous peaks was observed, this supports that BV-2 express the gene
581 of 11 β -HSD1 and that the primer sequences used are appropriated (Supplementary Fig. 3). According
582 to these data, our results do not support a conversion of 7KC in 7 β -OHC, and reciprocally via 11 β -
583 HSD1, in 158N cells. Since 7 β -OHC can be converted to 7KC by the enzyme 11 β -HSD2, the
584 expression of the HSD11B2 gene encoding this enzyme was quantified on both 158N ($Ct=26.0 \pm 0.6$)
585 and BV-2 ($Ct=29.3 \pm 0.3$) (Supplementary Fig. 4). The Ct values and the appearance of the melt
586 curves are in favor of an expression of the HSD11B2 gene in these cells. This suggests that in 158N
587 cells, 7 β -OHC could be converted to 7KC, especially when the cells are co-treated with 7 β -OHC
588 associated with DMF or MMF. The expression of the HSD11B2 gene, which is decreased in the
589 presence of 7 β -OHC, evokes that of the control when the cells are cultured in the presence of 7 β -OHC
590 associated with DMF or MMF (Supplementary Fig. 4).

591 592 3.5.3. Effects of 7 β -hydroxycholesterol, dimethyl fumarate and monomethyl fumarate on the 593 cellular fatty acid profile

594 In order to identify the effects of 7 β -OHC, DMF, MMF, and 7 β -OHC with or without DMF and MMF
595 on fatty acid metabolism, fatty acid profiles were determined.

596 As shown in **Table 3**, 7 β -OHC induced a significant decrease in the total long chain saturated fatty
 597 acids (\sum SFA (C<22)) compared to vehicle-treated cells. A significant decrease in myristic acid
 598 (C14:0), palmitic acid (C16:0), stearic acid (C18:0), and arachidic acid (C20:0) levels was observed in
 599 7 β -OHC-treated 158N cells compared to the vehicle-treated cells. However, under treatment with 7 β -
 600 OHC, the total very long chain saturated fatty acid (\sum VLC SFA C \geq 22) level was 3 times higher than
 601 in the vehicle treated cells. Thus, behenic acid (C22:0), tetracosanoic acid (C24:0), and cerotic acid
 602 (C26:0) levels were significantly enhanced in 7 β -OHC-treated cells compared to vehicle. When 158N
 603 cells were co-treated with MMF and 7 β -OHC, all SFA levels were in the range of untreated and
 604 vehicle-treated cells (**Table 3**). However, when 158N cells were co-treated with DMF and 7 β -OHC,
 605 only C18:0, C22:0 and C26:0 levels were normalized (**Table 3**).

606 As shown in **Table 4**, 7 β -OHC also induced a significant decrease in the total mono-unsaturated fatty
 607 acid (\sum MUFA) compared to vehicle-treated cells. Thus, myristoleic acid (C14:1 n-5), sapienic acid
 608 (C16:1 n-10), palmitoleic acid (C16:1 n-7), eicosenoic acid (C20:1 n-9), and eicosenoic acid (C20:1 n-
 609 7) levels were significantly decreased in 158N treated with 7 β -OHC compared to vehicle-treated cells;
 610 however, under the same conditions, very long chain monounsaturated fatty acids such as nervonic
 611 acid (C24:1 n-9) and hexacosanoic acid (C26:1 n-9) levels were enhanced (**Table 4**). MMF and DMF
 612 had almost similar effects on the MUFA profile when used 2 h prior the incubation with 7 β -OHC. In
 613 fact, all the MUFA levels came closer to those of the untreated cells or vehicle treated cells, with the
 614 exception of C16:1 n-10 or n-9 and C16:1 n-7, of which levels were higher even than control and
 615 vehicle treated cells. Interestingly when added alone, these molecules (DMF and MMF) and in
 616 particular MMF were able to significantly enhance levels of several MUFA compared to the
 617 corresponding vehicle.

618 As shown in **Table 5**, 7 β -OHC induced a significant decrease of all polyunsaturated fatty acid (PUFA)
 619 levels compared to vehicle-treated cells; thus, total PUFA content was reduced by about half in the
 620 cells. When DMF and MMF were used alone on 158N cells, linoleic acid (C18:2 n-6), arachidonic
 621 acid (AA; C20:4 n-6), and docosahexaenoic acid (DHA; C22:6 n-3) were enhanced, compared to
 622 corresponding to the vehicle. Co-treatment of 7 β -OHC with DMF and MMF induced a correction of all
 623 PUFA levels which are in the range of vehicle treated cells (**Table 5**), with the exception of (C20:4 n-
 624 6) and eicosapentaenoic acid (EPA; C20:5 n-3), which remained lower than in vehicle- and 7 β -OHC-
 625 treated cells.

626 Furthermore, Δ 4-desaturase (C22:6 n-3/C22:5 n-3), Δ 8-desaturase (C20:3 n-6 /C20:2 n-6), Δ 9-
 627 desaturase (C18:1 n-9/ C18:0), and elongase activity index (C22:5 n-3 /C20:5 n-3) activity index were
 628 significantly increased in 7 β -OHC-treated cells (**Table 5**). Compared to 7 β -OHC, these indices were
 629 significantly reduced when the cells were co-treated with 7 β -OHC associated with DMF and MMF.

630 The elongase (C18:0/C16:0) activity index was higher in 7 β -OHC cells comparatively to (7 β -OHC +
631 DMF); however, it was reduced in (7 β -OHC + MMF) compared to 7 β -OHC (Table 5).

632 Our results demonstrated the impact of 7 β -OHC on fatty acid metabolism, which could be at least in
633 part, due to mitochondrial damage, since this organelle plays important roles in fatty acid anabolism
634 and catabolism. Our data also demonstrate that DMF and MMF prevent fatty acidsmetabolic disorders
635 in 7 β -OHC-treated cells.
636

637 **3.5.4. Effects of 7 β -hydroxycholesterol, dimethyl fumarate and monomethyl fumarate on** 638 **sphingomyelin and phosphatidylcholine content**

639 The effects of 7 β -OHC, DMF, MMF, and 7 β -OHC with or without DMF orMMF on the cell content in
640 sphingomyelin (SM) and phosphatidylcholine (PC) were determined by GC/MS. In the presence of
641 7 β -OHC, significant decreases in SM and PC were observed comparatively to control (untreated cells),
642 vehicle-treated cells (EtOH 0.6% or DMSO 0.05%), α -tocopherol, DMF and MMF (Fig. 8). Whereas
643 α -tocopherol either failed to, or had a minor effect in preventing the 7 β -OHC-induced decreased of SM
644 and PC, respectively, DMF was more efficient than MMF at attenuating the decrease of SM and PC
645 under treatment with 7 β -OHC (Fig. 8).
646

647 **3.6. Effects of 7 β -hydroxycholesterol with or without dimethyl fumarate and monomethyl** 648 **fumarate on apoptosis and autophagy**

649 The oxidative stress induced by 7 β -OHC in 158N cells was associated with characteristic features of
650 apoptotosis and autophagy, in agreement with previous studies showing that 7 β -OHC triggers an
651 oxiapoptophagic mode of cell death on 158N cells [8] (Fig. 9). Apoptosis is morphologically
652 characterized by the presence of cells with condensed and/or fragmented nuclei (Fig. 9A) and by an
653 induction of cleaved caspase-3 (Fig. 10). The occurrence of autophagy is supported by an
654 enhancement of the percentage of acridine orange (AO)-positive cells (Fig. 9B), which could
655 correspond to the presence of cells with large and/or numerous autophagic vesicles, as well as by an
656 activation of LC3-I into LC3-II leading to an enhancement of the [(LC3-II) / (LC3-I)] ratio (Fig. 10).
657 Of note, DMF and MMF were able to counteract 7 β -OHC-induced apoptosis and autophagy. Thus, in
658 (7 β -OHC + DMF)- and (7 β -OHC + MMF)-treated cells, the percentage of apoptotic cells was strongly
659 and significantly decreased, and the cleavage of caspase-3 was also reduced; in addition, the
660 percentage of AO positive cells was also strongly decreased and the activation of LC3-I into LC3-II
661 was diminished (Fig. 10). As little is known on the signaling pathway leading to 7 β -OHC-induced cell
662 death, various inhibitors were used: H89 (20 μ M), U73122 (1 μ M), chelerythrine (1 μ M), and U0126
663 (20 μ M), which inhibit protein kinase A (PKA), phospholipase C (PLC), protein kinase C (PKC) and

664 MEK, respectively. Undetectable or slight inhibition of cell death evaluated with the FDA assay was
665 observed when 7 β -OHC was associated with U73122 and H89, respectively (Fig. 11). However, 7 β -
666 OHC-induced cell death was markedly reduced in the presence of chelerythrine and U0126 (Fig. 11)
667 supporting an involvement of PKC and MEK in the deleterious effects of 7 β -OHC in 158N cells.

668 3.7. Effect of dimethyl fumarate and monomethyl fumarate on the recovery of 7 β - 669 hydroxycholesterol-induced cell death

670 We also studied the effect of DMF (25 μ M) and MMF (25 μ M) on 7 β -OHC (50 μ M)-induced cell
671 death when DMF and MMF were added after 7 β -OHC. With the FDA assay, the ability of α -
672 tocopherol (400 μ M) to prevent 7 β -OHC-induced cell death was the most efficient when it was
673 introduced in the culture medium 6 h after 7 β -OHC; whereas significant, the cytoprotection found
674 when α -tocopherol was introduced 10 h after 7 β -OHC, was very slight (Supplementary Fig. 5). Based
675 on these data, the effects of DMF and MMF were evaluated when they were added 6 h after 7 β -
676 OHC; of note, under these conditions, data obtained by cell counting with trypan blue, and nuclear
677 staining with Hoechst 33342 show that DMF and MMF prevent 7 β -OHC-induced cell death and
678 apoptosis (Fig. 12). Therefore, DMF and MMF also have cytoprotective effects on 7 β -OHC-induced
679 cell death when they are added after this oxysterol.

680

681 3.8. Analysis of myelin proteins expression (PLP, MBP) in 7 β -hydroxycholesterol-treated murine 682 oligodendrocytes 158N without or with dimethyl fumarate and monomethyl fumarate

683 Myelin proteins such as proteolipid protein (PLP) and myelin basic protein (MBP) correspond to 50
684 and 30% of myelin proteins, respectively [30], and are expressed by 158N cells [29]. Therefore, we
685 determined whether PLP and MBP expression was affected by 7 β -OHC (50 μ M), and the impact of
686 DMF (25 μ M), MMF (25 μ M) and α -tocopherol (400 μ M; used as cytoprotective agent) on these
687 proteins.

688 Compared to control cells, PLP expression was either similar or slightly lower in vehicles-treated cells
689 (supplementary Fig. 6); it was similar in, α -tocopherol-, DMF- and MMF-treated cells
690 (supplementary Fig. 6). However, PLP expression was higher in 7 β -OHC-, (7 β -OHC + α -
691 tocopherol)-, and (7 β -OHC + MMF)-treated cells (supplementary Fig. 6). In the mouse, MBP is
692 present under 2 major (around 18.5 and 14 kDa) and two minor (around 21.5 and 17 kDa) forms [60].
693 In 158N cells, the 21 kDa (minor form) and 18 kDa (major form) were easily detected
694 (supplementary Fig. 6). Compared to control cells, MBP expression was enhanced by DMF
695 (supplementary Fig. 6). MBP expression was also higher in 7 β -OHC-, (7 β -OHC + α -tocopherol),
696 (7 β -OHC + DMF), and (7 β -OHC + MMF)-treated cells (supplementary Fig. 6). Thus, an increase in

697 the expression of PLP and MBP is observed with 7 β -OHC; the increase in PLP is not corrected by α -
698 tocopherol, and DMF but normalized with MMF; the increase in MBP observed with 7 β -OHC is
699 slightly and non significantly accentuated in the presence of α -tocopherol (supplementary Fig. 6).
700 DMF is more efficient than MMF to reduce the overexpression of PLP, mainly the 18 kDa
701 (supplementary Fig. 6).

702

703 Discussion

704 Oxidative stress and mitochondrial dysfunction are involved in numerous neurodegenerative diseases
705 [61]. These dysfunctions favor lipid peroxidation leading to increased levels of 7-ketocholesterol
706 (7KC) and 7 β -OHC [7, 19]. These oxysterols are found at significantly elevated levels in the brain,
707 CSF and / or plasma of patients with AD [3], MS [9] as well as in patients with X-linked
708 adrenoleukodystrophy [20]. These modifications could play a critical role in the evolution of
709 neurodegenerative diseases since these oxysterols are able to modify numerous cellular functions [3].
710 Indeed, it has been reported that 7 β -OHC as well as 7KC, known as indicators of oxidative stress [62],
711 contribute to disruption of Redox homeostasis and are potent inducers of inflammation and cell death
712 in different cell types of the CNS [3, 16]. This simultaneous induction of cell death associated with
713 oxidative stress, and presenting apoptotic and autophagic criteria, has been described in
714 oligodendrocytes and microglial cells treated with 7KC, 7 β -OHC and 24S-hydroxycholesterol, and
715 was defined as oxiaoptophagy [8]. To prevent neurodegenerative diseases associated with these
716 molecules, it is therefore important to better know their signaling pathways to develop therapeutic
717 strategies to oppose their deleterious effects. Currently, the ability of several molecules to attenuate
718 oxysterols-induced oxiaoptophagy was mainly studied on oligodendrocytes 158N and the most
719 powerful compound identified at the moment is α -tocopherol [8, 17, 19, 39]. In the present study,
720 DMF and its main metabolite, MMF, have been chosen to counteract 7 β -OHC-induced oxidative
721 stress, mitochondrial damage and cell death given their ability to prevent oxidative stress via
722 upregulation of anti-oxidative mechanisms [63]. DMF, marketed under the name of Tecfidera
723 (Biogen), is used to treat the relapsing-remitting form of MS. In this study, 158N murine cells were
724 used since they present several characteristics of differentiated oligodendrocytes, which are myelin
725 producing cells in the CNS and consequently essential for an efficient transmission of the nervous
726 impulse. In addition, 158N cells express major myelin proteins (PLP, MBP) [29]. Therefore, the
727 effects of 7 β -OHC with or without DMF and MMF on PLP and MBP expression was also evaluated.
728 Our data show important cytoprotective effects of DMF and MMF on 7 β -OHC-treated 158N cells, and
729 demonstrate the ability of DMF and MMF to prevent 7 β -OHC-induced oxiaoptophagy.

730 In 158N cells, 7 β -OHC exhibits cytotoxic effects. An increased percentage of floating round cells and
731 a decreased of adherent cells associated with a loss of membrane integrity were revealed, suggesting
732 an alteration of membrane constituents associated with cell death. These alterations could be explained
733 by Redox disequilibrium induced by 7 β -OHC, which could affect the different cellular compartments
734 as a consequence of lipid peroxidation. Indeed, an overproduction of ROS and of mitochondrial O₂^{•-}
735 associated with an enhancement of antioxidant enzyme activities (SOD, catalase, GPx), as well as lipid
736 and protein oxidized derivatives were revealed under treatment with 7 β -OHC. The enhanced activity
737 of SOD evokes the data obtained by **Yuan et al.**, who showed that human macrophages exposed to
738 7KC have increased levels of MnSOD mRNA [50]. The increased levels of GPx, catalase, and SOD
739 activities, were considered as a cellular reaction involved in the defense against the free radicals. The
740 overproduction of ROS could favor the increase of SOD, catalase and GPx activities in a cascade of
741 events to reduce oxidative stress. The disruption of Redox equilibrium observed under treatment with
742 7 β -OHC, in agreement with previous studies [8, 19, 34, 64], is also associated with an increase of lipid
743 and protein oxidation (MDA, CDs and CPs) which could be used as biomarkers of oxidative stress in
744 diseases associated with increased levels of this oxysterol.

745 In 158N cells exposed to 7 β -OHC, as observed under treatment with 7KC [39], the overproduction of
746 ROS was associated with a loss of transmembrane mitochondrial potential ($\Delta\Psi_m$). Thus, we were
747 interested in the characterization of mitochondrial functions in the presence of 7 β -OHC. In agreement
748 with previous studies, we found that 7 β -OHC is an inducer of cell death associated with mitochondrial
749 dysfunction, including morphological, functional and metabolic mitochondrial alterations [8]. This
750 includes: a loss of succinate dehydrogenase activity, a loss of $\Delta\Psi_m$, an increase of mitochondrial mass,
751 and an overproduction of O₂^{•-} at the mitochondria level. These findings are in accordance with studies
752 conducted on various cell types from different species [16, 17]. The increase of mitochondrial mass
753 could be an adaptative response of the mitochondria to prevent the loss of transmembrane
754 mitochondrial potential and preserve mitochondrial metabolism [65]. It has been reported that an
755 increase of mitochondrial mass was correlated to the hyperactivity of the mitochondrial complex IV in
756 the axons of patients with MS [66, 67]. As it is known, the main mitochondrial function is to control
757 respiratory chain in order to produce energy in the form of ATP via TCA cycle which is primordial for
758 numerous cellular functions such as carbohydrates, proteins and lipids metabolism [68]. Here, we
759 remarked an enhancement of cellular lactate (lactic acid), associated with a decrease of pyruvate level
760 in the presence of 7 β -OHC. This suggests a defect of carbohydrate metabolism and in particular of
761 glycolysis. The accumulation of lactic acid could result from pyruvate conversion to regenerate NAD⁺
762 used to restore glycolysis. Since pyruvate is the precursor of acetyl-CoA, the decreased amount of
763 pyruvate could be at the origin of TCA cycle impairment. The alteration of the TCA cycle is supported

764 by the reduced concentrations of some organic acids of the TCA cycle, citrate, fumarate, succinate, and
765 malate in the presence of 7 β -OHC. TCA cycle impairment can lead to oxidative phosphorylation
766 alteration, and consequently to mitochondrial failure [39]. On the other hand, 7 β -OHC induces a
767 decrease in the cardiolipin levels. Similar observations were made in 158N exposed to 7KC [69].
768 Cardiolipins are mitochondrial phospholipids synthesized and located in the inner mitochondrial
769 membrane, and are involved in various mitochondrial functions and in bioenergetics [58]. The loss of
770 cardiolipin content in mitochondria may also reflect biochemical modifications of mitochondrial
771 membrane. It is suggested that the observed decrease of cardiolipin content could be consequence of
772 their increased hydrolysis by endogenous phospholipases, and/or by a decreased de novo synthesis
773 resulting from impaired function of the enzymes involved in their synthesis, or by a decreased
774 bioavailability of cardiolipin precursors. During cell death, release of cardiolipins outside the
775 mitochondria and outside the cells is also possible. These data underline the major role of 7 β -OHC in
776 mitochondrial dysfunction.

777 Given the important role of lipids, including cholesterol in myelin structure and brain health [70], it
778 was important to evaluate the impact of 7 β -OHC on cholesterol metabolism via the quantification of
779 some of its precursors and derivatives. Under treatment with 7 β -OHC, reduced levels of total
780 cholesterol and its precursors, lathosterol, lanosterol, and desmosterol were observed, arguing in favor
781 a defect on cholesterol synthesis. Our results support previous findings reported on different cellular
782 lines treated with 24(S)-hydroxycholesterol or 7KC: these studies have shown that cholesterol
783 synthesis alteration was associated with other events such as mitochondrial damage and cell death
784 [39]. As our study reveals oxidative stress along with severe mitochondrial dysfunctions, under
785 treatment with 7 β -OHC, this could explain even in part, the cellular accumulation of 7KC formed by
786 an oxidation of cholesterol induced by ROS overproduction.

787 Our results support the idea that the alteration of cholesterol synthesis, lead to cholesterol homeostasis
788 disturbance, which in oxidative stress conditions might be associated with increased levels of some
789 oxysterols. In addition, it has been reported that oxysterols can disturb lipid homeostasis through the
790 activation of transcription factors. They can act on sterol responsive element binding proteins
791 (SREBPs), which activate several genes involved in cholesterol, triglycerides, phospholipids, and fatty
792 acid synthesis [71]. In addition, fatty acids, which are components of phospholipids, are essential
793 constituents in the structure and functions of myelin sheath [72]. Thus, it was important to evaluate
794 both the fatty acid profile and the phospholipid content (sphingomyelin, phosphatidylcholine) when
795 158N cells were exposed to 7 β -OHC. In this condition, a reduction of saturated fatty acids as myristic
796 acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0), structural elements of membrane
797 phospholipids, as well as of phosphatidylcholine and sphingomyelin were observed. In addition, an

798 accumulation of total and very long chain saturated fatty acids (VLCSFAs) has been remarked under
799 treatment with 7 β -OHC, which points towards peroxisomal defect, since β -oxidation of VLCSFAs
800 leading to their degradation takes place in the peroxisome [73]. Thus, as reported with 7KC, the
801 treatment with 7 β -OHC also induces peroxisomal dysfunction [69]. Furthermore, it has been
802 demonstrated that the VLCSFAs are able to induce cell death, mitochondrial impairment, and
803 oxidative stress in nerve cells [74], which could aggravate the cytotoxic effect of 7 β -OHC. Moreover,
804 MUFA and PUFA levels were also reduced, which is in accordance with other studies, conducted on
805 158N cells in the presence of 7 β -OHC [34]. It is also well established that MUFA and PUFA are
806 indispensable for neuronal membrane fluidity [75], and have neuroprotective, anti-inflammatory, and
807 antioxidant properties [76, 77].

808 According to previous findings on different cell lines, 7 β -OHC was considered as a strongly cytotoxic
809 oxysterol. So, we intended to characterize 7 β -OHC-induced cell death. Our data show that 7 β -OHC-
810 induced cell death triggers caspase-3 activation associated with fragmented and/or condensed nuclei.
811 This supports that 7 β -OHC induces an apoptotic mode of cell death. Moreover, our data confirm that
812 7 β -OHC also triggers autophagy. Indeed, a higher percentage of cells with altered lysosomes was
813 observed. These data underline that 7 β -OHC-induced cell death is characterized by lysosomal
814 dysfunction [78] which could favour mitochondrial alteration [79]. Furthermore, a conversion of LC3-I
815 to LC3-II, and an increase of (LC3-II / LC3-I) ratio, which are characteristics of autophagy, were
816 detected demonstrating that 7 β -OHC simultaneously triggers apoptosis and autophagy, and induces an
817 oxiaoptophagic process [64]. Altogether, our data highlight the major role played by 7 β -OHC in
818 mitochondrial dysfunction, oxidative stress induction, cellular metabolism alteration and cell death
819 induction which are common features observed during neurodegeneration. In addition, our study
820 brings new information on the signalling pathway induced during 7 β -OHC-induced cell death and
821 supports the hypothesis of an activation of the PKC / p38 / MEK signalling pathway [18].

822 When 158N cells were simultaneously treated with DMF or MMF and 7 β -OHC, our data demonstrate
823 that DMF, a therapeutic agent (prescribed under the name of Tecfidera used for the treatment of
824 relapsing remitting MS), as well as MMF, which is its major metabolite, known to have antioxidant
825 and neuroprotective activities are able to prevent 7 β -OHC-induced cytotoxicity [22, 23]. Under these
826 conditions, we observed an attenuation of cell detachment and a preservation of plasma membrane
827 integrity. It has also been shown that DMF induces an increase of antioxidant molecules such as
828 glutathione (GSH), carnitine, and ascorbic acid above basal levels [24]. These molecules, especially
829 ascorbic acid, are able to maintain membrane integrity in a pro-oxidative environment by preventing
830 lipid peroxidation [80]. In our study, the ability of DMF to increase the levels of SH-groups suggests
831 that this compound might favor this mechanism. It may act through the activation of Nrf2 pathway

832 [81]. Thus, it was of interest to evaluate the part taken by DMF and its major metabolite MMF on
833 Redox status. We therefore determine the ability of DMF and MMF to inhibit the overproduction of
834 ROS, to increase the antioxidant enzyme activities (SOD, catalase, GPx), to increase SH-groups level,
835 and to reduce lipid peroxidation and protein oxidation products. Overall, the cytotoxic effects induced
836 by 7 β -OHC on 158N cells were strongly attenuated by DMF and MMF. In line with these findings, on
837 human oligodendrocytes (MO3.13 cells), it has been reported that DMF prevent H₂O₂ overproduction-
838 induced cell death, and enhances antioxidant molecule levels [24]. In the presence of 7 β -OHC,
839 treatment with DMF and MMF favors the recovery of mitochondrial function through the restoration
840 of succinate dehydrogenase activity, and mitochondrial transmembrane potential. Furthermore, DMF
841 and MMF are able to counteract mitochondrial O₂^{•-} overproduction, supporting a role of DMF and
842 MMF on the control of cellular Redox potential [82]. In addition, DMF and MMF were able to
843 attenuate the increase of mitochondrial mass corresponding either to an increase of the number of
844 mitochondria and/or of their size [34]. Moreover, DMF and MMF normalize cardiolipin levels. Since a
845 loss of cardiolipin content or alterations in the composition of these molecules have been associated
846 with mitochondrial dysfunction, the restoration of their content argues in favor of the ability of DMF
847 and MMF to prevent and restore mitochondrial dysfunction. This conclusion is reinforced by the
848 ability of DMF and MMF to prevent TCA cycle alterations. These results underline that DMF and
849 MMF influence the activities of mitochondrial enzymes including those of the TCA cycle. Pyruvate
850 levels were also increased associated with a reduction of lactate after pretreatment with DMF or
851 MMF: this argues in favour a recovery on carbohydrate metabolism and especially glycolysis.

852 It is also well known that the myelin sheath is composed of approximately 70–80% of lipids [72]. The
853 synthesis and incorporation of phospholipids, sphingolipids, and cholesterol are critical for its
854 development and maintenance. Based on data demonstrating neuroprotection of DMF, we postulated
855 that this molecule and its metabolite (MMF) might increase the synthesis of lipids, which are major
856 components of myelin sheath. Of note, DMF and MMF were able to prevent 7 β -OHC-induced
857 modification of cholesterol synthesis through the restoration of the content of cholesterol precursors.
858 The formation of oxidized cholesterol derivatives (mainly 7KC) resulting chiefly from cholesterol
859 autoxidation was also reduced in the presence of DMF or MMF. However, DMF and MMF were
860 unable to prevent the intracellular accumulation of 7 β -OHC. As the expression of the gene HSD11B1
861 encoding for the enzyme 11 β -HSD1 is considered unlikely, a conversion of 7 β -OHC to 7KC by this
862 enzyme, is excluded. On the other hand, as the gene HSD11B2 encoding for the enzyme 11 β -HSD2
863 catalyzing the conversion of 7 β -OHC to 7KC is expressed, this may explain the persistent presence of
864 7KC despite the powerful antioxidant effects of DMF and MMF. In addition, we report the ability of
865 DMF and MMF to normalize the fatty acid profile. The reduction of VLCSFAs levels observed when

866 7 β -OHC was associated with DMF and MMF support that DMF and MMF not only tend to normalize
867 mitochondrial activity, but also peroxisomal activity. Of note, DMF and MMF were also able to
868 attenuate the decrease of phospholipids (sphingomyelin, phosphatidylcholine) which are important
869 component of myelin. Indeed, whereas the molar ratio of cholesterol, phospholipids and
870 glycosphingolipids in most membranes is in the order of 25%:65%:10%, the molar ratios in myelin are
871 in the range of 40%:40%:20% [83]. As DMF and MMF contribute to restore a normal lipid profile,
872 concerning myelin associated-lipids (cholesterol, phospholipids), it was important to determine how
873 major myelin proteins such as proteolipid protein (PLP) and myelin basic protein (MBP), which
874 correspond to 50 and 30% of myelin proteins, respectively [30], and which are expressed by the 158N
875 cells [29], were affected by 7 β -OHC and what was the impact of DMF and MMF on these proteins.
876 Noteworthy, the increased expression of PLP was normalized with MMF, and the increase expression
877 of MBP observed with 7 β -OHC was more or less attenuated by DMF and MMF.
878 Finally, DMF and MMF are also able to counteract 7 β -OHC-induced apoptosis as well as autophagy.
879 At the moment, on 158N cells, similar cytoprotective effects were only observed with α -tocopherol,
880 DHA, and biotin which allow attenuation of 7 β -OHC-induced oxiaoptophagy [8, 31].

881

882 **Conclusion**

883 Our data obtained on oligodendrocytes 158N demonstrate that 7 β -OHC induces ROS overproduction
884 subsequently leading to increased antioxidant enzyme activity, and oxidation of cellular
885 macromolecules. In addition, 7 β -OHC triggers important mitochondrial dysfunctions: decreases of
886 $\Delta\Psi_m$, increases of mitochondrial mass, decreased cardiolipin content and alteration of the TCA cycle,
887 which can influence the cellular metabolism, especially lipid metabolism. These different alterations
888 contribute to cell death through oxiaoptophagy. The different events associated with oxiaoptophagy,
889 revealed from data obtained in the present study and in previous studies, are summarized in
890 **Supplementary Fig. 7**. Our data also show that DMF and MMF attenuate 7 β -OHC-induced oxidative
891 stress, mitochondrial dysfunction, lipid metabolism alteration and cell death, which are hallmarks of
892 neurodegenerative diseases. As 7 β -OHC is associated with neurodegeneration, the ability of DMF and
893 MMF to counteract the toxicity of this oxysterol reinforces the interest of DMF for the treatment of
894 neurodegenerative diseases as well as of other diseases associated with increased levels of 7 β -OHC [3,
895 16].

896

897 **Conflict of interest:** The authors have no conflict of interest to declare.

898

899 **Acknowledgments:** This work was presented as a poster at the 8th ENOR Symposium “Oxysterols and
900 Sterols: from Lipidomics to Food Sciences”, September 20–21, 2018, University of Bologna, Bologna,
901 Italy (<https://www.oxysterols.net/>). This work was supported by grants from: Univ. Bourgogne (Dijon,
902 France), Univ. Monastir (Monastir, Tunisia), and Univ. Manouba (Tunis, Tunisia). We acknowledge
903 Dr Delphine Meffre (Inserm UMR 1124, Paris, France) for her valuable advice on the choice of anti-
904 PLP and anti-MBP antibodies, and for the conditions of analysis of these proteins by Western
905 blotting.

906

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1139 **Figure legends**

1140 **Fig. 1: Effect of 7 β -OHC, dimethyl fumarate and monomethyl fumarate on cell growth and cell**
1141 **adhesion.** 158N cells were cultured for 24 h with or without 7 β -hydroxycholesterol (7 β -OHC, 50 μ M)
1142 in the presence or absence of DMF (25 μ M), MMF (25 μ M) or α -tocopherol (400 μ M). The effects on
1143 cell growth and cell adhesion were determined by phase contrast microscopy (A) and crystal violet
1144 staining (B). Data shown are mean \pm SD of three independent experiments performed in triplicate.

1145 A two way ANOVA followed by a Student's t-test was realized. Significance of the differences
1146 between 7 β -OHC-treated cells and its vehicle (Ethanol (EtOH) 0.6%); # $P \leq 0.05$. Significance of the
1147 differences between 7 β -OHC-treated cells, (7 β -OHC + DMF), (7 β -OHC + MMF) or (7 β -OHC + α -
1148 tocopherol)-treated cells; * $P \leq 0.05$. No significant differences were observed between control
1149 (untreated cells), vehicle (EtOH 0.6%), and vehicle (DMSO 0.05%).

1150

1151 **Fig. 2: Effect of 7 β -OHC, dimethyl fumarate and monomethyl fumarate on cell viability and**
1152 **plasma membrane integrity.** 158N cells were cultured for 24 h with or without 7 β -
1153 hydroxycholesterol (7 β -OHC, 50 μ M) in the presence or absence of DMF (25 μ M), MMF (25 μ M) or
1154 α -tocopherol (400 μ M). The effect of 7 β -OHC (50 μ M) with or without DMF (25 μ M), MMF (25
1155 μ M) or α -tocopherol (400 μ M) was determined on cell viability and plasma membrane integrity with
1156 complementary criteria: plasma membrane integrity was measured with the FDA assay (A), plasma
1157 membrane permeability was measured after staining with propidium iodide (PI) (% of PI positive
1158 cells) (B), the impact on cell viability was evaluated by the counting of living cells after staining with
1159 trypan blue (dead cells are blue whereas living cells are not) (C) as well as by the measurement LDH
1160 activity in the culture medium (D). Data shown are mean \pm SD of three independent experiments
1161 performed in triplicate.

1162 A two way ANOVA followed by a Student's t-test was realized. Significance
1163 of the differences between 7 β -OHC-treated cells and its vehicle (Ethanol (EtOH) 0.6%); # $P \leq 0.05$.
1164 Significance of the differences between 7 β -OHC-treated cells and (7 β -OHC + DMF)-, (7 β -OHC +
1165 MMF)- or (7 β -OHC + α -tocopherol)-treated cells; * $P \leq 0.05$. No significant differences were observed
1166 between control (untreated cells), vehicle (EtOH 0.6%), and vehicle (DMSO 0.05%).

1166

1167 **Fig. 3: Effect of dimethyl fumarate and monomethyl fumarate on 7 β -hydroxycholesterol-induced**
1168 **reactive oxygen species (ROS) overproduction.** 158N cells were cultured for 24 h with or without
1169 7 β -hydroxycholesterol (7 β -OHC; 50 μ M) in the presence or absence of DMF (25 μ M), MMF (25 μ M),
1170 or α -tocopherol (400 μ M). ROS overproduction was measured by flow cytometry; ROS

1171 overproduction, including superoxide anion ($O_2^{\bullet-}$), was evaluated with DHE (% DHE positive cells)
1172 (A), and with DHR123, which takes into account hydrogen peroxide (H_2O_2) overproduction (% DHR
1173 123 positive cells) (B). Data shown are mean \pm SD of three independent experiments performed in
1174 triplicate. A two way ANOVA followed by a Student's t-test was realized. Significance of the
1175 difference between vehicle (ethanol (EtOH) 0.6%) and 7β -OHC-treated cells; # $p \leq 0.05$ or less.
1176 Significance of the differences between 7β -OHC-treated cells and (7β -OHC + DMF)-, (7β -OHC +
1177 MMF)- or (7β -OHC + α -tocopherol)-treated cells; * $P \leq 0.05$. No significant differences were observed
1178 between control (untreated cells), vehicle (EtOH 0.6%), and vehicle (DMSO 0.05%).

1179
1180 **Fig. 4: Effect of dimethyl fumarate and monomethyl fumarate on 7β -hydroxycholesterol-induced**
1181 **oxidative stress.** 158N cells were cultured for 24 h with or without 7β -hydroxycholesterol (7β -OHC,
1182 50 μ M) in the presence or absence of DMF (25 μ M), MMF (25 μ M) or α -tocopherol (400 μ M). The
1183 effects on antioxidant enzyme activities were determined by colorimetric assays with the measurement
1184 of glutathione peroxidase (GPx) activity (A) superoxide dismutase (SOD) activity (B) and catalase
1185 (CAT) activity (C) as well as by the measurement of thiol-SH group (D). Data shown are mean \pm SD
1186 of three independent experiments conducted in triplicate. A two way ANOVA followed by a Student's
1187 t-test was realized. Significance of the differences between 7β -OHC-treated cells and its vehicle
1188 (Ethanol (EtOH) 0.6%); # $P \leq 0.05$ or less. Significance of the differences between 7β -OHC-treated
1189 cells, (7β -OHC + DMF), (7β -OHC + MMF) or (7β -OHC + α -toco)-treated cells; * $P \leq 0.05$ or less. No
1190 significant differences were observed between control (untreated cells), vehicle (EtOH 0.6%), and
1191 vehicle (DMSO 0.05%).

1192
1193 **Fig. 5: Effect of dimethyl fumarate and monomethyl fumarate on 7β -hydroxycholesterol-induced**
1194 **lipid peroxidation and carbonylated protein formation.** 158N cells were cultured for 24 h with or
1195 without 7β -hydroxycholesterol (7β -OHC, 50 μ M) in the presence or absence of DMF (25 μ M), MMF
1196 (25 μ M) or α -tocopherol (400 μ M). The effects on lipid peroxidation product formation were
1197 evaluated by the measurement of CDs (A), and MDA (B) levels. The impact on protein oxidation was
1198 evaluated by the measurement of carbonylated protein (CPs) levels (C). Data shown are mean \pm SD of
1199 three independent experiments performed in triplicate. A two way ANOVA followed by a Student's t-
1200 test was realized. Significance of the differences between 7β -OHC-treated cells and its vehicle
1201 (Ethanol (EtOH) 0.6%); # $P \leq 0.05$ or less. Significance of the differences between 7β -OHC-treated
1202 cells, (7β -OHC + DMF)-, (7β -OHC + MMF) or (7β -OHC + α -toco)-treated cells; * $P \leq 0.05$ or less. No
1203 significant differences were observed between control (untreated cells), vehicle (EtOH 0.6%), and
1204 vehicle (DMSO 0.05%).

1205
1206 **Fig. 6: Evaluation of the effect of dimethyl fumarate and monomethyl fumarate on 7 β -**
1207 **hydroxycholesterol-induced mitochondrial damage.** 158N cells were cultured for 24 h with or
1208 without 7 β -hydroxycholesterol (7 β -OHC, 50 μ M) in the presence or absence of DMF (25 μ M), MMF
1209 (25 μ M) or α -tocopherol (400 μ M). Under these conditions, the effects succinate deshydrogenase
1210 activity were evaluated with the MTT test (A). The transmembrane mitochondrial potential ($\Delta\Psi_m$) was
1211 measured by flow cytometry after staining with DiOC₆(3) (B). The mitochondrial mass was
1212 determined by flow cytometry after staining with MitoTracker Red (C). Cardiolipin levels were
1213 determined by GC-MS (D) and mitochondrial superoxide anion (O₂^{•-}) production was evaluated by
1214 flow cytometry after staining with MitoSOX (E). Data shown are mean \pm SD of three independent
1215 experiments performed in triplicate. A two way ANOVA followed by a Student's t-test was realized.
1216 Significance of the differences between 7 β -OHC-treated cells and its vehicle (Ethanol (EtOH) 0.6%); #
1217 P \leq 0.05 or less. Significance of the differences between 7 β -OHC-treated cells, (7 β -OHC + DMF), (7 β -
1218 OHC + MMF)- or (7 β -OHC + α -toco)-treated cells; * P \leq 0.05 or less. No significant differences were
1219 observed between control (untreated cells), vehicle (EtOH 0.6%), and vehicle (DMSO 0.05%).

1220
1221 **Fig. 7: Ultrastructural characterization by transmission electron microscopy of mitochondria**
1222 **and peroxisomes on 158N murine oligodendrocytes incubated with or without 7 β -**
1223 **hydroxycholesterol in the presence or absence of dimethyl fumarate (DMF).** 158N cells were
1224 cultured for 24 h with or without 7 β -hydroxycholesterol (7 β -OHC, 50 μ M) in the presence or absence
1225 of DMF (25 μ M). Ultrastructural aspects of mitochondria and peroxisomes in untreated (control) (A),
1226 DMF (B), 7 β -OHC (C), and (7 β -OHC + DMF) (D)-treated 158N cells. In the presence of 7 β -OHC,
1227 several peroxisomes and mitochondria smaller than in the control were observed (C); these differences
1228 were no longer observed when 7 β -OHC was associated with DMF (D). One experiment was
1229 performed.

1230
1231 **Fig. 8: Phospholipid (sphingomyelin, phosphatidylcholine) content in 158N cells exposed to 7 β -**
1232 **hydroxycholesterol with or without dimethyl fumarate or monomethyl fumarate.** 158N cells were
1233 cultured for 24 h with or without 7 β -hydroxycholesterol (7 β -OHC, 50 μ M) in the presence or absence
1234 of DMF (25 μ M), MMF (25 μ M) or α -tocopherol (400 μ M). Under these conditions, the quantity of
1235 sphingomyelin (SM) and phosphatidylcholine (PC) per 10⁶ cells was determined by GC/MS. Data
1236 shown are mean \pm SD of three independent experiments performed in triplicate. A two way ANOVA
1237 followed by a Student's t-test was realized. Significance of the differences between 7 β -OHC-treated
1238 cells and its vehicle (Ethanol (EtOH) 0.6%); # P \leq 0.05 or less. Significance of the differences between

1239 7 β -OHC-treated cells, (7 β -OHC + DMF)-, (7 β -OHC + MMF)- or (7 β -OHC + α -toco)-treated cells; *
1240 P \leq 0.05 or less. No significant differences were observed between control (untreated cells), vehicle
1241 (EtOH 0.6%), and vehicle (DMSO 0.05%).

1242
1243 **Fig. 9:** Effect of dimethyl fumarate and monomethyl fumarate on 7 β -hydroxycholesterol-induced
1244 morphological nuclear changes characteristic of apoptosis and lysosomal modifications evocating
1245 autophagy. 158N cells were cultured for 24 h with or without 7 β -hydroxycholesterol (7 β -OHC, 50
1246 μ M) in the presence or absence of DMF (25 μ M), MMF (25 μ M) or α -tocopherol (400 μ M). Under
1247 these conditions, the quantification of the percentage of apoptotic cells was evaluated by nuclear
1248 morphologic criteria after staining with Giemsa and Hoechst 33342; apoptotic cells are characterized
1249 by condensed and/or fragmented nuclei whereas normal cells have round and regular nuclei (A). The
1250 impact of 7 β -OHC (50 μ M) with or without DMF (25 μ M), MMF (25 μ M) or α -tocopherol (400 μ M)
1251 on the lysosome, which is involved in the autophagic process, was determined by flow cytometry after
1252 staining with acridine orange (AO); under these conditions, the percentage of AO positive cells was
1253 quantified (B). Data shown are mean \pm SD of three independent experiments performed in triplicate. A
1254 two way ANOVA followed by a Student's t-test was realized. Significance of the differences between
1255 7 β -OHC-treated cells and its vehicle (Ethanol (EtOH) 0.6%); # P \leq 0.05 or less. Significance of the
1256 differences between 7 β -OHC-treated cells, (7 β -OHC + DMF)-, (7 β -OHC + MMF)- or (7 β -OHC + α -
1257 toco)-treated cells; * P \leq 0.05 or less. No significant differences were observed between control
1258 (untreated cells), vehicle (Ethanol 0.6%), and vehicle (DMSO 0.05%).

1259
1260 **Fig. 10:** Western blotting analysis of the effects dimethyl fumarate and monomethyl fumarate on
1261 7 β -hydroxycholesterol-induced apoptosis and autophagy on 158N murine oligodendrocytes.
1262 158N cells were cultured for 24 h with or without 7 β -hydroxycholesterol (7 β -OHC, 50 μ M) in the
1263 presence or absence of DMF (25 μ M), MMF (25 μ M) or α -tocopherol (400 μ M). Apoptosis and
1264 autophagy were characterized by Western blotting with appropriate antibodies raised against uncleaved
1265 and cleaved caspase-3 and LC3-I / LC3-II, respectively. Autophagy is characterized by an enhanced
1266 (LC3-II / LC3-I) ratio. Data shown are characteristic of at least three independent experiments. A two
1267 way ANOVA followed by a Student's t-test was realized. Significance of the differences between 7 β -
1268 OHC-treated cells and its vehicle (Ethanol (EtOH), 0.6%); # P \leq 0.05 or less. Significance of the
1269 differences between 7 β -OHC-treated cells, (7 β -OHC + DMF)-, (7 β -OHC + MMF)- or (7 β -OHC + α -
1270 toco)-treated cells; * P \leq 0.05 or less. No significant differences were observed between control
1271 (untreated cells), vehicle (EtOH 0.6%), and vehicle (DMSO 0.05%).

1272

1273 **Fig. 11: Evaluation of protein kinase A (PKA), phospholipase C (PLC)/protein kinase C (PKC)**
1274 **and MEK / ERK signaling pathways in 7 β -OHC-induced cell death on 158N cells.** Murine
1275 oligodendrocyte 158N cells previously cultured for 24 h were further cultured for 24 h with or without
1276 7 β -OHC (50 μ M) in the presence or absence of different inhibitors, H89, U0126, U73122 and
1277 chelerythrine, introduced 30 min before the addition of 7 β -OHC. Data shown are mean \pm SD of two
1278 independent experiments performed in triplicate. **A two way ANOVA followed by a Student's t-test**
1279 **was realized.** No significant difference was observed between control (untreated cells), vehicle
1280 (ethanol (EtOH): 0.6%)-treated cells and inhibitors (H89, U0126, U73122 or chelerythrine)-treated
1281 cells. Significance of the differences between 7 β -OHC-treated cell and vehicle (EtOH 0.6%); # $P \leq 0.05$
1282 or less. Significant of the differences between 7 β -OHC-treated cells and (7 β -OHC + (H89, U0126,
1283 U73122, or chelerythrine))-treated cells: * $P \leq 0.05$.

1284
1285 **Fig. 12: Effects of dimethyl fumarate and mono methyl fumarate on the recovery of 7 β -**
1286 **hydroxycholesterol-induced cell death.** In these experiments, after 24 h of culture, 158N cells were
1287 incubated with 7 β -OHC (50 μ M) for 6 h before the addition of DMF (25 μ M), MMF (25 μ M), or α -
1288 tocopherol (400 μ M). The cells were subsequently incubated with 7 β -OHC (50 μ M) associated with
1289 DMF, MMF or α -tocopherol for an 18 h additional period of time. **A: total number of living cells**
1290 **evaluated after staining with trypan blue; B: percentage of cells with apoptotic nuclei determined after**
1291 **staining with Hoechst 33342. Data shown are mean \pm standard deviation (SD) of three independent**
1292 **experiments performed in triplicate. A two way ANOVA followed by a Student's t-test was realized.**
1293 **Significant differences between 7 β -OHC-treated cells and the vehicle (ethanol (EtOH, 0.6%): #**
1294 **$P \leq 0.05$. Significant differences between 7 β -OHC-treated cells and (7 β -OHC + DMF)-, (7 β -OHC +**
1295 **MMF)- or (7 β -OHC + α -tocopherol)-treated cells; * $P \leq 0.05$. No significant differences were observed**
1296 **between control (untreated cells), vehicle (EtOH 0.6%), and vehicle (DMSO 0.05%).**

1297
1298 **Supplementary Fig. 1: Estimation of the 50% inhibiting concentration of 7 β -hydroxycholesterol**
1299 **on the viability of murine oligodendrocytes 158N.** 158N cells previously cultured for 24 h were
1300 further incubated for 24 h with or without 7 β -hydroxycholesterol (7 β -OHC: 1, 6.25, 12.5, 25, 50, 100,
1301 200 μ M). In those conditions, the cytotoxicity was determined with the MTT test. Vehicle: EtOH
1302 0.6%. Data shown are mean \pm SD (Two experiments realized in triplicate). **A one way ANOVA**
1303 **followed by a Student's t-test was realized.** Significance of the differences between 7 β -OHC-treated
1304 cells and vehicle (Ethanol (EtOH) 0.6%)-treated cells; # $P \leq 0.05$ or less. No difference was observed
1305 between control (untreated cells) and vehicle (EtOH 0.6%)-treated cells.

1306

1307 **Supplementary Fig. 2: Evaluation of the optimal concentration of DMF and MMF preventing**
1308 **7 β -hydroxycholesterol-induced cytotoxicity on murine oligodendrocytes 158N.** 158N cells
1309 previously cultured for 24 h were further incubated for 24 h with or without 7 β -hydroxycholesterol
1310 (7 β -OHC, 50 μ M) in the presence or absence of DMF or MMF (1, 12.5, 25, 50 and 100 μ M). In these
1311 conditions, the cytotoxicity was determined with the MTT test. Vehicles: ethanol (EtOH, 0.6%) or
1312 DMSO (0.002, 0.025, 0.05, 0.1, 0.2%). Data shown are mean \pm SD of three independent experiments
1313 realized in triplicate. A two way ANOVA followed by a Student's t-test was realized. No significant
1314 differences were observed between control (untreated cells), and vehicle (EtOH or DMSO). Significant
1315 differences between 7 β -OHC-treated cells and the vehicle (EtOH: 0.6%): # $P \leq 0.05$. Significant
1316 differences between 7 β -OHC-treated cells and (7 β -OHC + DMF)- or (7 β -OHC + MMF)-treated cells;
1317 * $P \leq 0.05$.

1318
1319 **Supplementary Fig. 3: Determination of the Ct values of the gene HSD11B1 encoding for the**
1320 **enzyme 11 β -HSD1 in murine oligodendrocytes 158N and murine microglial BV-2 cells.** The
1321 amplification plots and the melt curves of HSD11B1 are shown for 158N and BV-2 cells. The Ct
1322 values of HSD11B1 and of the reference gene 36B4 are also shown. Data shown are representative of
1323 3 independent experiments realized in triplicate.

1324
1325 **Supplementary Fig. 4: Determination of the Ct values of the gene HSD11B1 encoding for the**
1326 **enzyme 11 β -HSD1 in murine oligodendrocytes 158N and murine microglial BV-2 cells.** The
1327 amplification plots and the melt curves of HSD11B2 are shown for 158N and BV-2 cells. The Ct
1328 values of HSD11B2 and of the reference gene 36B4 are also shown as well as the expression of
1329 HSD11B2 in 158N cells (158N cells previously cultured for 24 h were further incubated for 24 h with
1330 or without 7 β -OHC (50 μ M) in the presence or absence of DMF or MMF (25 μ M); the quantitative
1331 induction of HSD11B2 was determined as fold induction of the control; a two way ANOVA followed
1332 by a Student's t-test was realized: no significant difference was observed). Data shown are
1333 representative of 1 experiment realized in triplicate.

1334
1335 **Supplementary Fig.5 :** **Determination of the time of treatment with dimethyl fumarate and**
1336 **monomethyl fumarate for recovery of 7 β -hydroxycholesterol-induced cell death.** The effect of
1337 DMF and MMF (25 μ M) or α -tocopherol (400 μ M) on 7 β -OHC (50 μ M)-induced cell death was
1338 determined with the FDA test when biotin or α -tocopherol were added after 7 β -OHC (6 and 10 h). The
1339 most efficient time to prevent 7 β -OHC-induced cell death when α -tocopherol and biotin were
1340 introduced in the culture medium after 7 β -OHC was 6 h. Data shown are mean \pm SD (Two

1341 independent experiments realized in triplicate). A two way ANOVA followed by a Student's t-test was
1342 realized. No significant differences were observed between control (untreated cells), and vehicle
1343 (ethanol (EtOH) 0.6% or DMSO 0.05%). Significant differences between treated cells and the vehicle
1344 (EtOH: 0.6%): # $P \leq 0.05$. Significant differences between 7β -OHC-treated cells and (7β -OHC + DMF),
1345 (7β -OHC + MMF) or (7β -OHC + α -tocopherol)-treated cells; * $P \leq 0.05$.

1346
1347 **Supplementary Fig. 6: Analysis of myelin protein expression (PLP, MBP) in 158N cells.** Murine
1348 oligodendrocytic 158N cells were cultured for 24 h with or without 7β -hydroxycholesterol (7β -OHC,
1349 50 μ M) in the presence or absence of DMF (25 μ M), MMF (25 μ M), or α -tocopherol (400 μ M). The
1350 major myelin proteins PLP and MBP as well as actin (used as internal reference) were characterized by
1351 Western blotting with appropriate antibodies. Vehicles: ethanol (ethanol (EtOH) 0.6%; DMSO 0.05%).
1352 Data are mean \pm SD of three independent experiments. A two way ANOVA followed by a Student's t-
1353 test was realized. Significance of the differences between 7β -OHC-treated cells and its vehicle (EtOH
1354 0.6%); # $P \leq 0.05$ or less. Significance of the differences between 7β -OHC-treated cells, (7β -OHC +
1355 DMF)-, (7β -OHC + MMF)- or (7β -OHC + α -toco)-treated cells; * $P \leq 0.05$ or less.

1356
1357 **Supplementary Fig. 7: Schematic signalling pathways associated with 7β -hydroxycholesterol-**
1358 **induced oxiaoptophagy.** The schematic signalling pathways presented summarizes the data obtained
1359 from different cell types [3, 8, 16, 18, 19, 84]. In the different cell types studied, 7β -OHC is a strong
1360 inducer of reactive oxygen species (ROS) overproduction. In 158N cells, 7β -OHC also favors the
1361 disturbance of Redox homeostasis by increasing the formation of lipid peroxidation products
1362 (malondialdehyde (MDA), conjugated dienes (CDs)) and of carbonylated proteins (CPs) which can
1363 further contribute to cell death. Important impact on the mitochondria was also observed whatever the
1364 cells are considered. In human monocytic THP-1 and U937 cells, a down-regulation of Bcl-2
1365 expression was also detected as well as an activation of the pro-apoptotic proteins (Bid, Bax),
1366 associated with a release of cytochrome c and an activation of caspase-9, caspase-8, caspase-3 and
1367 caspase-7. In U937 cells, 7β -OHC also induced an increase in cytosolic Ca^{2+} concentration, associated
1368 with a decrease of Akt activation and a mitochondrial release of various proteins such as cytochrome c,
1369 apoptosis-inducing factor (AIF), and endonuclease-G (Endo-G), associated with caspase-3, -7, -8, and
1370 -9 activation, Bid cleavage and poly(ADP-ribose)polymerase (PARP) degradation. In C6 glioblastoma
1371 cells, 7β -OHC induces apoptosis through the decrease of ERK signalling, the transient PI3K / Akt
1372 activation, the loss of GSK3 β activation and the activation of p38. The data obtained in the present
1373 study on 158N cells support that p38 activation could be triggered by PKC. On U937 cells as well as

1374 on human retinal pigment epithelial cells (ARPE-19), large myelin figures (evocating reticulophagy)
1375 were observed. On ARPE-19 cells, a link between lysosome and cell death was also established. In
1376 158N cells, the complex mode of cell death induced by 7 β -OHC (oxiaptophagy) is characterized by
1377 a dephosphorylation of PKB / Akt, an activation of GSK3, and by a reduced expression of Bcl-2;
1378 altogether these events contribute to mitochondrial depolarization leading to caspase-3 activation,
1379 PARP degradation and internucleosomal DNA fragmentation. Moreover, 7 β -OHC promotes the
1380 conversion of microtubule-associated protein light chain 3 (LC3-I) to LC3-II which is a criteria of
1381 autophagy. Altogether, these data establish that 7 β -OHC is a potent inducer of oxiaptophagy
1382 through the concomittent activation of several signalling pathways involved in oxidative stress,
1383 apoptosis and autophagy.

1384

1385

1386

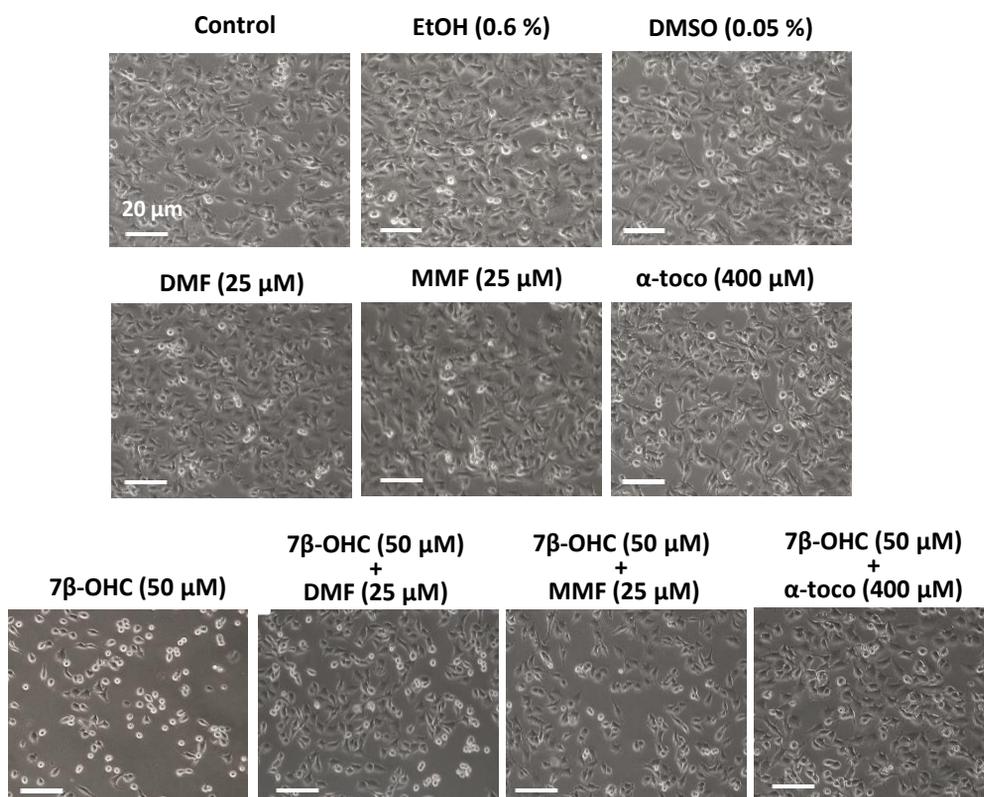
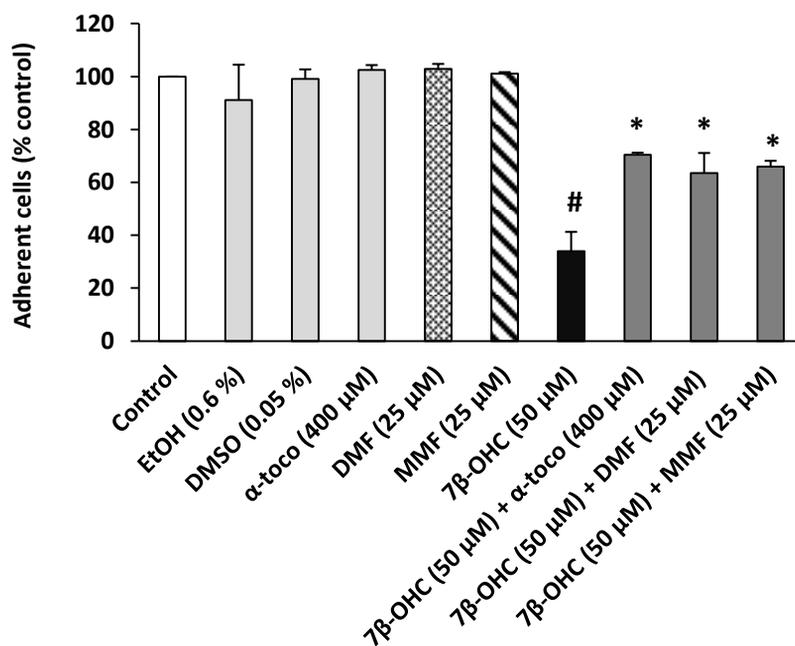
A**B**

Fig. 1; Sghaier R et al

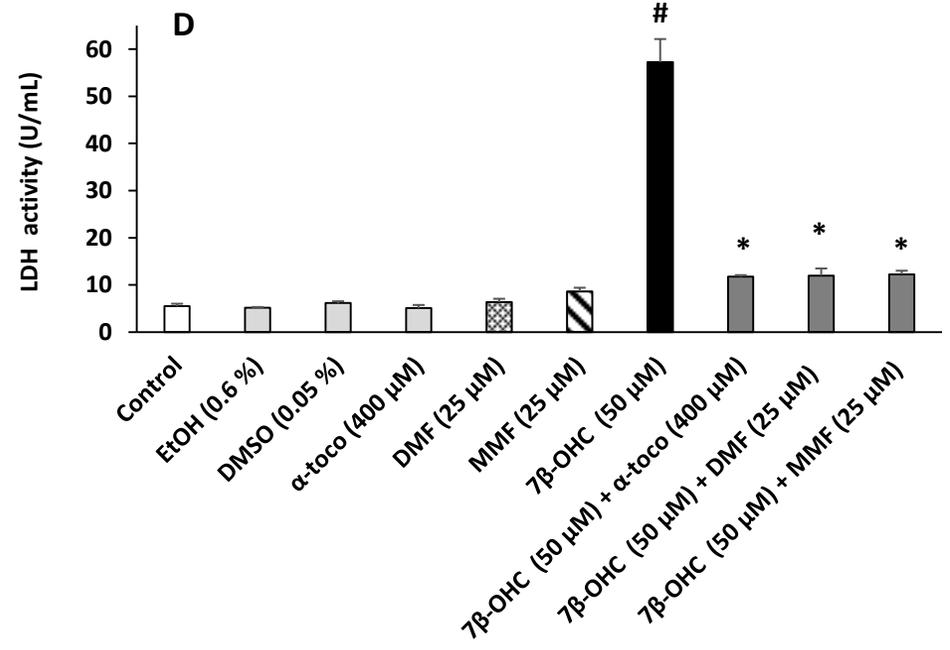
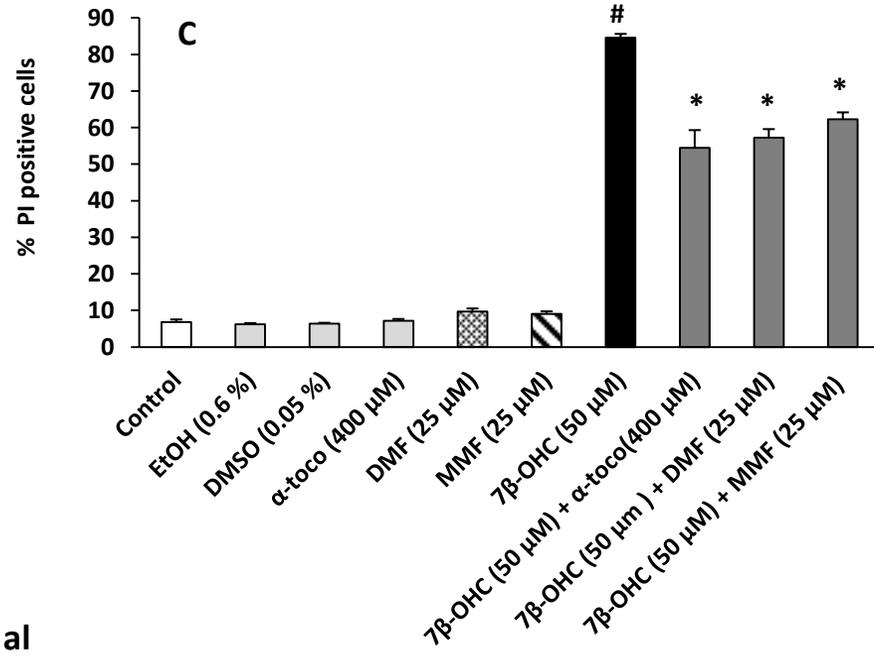
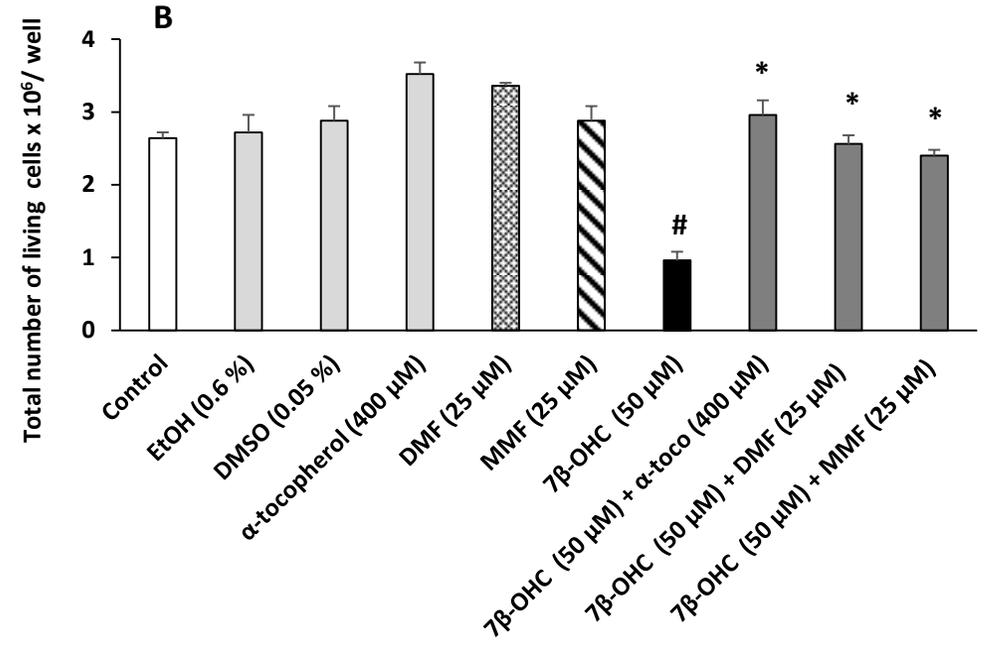
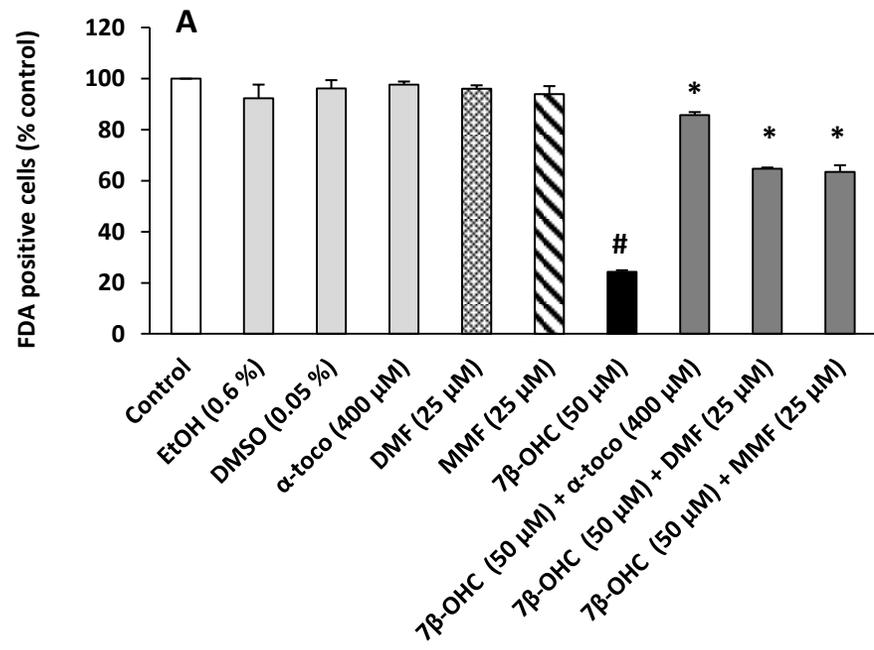


Fig. 2; Sghaier R et al

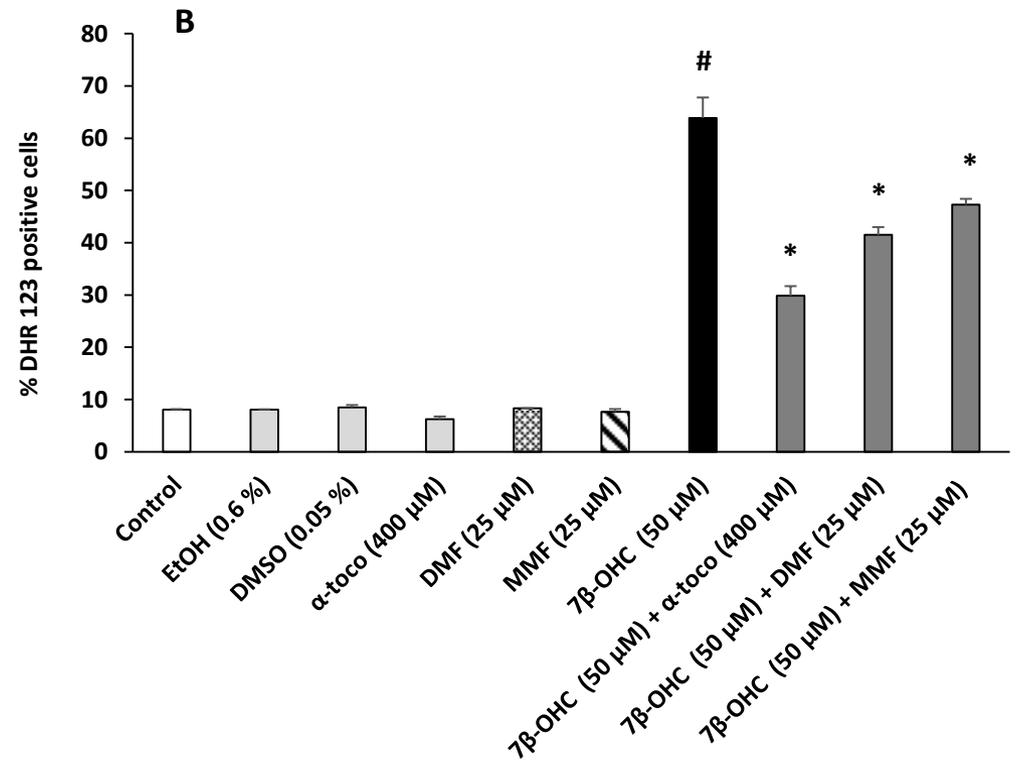
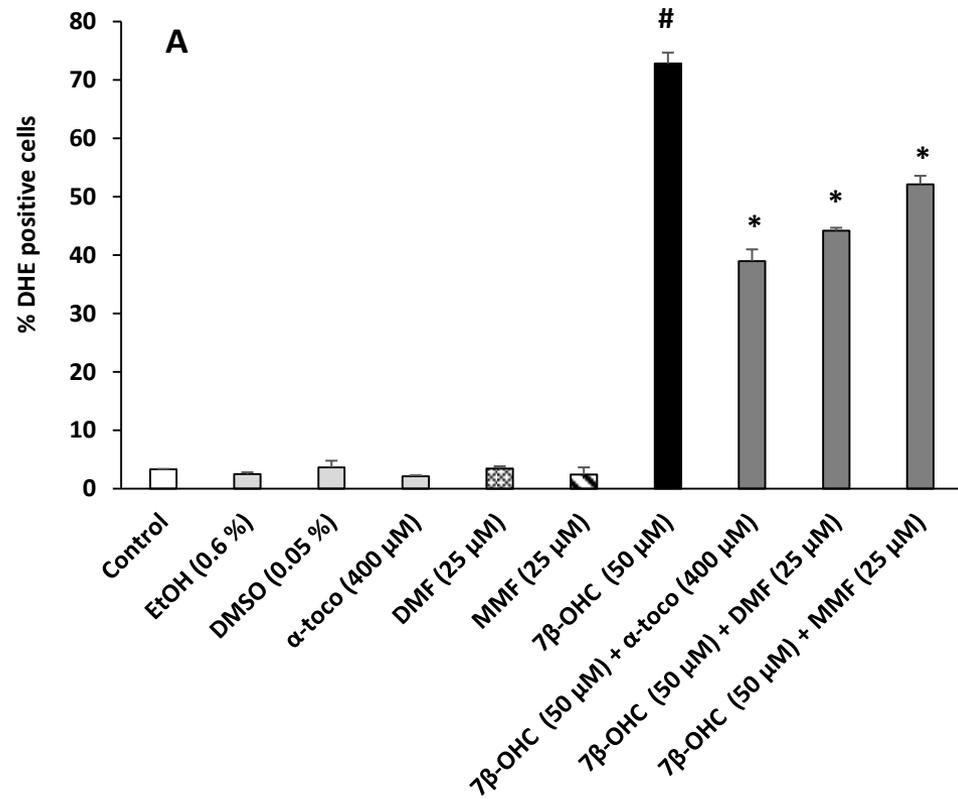


Fig. 3; Sghaier R et al

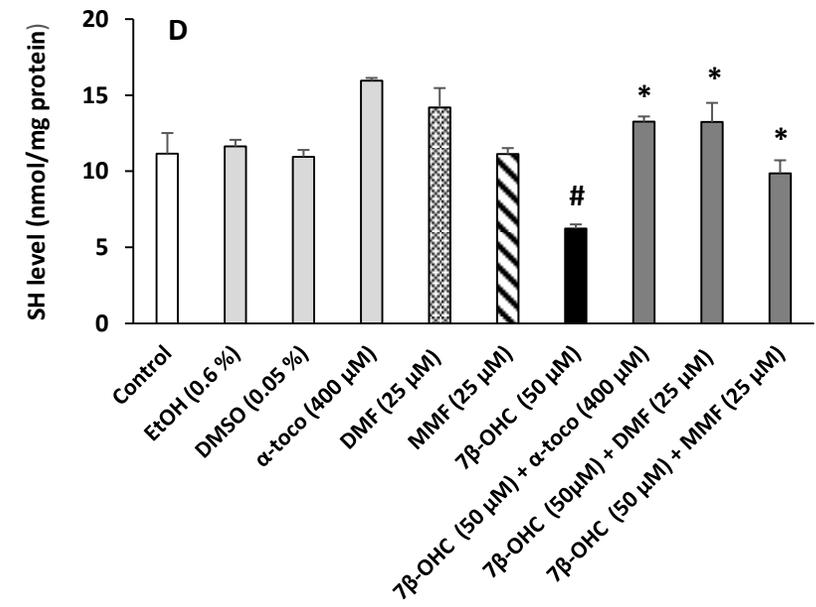
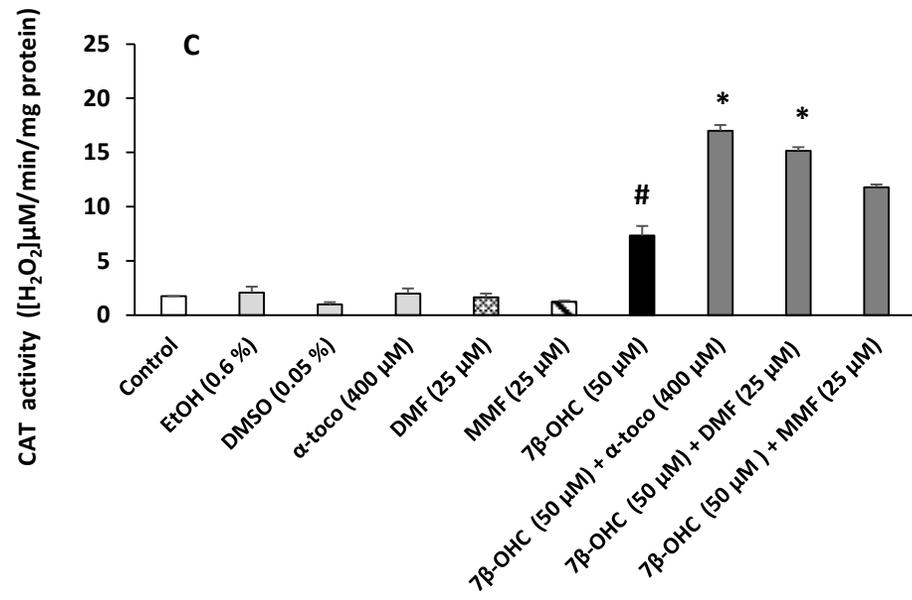
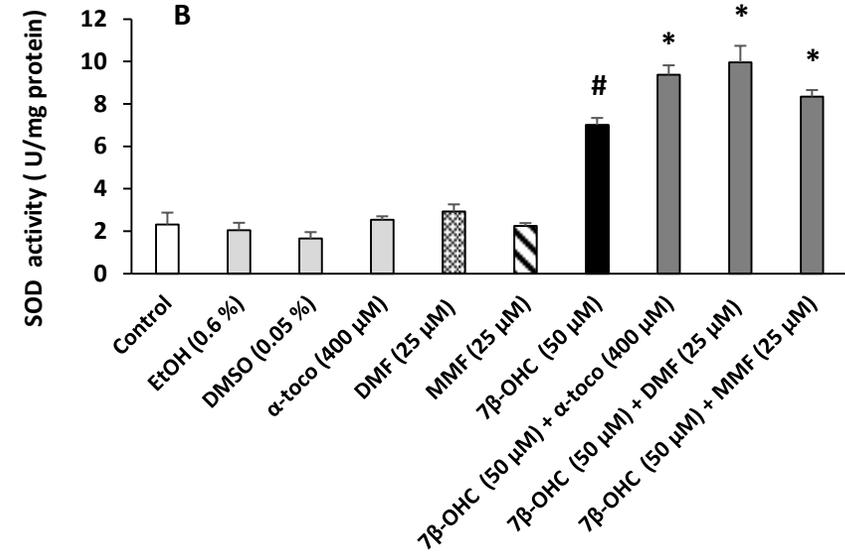
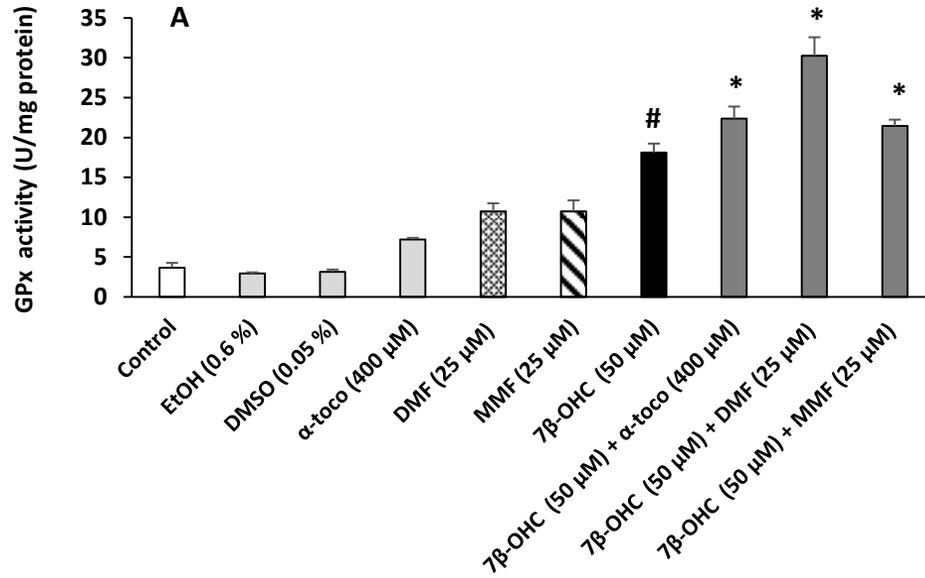


Fig. 4; Sghaier R et al

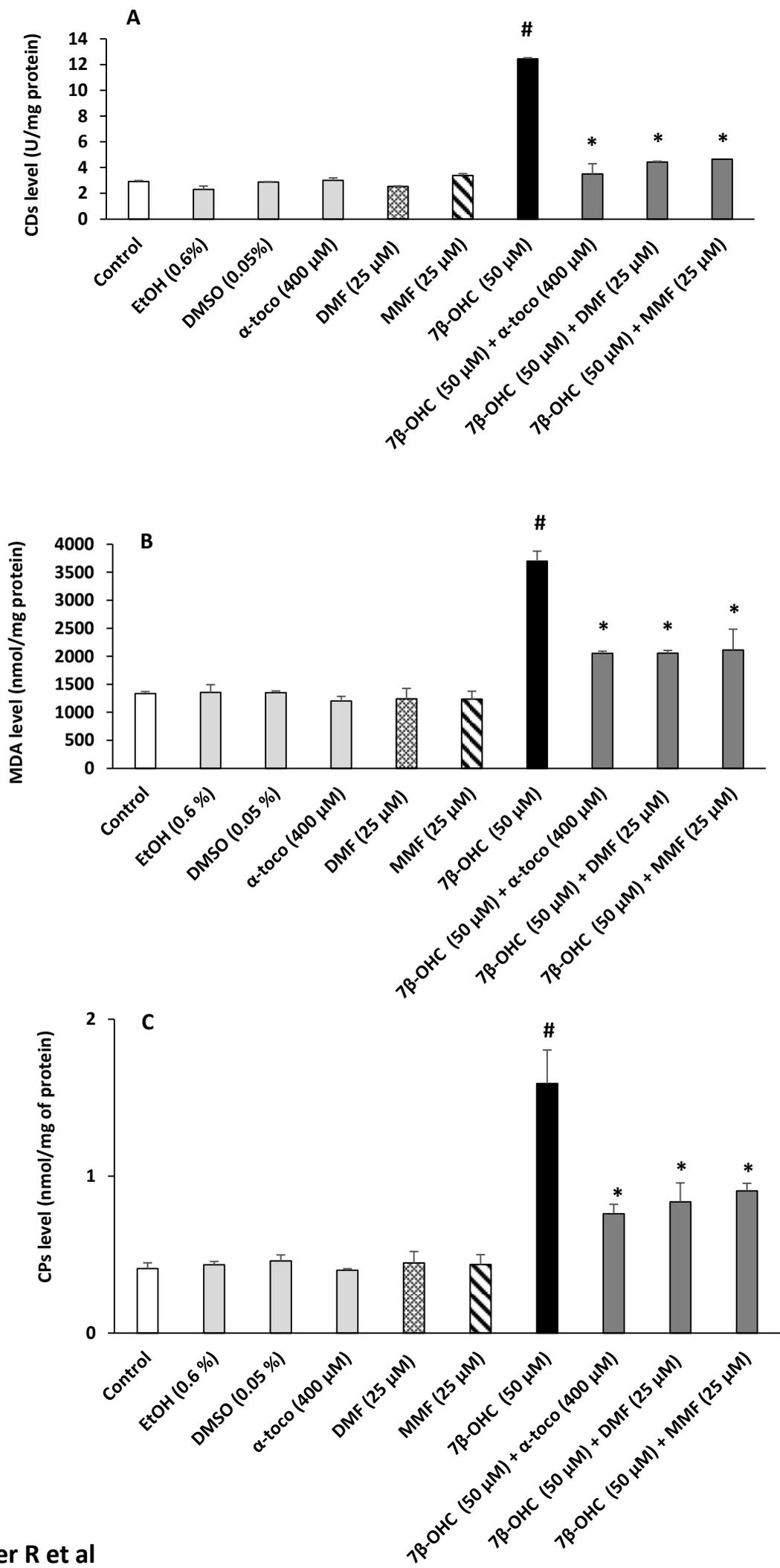


Fig. 5; Sghaier R et al

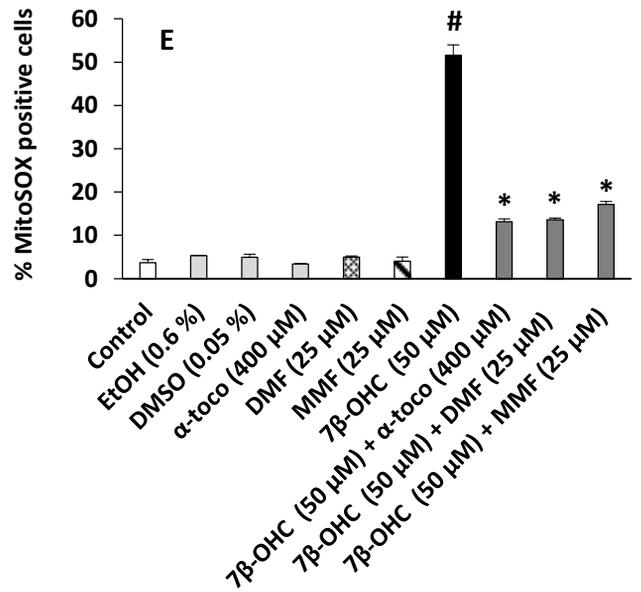
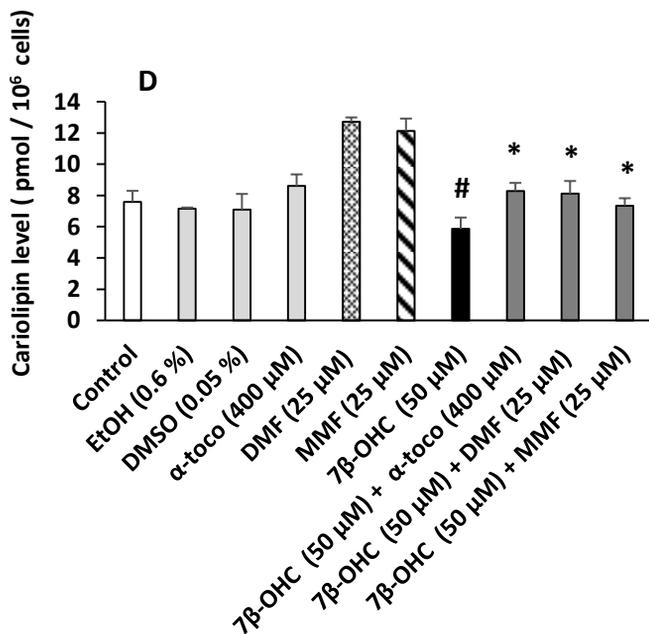
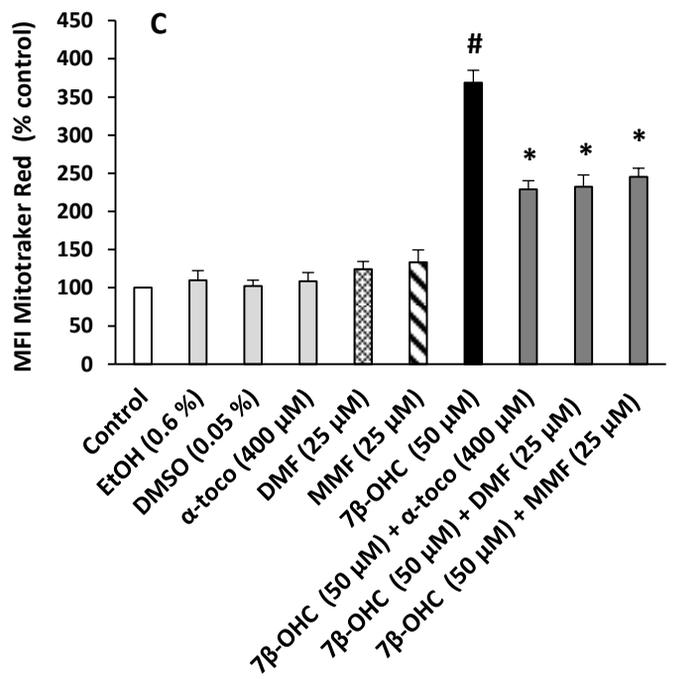
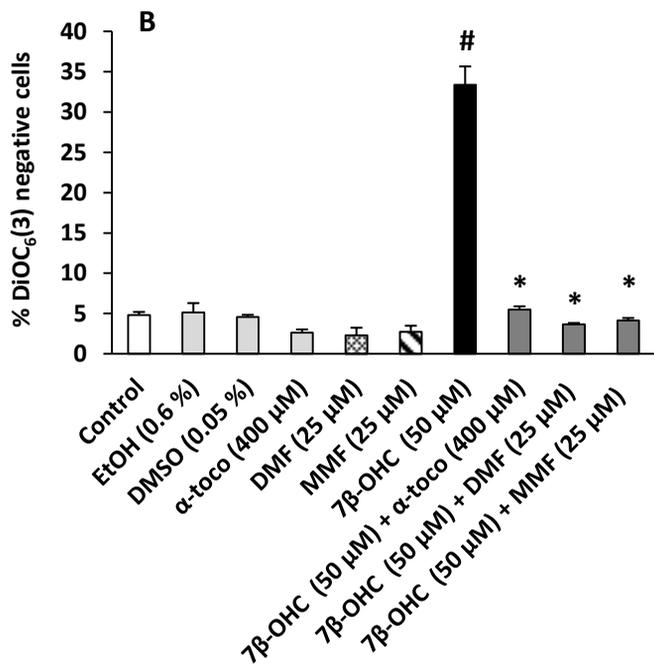
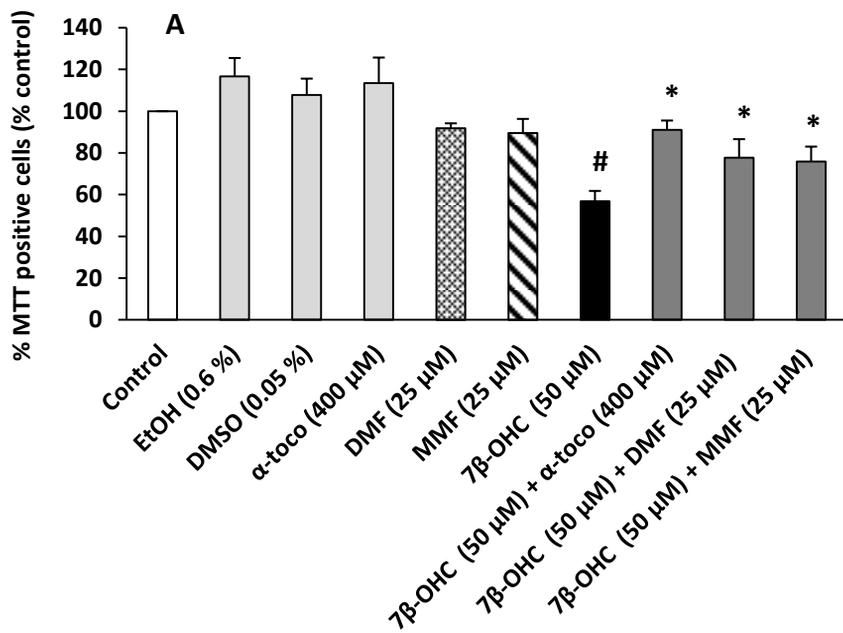


Fig. 6; Sghaier R et al

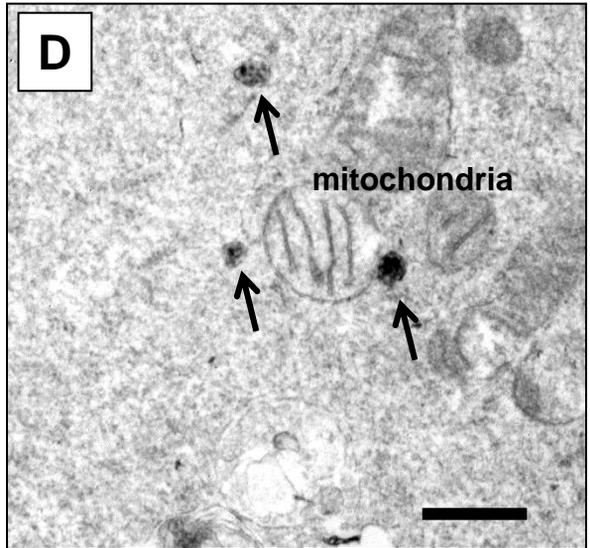
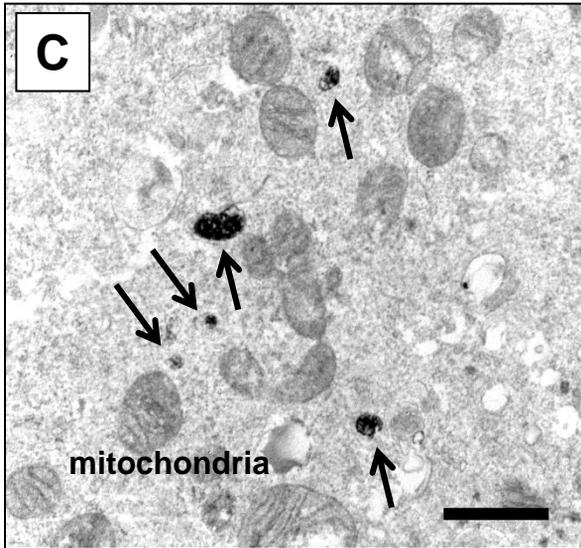
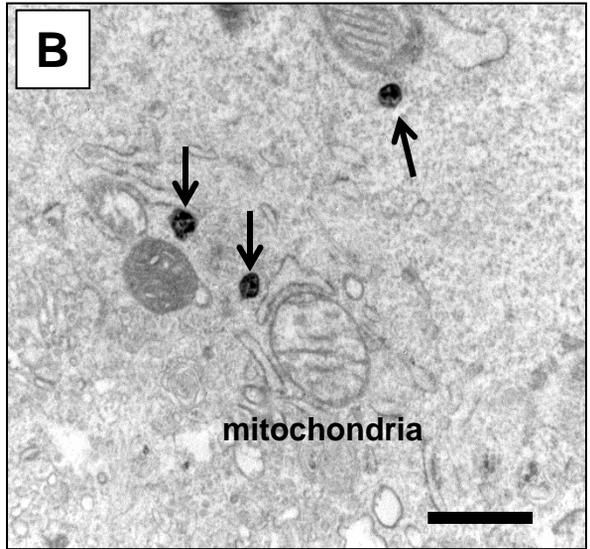
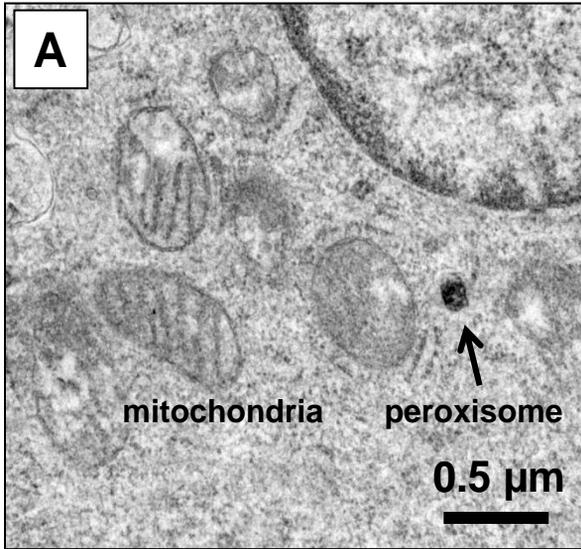


Fig. 7; Sghaier R et al

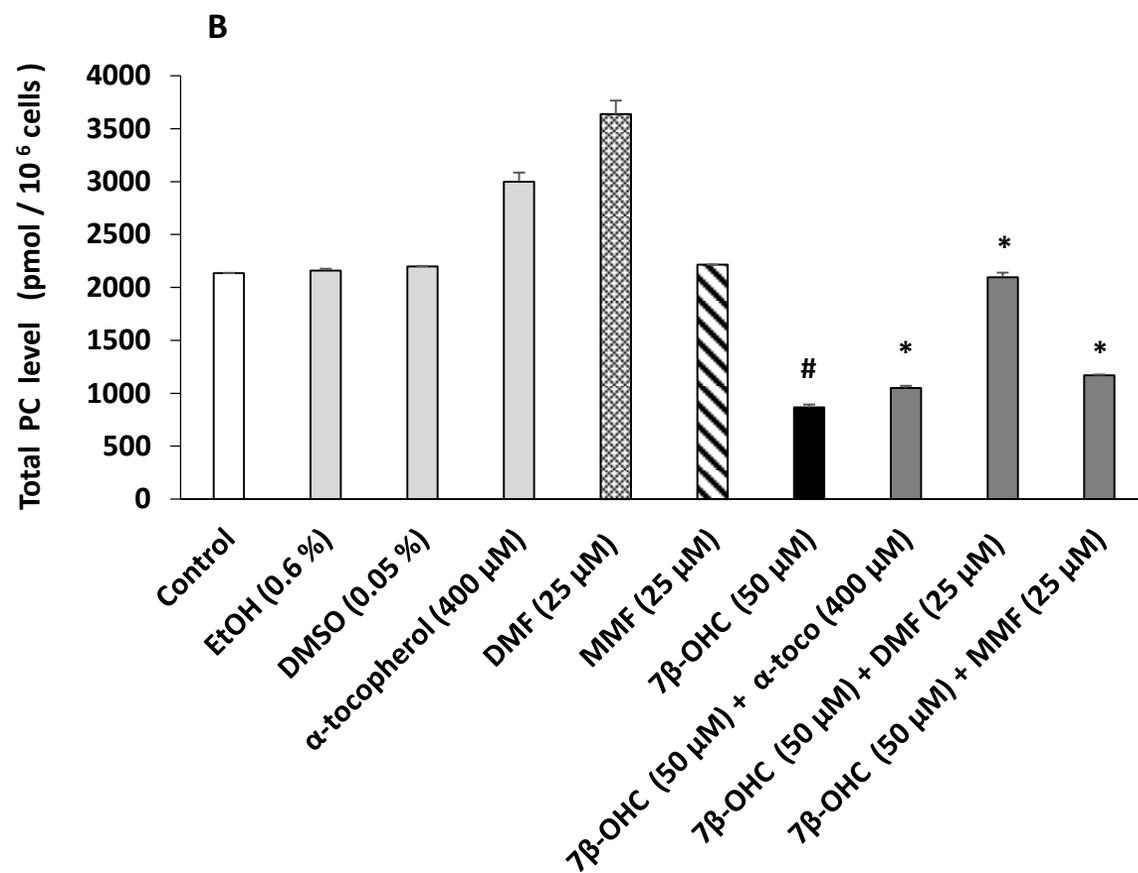
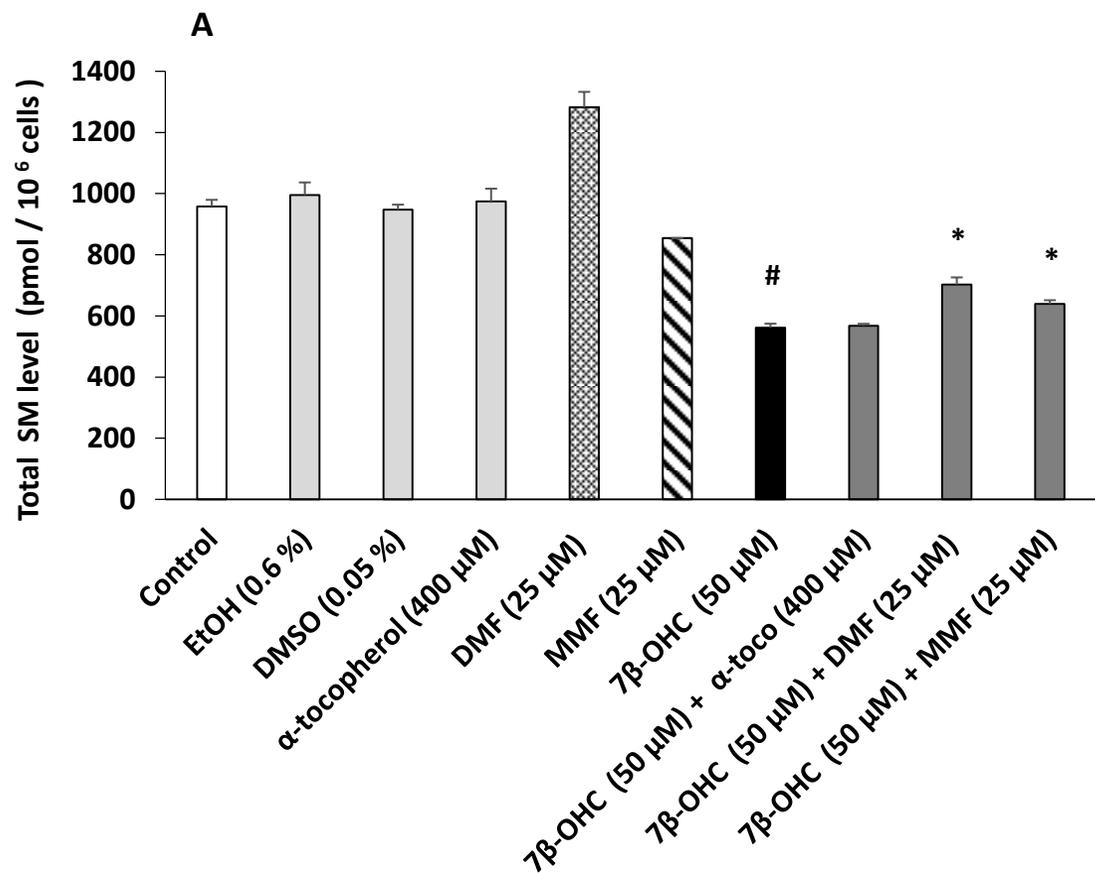


Fig. 8; Sghaier R et al

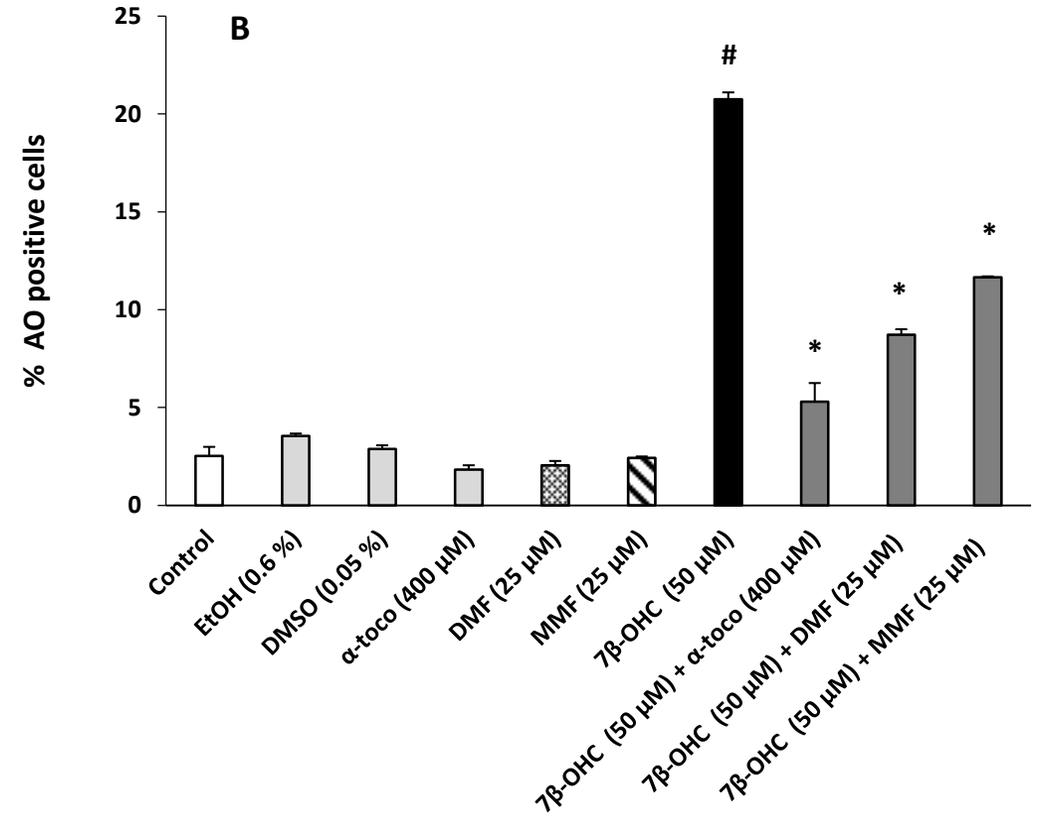
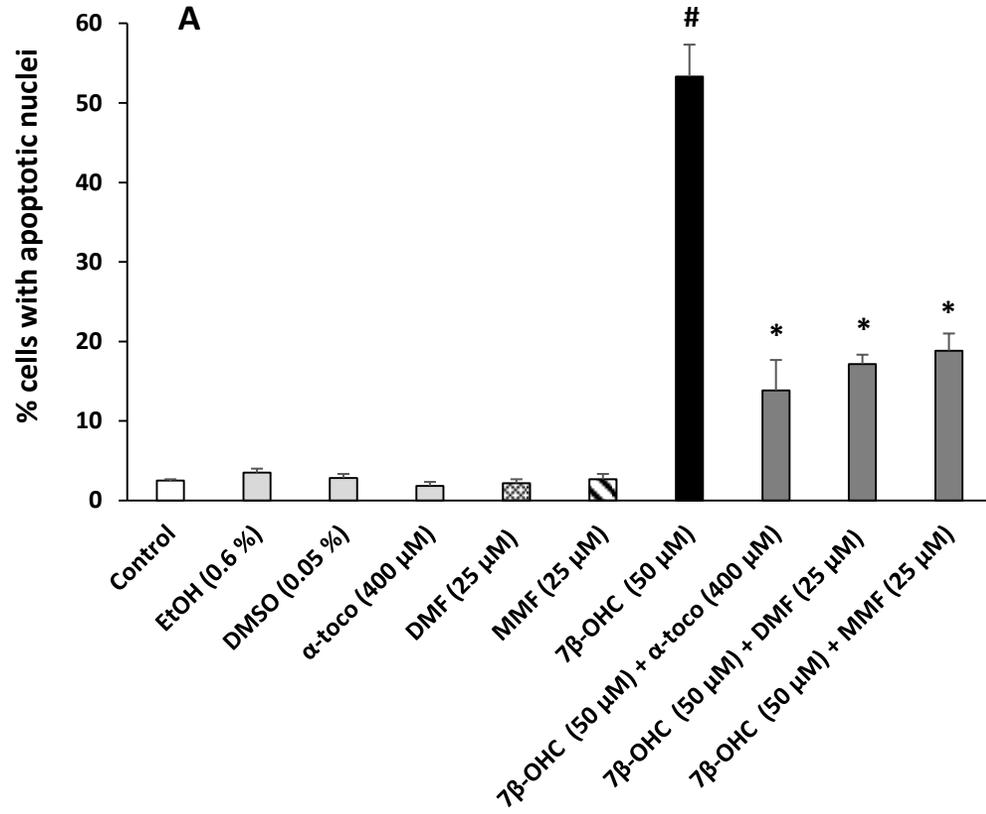


Fig. 9; Sghaier R et al

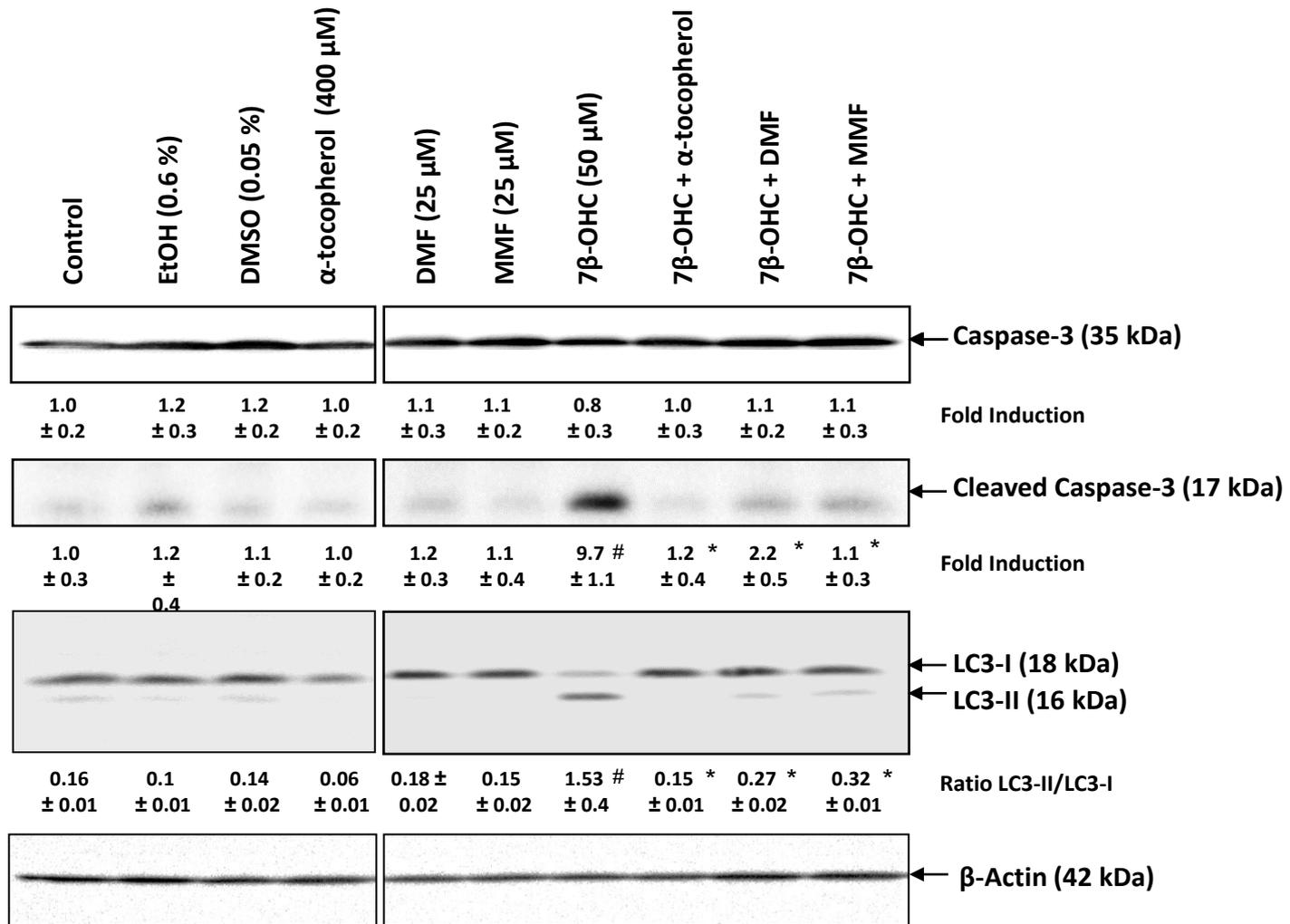


Fig. 10; Sghaier R et al

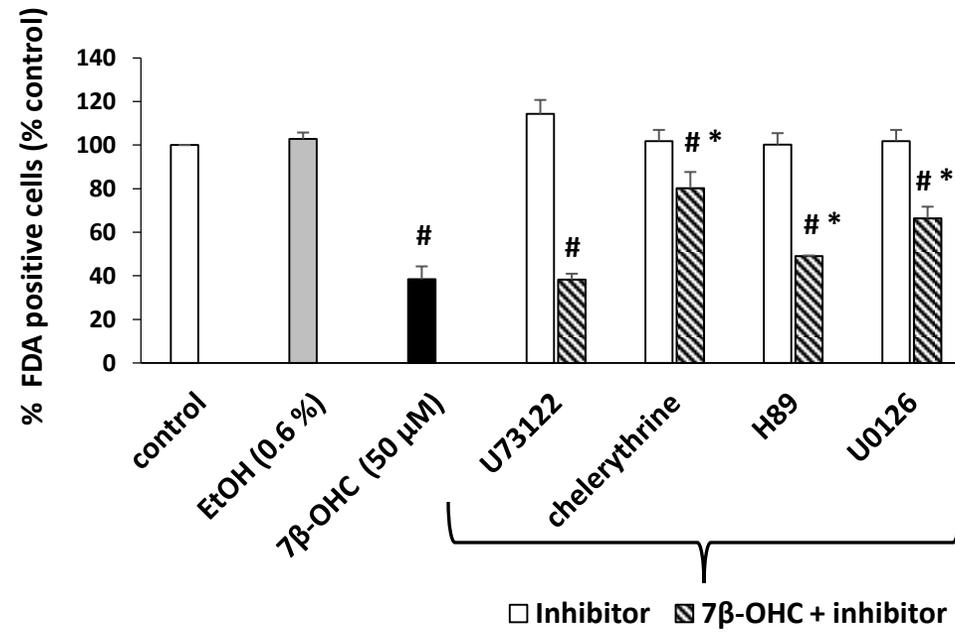


Fig. 11; Sghaier R et al

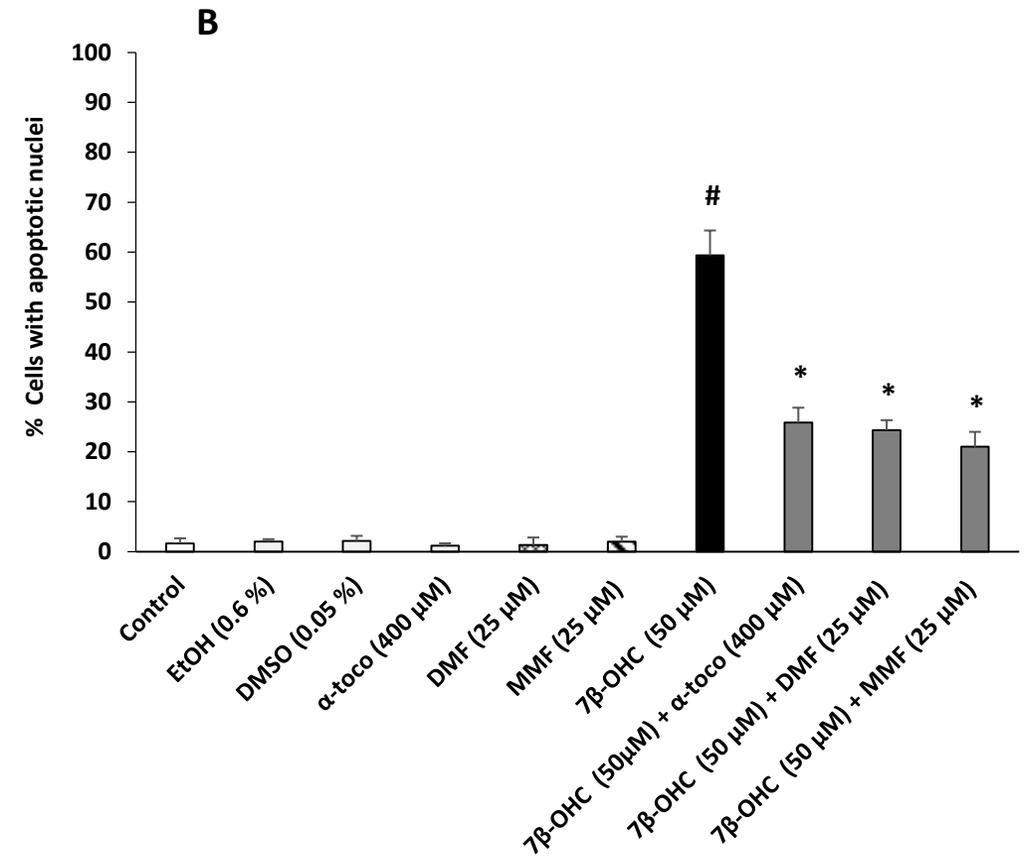
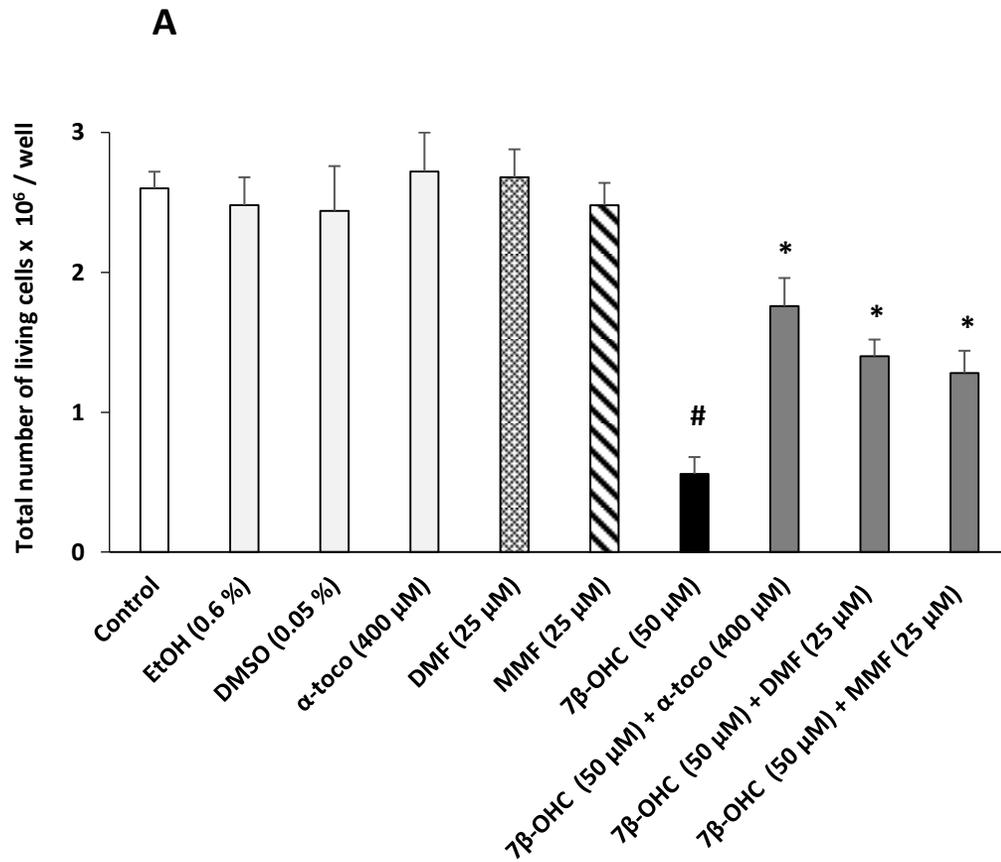


Fig. 12; Sghaier R et al

Table 1: Profile of organic acids in 158N cells exposed to 7 β -hydroxycholesterol with or without dimethyl fumarate (DMF) or monomethyl fumarate (MMF)

	Lactic acid	Pyruvic acid	Succinic acid	Fumaric acid	Malic acid	Citric acid
Control	126.98 \pm 7.99	15.27 \pm 0.36	7.55 \pm 0.11	1.33 \pm 0.04	3.69 \pm 0.10	4.79 \pm 0.20
EtOH (0.6%)	122.64 \pm 3.53	14.13 \pm 0.50	6.82 \pm 0.05	1.22 \pm 0.06	2.54 \pm 0.08	3.84 \pm 0.11
DMSO (0.05%)	125.90 \pm 12.62	13.43 \pm 0.78	6.88 \pm 0.11	1.30 \pm 0.03	2.36 \pm 0.03	4.60 \pm 0.06
DMF (25 μ M)	97.30 \pm 1.51	16.43 \pm 1.48	6.97 \pm 0.73	2.66 \pm 0.21	3.88 \pm 0.22	5.89 \pm 0.16
MMF (25 μ M)	104.84 \pm 3.45	15.46 \pm 0.01	7.18 \pm 0.11	2.72 \pm 0.05	4.93 \pm 0.17	6.34 \pm 0.04
7 β -OHC (50 μ M)	220.59 \pm 2.50 #	8.11 \pm 0.06 #	5.46 \pm 0.01 #	0.93 \pm 0.02 #	1.75 \pm 0.08 #	2.80 \pm 0.25 #
7 β -OHC (50 μ M) + DMF (25 μ M)	147.63 \pm 6.46*	15.64 \pm 0.87*	7.41 \pm 0.58*	1.35 \pm 0.16 *	4.67 \pm 0.11*	3.60 \pm 0.05*
7 β -OHC (50 μ M) + MMF (25 μ M)	120.84 \pm 4.03*	12.55 \pm 1.35*	11.48 \pm 0.45*	1.78 \pm 0.00*	3.59 \pm 0.08*	4.07 \pm 0.08*

Concentrations are expressed as ng/million cells.

Results are expressed as mean \pm SD of three independent experiments. A two way ANOVA followed by a Student's *t*-test was realized. Significant difference between vehicle (EtOH 0.6%) and 7 β -OHC-treated cells is indicated by #; *P* < 0.05; significant difference between 7 β -OHC and (7 β -OHC + DMF) or (7 β -OHC + MMF) is indicated by *; *P* < 0.05.

No significant differences are observed between Control, EtOH and DMSO. 7 β -hydroxycholesterol: 7 β -OHC.

Table 2: Profiles of cholesterol, cholesterol precursors (lathosterol, desmosterol, lanosterol) and oxysterols (triol, 7-ketocholesterol, 7 β -hydroxycholesterol) in 158N cells exposed to 7 β -hydroxycholesterol with or without dimethyl fumarate (DMF) or monomethyl fumarate (MMF)

	Cholesterol	Cholesterol precursors			Oxysterols		
		<i>Lathosterol</i>	<i>Desmosterol</i>	<i>Lanosterol</i>	<i>Triol</i>	<i>7KC</i>	<i>7β-OHC</i>
Control	2.84 \pm 0.01	14.80 \pm 0.15	12.93 \pm 0.37	0.63 \pm 0.00	0.17 \pm 0.01	0.56 \pm 0.02	0.62 \pm 0.00
EtOH (0.6%)	2.86 \pm 0.02	14.08 \pm 0.02	12.08 \pm 0.13	0.58 \pm 0.02	0.12 \pm 0.01	0.59 \pm 0.00	0.66 \pm 0.04
DMSO (0.05%)	2.64 \pm 0.03	13.64 \pm 0.36	10.01 \pm 0.42	0.45 \pm 0.03	0.08 \pm 0.01	0.42 \pm 0.06	0.56 \pm 0.02
DMF (25 μM)	2.72 \pm 0.11	14.93 \pm 0.19	9.58 \pm 0.26	0.65 \pm 0.02	0.09 \pm 0.02	0.51 \pm 0.00	0.62 \pm 0.07
MMF (25 μM)	2.83 \pm 0.10	17.10 \pm 0.94	10.07 \pm 0.50	0.69 \pm 0.03	0.05 \pm 0.01	0.60 \pm 0.09	2.94 \pm 0.07
7β-OHC (50 μM)	2.17 \pm 0.13 #	8.53 \pm 0.17 #	6.46 \pm 0.07 #	0.23 \pm 0.01 #	1.89 \pm 0.16 #	10.88 \pm 0.34 #	246.70 \pm 0.59 #
7β-OHC (50 μM) + DMF (25 μM)	2.56 \pm 0.05 *	5.33 \pm 0.14 *	6.20 \pm 0.07*	0.66 \pm 0.03 *	0.86 \pm 0.01 *	5.72 \pm 0.39 *	470.12 \pm 9.95 *
7β-OHC (50 μM) + MMF (25 μM)	2.16 \pm 0.12	6.72 \pm 0.34 *	5.08 \pm 0.21 *	0.32 \pm 0.00*	0.88 \pm 0.29 *	9.33 \pm 0.47 *	426.67 \pm 6.19 *

Concentrations are expressed as ng/million cells.

Results are expressed as mean \pm SD of three independent experiments. A two way ANOVA followed by a Student's t-test was realized. Significant difference between vehicle (EtOH 0.6%) and 7 β -OHC-treated cells is indicated by #; $P < 0.05$; significant difference between 7 β -OHC and (7 β -OHC + DMF) or (7 β -OHC + MMF) is indicated by *; $P < 0.05$. No significant differences are observed between Control, EtOH and DMSO.

7 β -hydroxycholesterol: 7 β -OHC.

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Table 3: Profile of saturated fatty acids in 158N cells exposed to 7 β -hydroxycholesterol with or without dimethyl fumarate (DMF) or monomethyl fumarate (MMF)

	Control	EtOH (0.6%)	DMSO (0.05%)	DMF (25 μ M)	MMF (25 μ M)	7 β -OHC (50 μ M)	7 β -OHC (50 μ M) + DMF (25 μ M)	7 β -OHC (50 μ M) + MMF (25 μ M)
C14:0	109.48 \pm 0.30	101.56 \pm 0.86	79.45 \pm 0.46	98.99 \pm 3.83 ¥	99.55 \pm 5.57 ¥	81.82 \pm 0.36 #	49.48 \pm 4.56 *	85.74 \pm 5.30
C16:0	2360.32 \pm 25.67	2300.14 \pm 40.89	2247.38 \pm 8.37	1934.86 \pm 20.36 ¥	2320.20 \pm 45.94 ¥	1727.54 \pm 29.59 #	1650.60 \pm 14.00 *	2553.74 \pm 23.91 *
C18:0	1240.25 \pm 21.80	1169.50 \pm 22.09	941.92 \pm 10.14	875.63 \pm 22.65 ¥	1338.75 \pm 7.60 ¥	722.54 \pm 16.83 #	805.03 \pm 6.67 *	829.81 \pm 2.14 *
C20:0	29.40 \pm 2.49	24.36 \pm 1.17	25.19 \pm 1.26	20.04 \pm 0.64 ¥	19.90 \pm 0.05 ¥	10.83 \pm 1.14 #	8.61 \pm 1.03 *	13.59 \pm 0.25 *
C22:0	4.61 \pm 0.54	4.49 \pm 0.18	4.69 \pm 0.01	4.38 \pm 0.12 ¥	4.79 \pm 0.53	8.19 \pm 0.26 #	5.97 \pm 0.06 *	6.68 \pm 0.49 *
C24:0	8.32 \pm 0.31	8.27 \pm 0.18	7.68 \pm 0.03	8.61 \pm 0.36 ¥	11.10 \pm 3.24 ¥	24.58 \pm 0.84 #	7.52 \pm 0.70 *	10.22 \pm 0.58 *
C26:0	1.47 \pm 0.13	1.51 \pm 0.28	1.56 \pm 0.04	3.78 \pm 0.24 ¥	2.54 \pm 0.07 ¥	11.85 \pm 0.91 #	5.79 \pm 0.45*	7.96 \pm 0.34 *
ΣSFA (C<22)	3739.44 \pm 44.69	3595.56 \pm 16.77	3293.94 \pm 0.05	2929.52 \pm 2.18 ¥	3778.41 \pm 59.16 ¥	2542.72 \pm 14.26 #	2513.71 \pm 26.26	3482.88 \pm 31.60 *
ΣVLCSFA C\geq22)	14.40 \pm 0.98	14.27 \pm 0.08	13.93 \pm 0.07	16.78 \pm 0.71 ¥	18.42 \pm 3.85¥	44.62 \pm 2.01 #	19.28 \pm 1.09 *	24.87 \pm 1.42 *
Σtotal SFA	3753.84 \pm 43.70	3609.83 \pm 16.69	3307.87 \pm 0.02	2946.30 \pm 1.47 ¥	3796.83 \pm 63.00 ¥	2587.34 \pm 12.25 #	2533.00 \pm 27.35 *	3507.75 \pm 30.18 *

Concentrations are expressed as ng/million cells.

Results are expressed as mean \pm SD of three independent experiments. A two way ANOVA followed by a Student's t-test was realized. Significant difference between vehicle (EtOH 0.6%) and 7 β -OHC-treated cells is indicated by #; $P < 0.05$; significant difference between 7 β -OHC and (7 β -OHC + DMF) or (7 β -OHC + MMF) is indicated by *; $P < 0.05$; significant difference between vehicle (DMSO 0.05%) and DMF or MMF treated cells is indicated by ¥; $P < 0.05$. No significant differences are observed between Control, EtOH and DMSO.

Σ SFA: Sum of saturated fatty acids; Σ VLCSFA: Sum of very long chain saturated fatty acids; 7 β -hydroxycholesterol: 7 β -OHC.

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Table 4: Profile of mono-unsaturated fatty acids profile in 158N cells exposed to 7 β -hydroxycholesterol with or without dimethyl fumarate (DMF) and monomethyl fumarate (MMF)

	Control	EtOH (0.6%)	DMSO (0.05%)	DMF (25 μ M)	MMF (25 μ M)	7 β -OHC (50 μ M)	7 β -OHC (50 μ M) + DMF (25 μ M)	7 β -OHC (50 μ M) + MMF (25 μ M)
C14:1 n-5	2.19 \pm 0.00	2.10 \pm 0.02	1.53 \pm 0.22	2.22 \pm 0.16 ¥	3.29 \pm 0.16 ¥	1.61 \pm 0.09 #	1.82 \pm 0.05 *	1.71 \pm 0.20
C16:1 n-10 or n-9	231.31 \pm 6.96	230.16 \pm 9.14	175.46 \pm 3.55	176.83 \pm 13.98	241.36 \pm 6.80 ¥	207.41 \pm 6.36 #	274.92 \pm 6.32 *	276.70 \pm 14.62 *
C16:1 n-7	454.04 \pm 26.71	420.16 \pm 28.80	352.91 \pm 10.09	400.29 \pm 4.27 ¥	553.16 \pm 11.81 ¥	313.62 \pm 11.98 #	373.66 \pm 2.06 *	545.29 \pm 4.92 *
C18:1 n-9	2239.11 \pm 16.15	2035.46 \pm 48.32	1812.39 \pm 30.35	2053.93 \pm 47.61 ¥	2487.96 \pm 8.81 ¥	1970.72 \pm 36.33	1746.52 \pm 0.25 *	1759.29 \pm 0.74 *
C18:1 n-7	502.24 \pm 8.92	464.92 \pm 46.02	448.74 \pm 28.15	546.50 \pm 18.31 ¥	718.05 \pm 51.47 ¥	403.71 \pm 2.75 #	452.47 \pm 31.14 *	439.80 \pm 1.16 *
C20:1 n-9	62.63 \pm 3.25	54.37 \pm 0.23	56.61 \pm 2.99	91.13 \pm 8.80 ¥	84.90 \pm 4.67 ¥	16.82 \pm 0.11 #	55.57 \pm 0.17 *	55.03 \pm 1.27 *
C20:1 n-7	32.03 \pm 0.04	34.63 \pm 0.47	35.26 \pm 0.16	24.77 \pm 2.21 ¥	27.13 \pm 0.48 ¥	5.65 \pm 1.00 #	12.83 \pm 0.45 *	14.74 \pm 0.40 *
C22:1 n-9	2.37 \pm 0.05	2.34 \pm 0.04	2.00 \pm 0.06	2.96 \pm 0.14 ¥	2.85 \pm 0.25 ¥	2.29 \pm 0.16	3.06 \pm 0.20 *	2.25 \pm 0.11
C24:1 n-9	12.58 \pm 0.53	13.01 \pm 0.01	14.61 \pm 0.51	22.37 \pm 0.58 ¥	24.19 \pm 1.78 ¥	32.71 \pm 0.96 #	23.52 \pm 1.44 *	23.99 \pm 2.85 *
C26:1 n-9	0.59 \pm 0.03	0.62 \pm 0.00	0.57 \pm 0.04	1.25 \pm 0.05 ¥	1.32 \pm 0.05 ¥	2.27 \pm 0.08 #	1.30 \pm 0.02 *	1.64 \pm 0.03 *
ΣMUFA	3539.09 \pm 47.48	3257.77 \pm 17.64	2900.09 \pm 13.39	3322.26 \pm 78.18 ¥	4144.22 \pm 55.05 ¥	2956.82 \pm 13.18 #	2945.67 \pm 20.57	3120.44 \pm 22.97 *

Concentrations are expressed as ng/million cells.

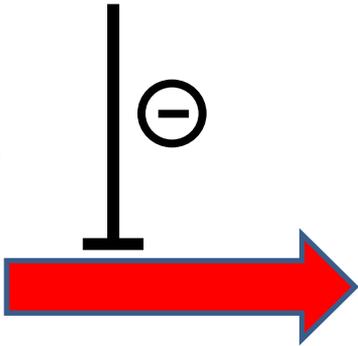
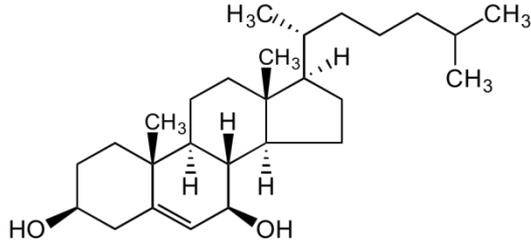
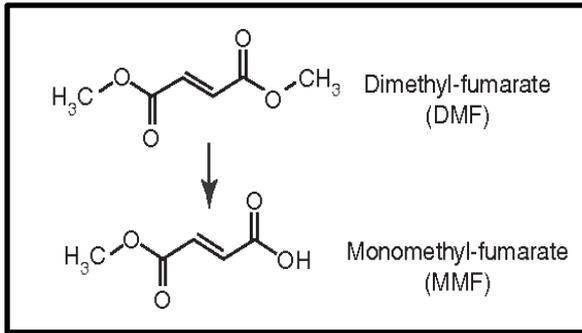
Results are expressed as mean \pm SD of 3 independent experiments. A two way ANOVA followed by a Student's t-test was realized. Significant difference between vehicle (EtOH 0.6%) and 7 β -OHC-treated cells is indicated by #; $P < 0.05$; significant difference between 7 β -OHC and (7 β -OHC + DMF) or (7 β -OHC + MMF) is indicated by *; $P < 0.05$; significant difference between vehicle (DMSO 0.05%) and DMF or MMF treated cells is indicated by ¥; $P < 0.05$. No significant differences are observed between Control, EtOH and DMSO. Σ MUFA: Sum of mono-saturated fatty acids; 7 β -hydroxycholesterol: 7 β -OHC.

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Table 5: Profile of polyunsaturated fatty acids in 158N cells exposed to 7 β -hydroxycholesterol with or without dimethyl fumarate (DMF) or monomethyl fumarate (MMF)

	Control	EtOH (0.6%)	DMSO (0.05%)	DMF (25 μ M)	MMF (25 μ M)	7 β -OHC (50 μ M)	7 β -OHC (50 μ M) +	7 β -OHC (50 μ M) +
							DMF (25 μ M)	MMF (25 μ M)
C18:2 n-6	32.66 \pm 2.10	28.06 \pm 0.94	29.72 \pm 0.41	36.30 \pm 1.14 ¥	36.77 \pm 3.82 ¥	23.14 \pm 1.48 #	26.58 \pm 1.44 *	26.33 \pm 1.95
C20:4 n-6 (AA)	314.84 \pm 6.38	277.13 \pm 2.48	269.37 \pm 1.78	338.36 \pm 44.67 ¥	317.85 \pm 15.25 ¥	203.91 \pm 2.73 #	185.39 \pm 5.99 *	195.29 \pm 13.48
C20:5 n-3 (EPA)	450.26 \pm 2.05	400.48 \pm 22.37	451.25 \pm 13.07	200.83 \pm 5.67 ¥	203.16 \pm 10.21 ¥	127.58 \pm 0.95 #	100.79 \pm 8.65 *	114.83 \pm 5.05 *
C20:3 n-6	87.23 \pm 7.07	84.27 \pm 2.34	67.61 \pm 0.67	81.62 \pm 2.39 ¥	89.69 \pm 1.34 ¥	65.15 \pm 2.24 #	81.93 \pm 2.81 *	72.33 \pm 3.41 *
C20:2 n-6	52.96 \pm 2.27	47.51 \pm 0.10	51.64 \pm 1.70	32.50 \pm 0.25 ¥	36.01 \pm 4.04 ¥	15.72 \pm 2.19 #	17.71 \pm 1.38*	34.50 \pm 1.08 *
C22:6 n-3 (DHA)	95.82 \pm 2.21	82.47 \pm 0.90	79.76 \pm 1.66	98.43 \pm 5.62 ¥	97.45 \pm 4.70 ¥	70.85 \pm 0.86 #	70.65 \pm 0.46	76.97 \pm 3.81 *
C22:5 n-3 (DPA)	92.21 \pm 1.70	90.63 \pm 3.51	79.05 \pm 1.42	93.39 \pm 5.68 ¥	89.42 \pm 2.41 ¥	63.52 \pm 0.87 #	76.81 \pm 0.17 *	75.06 \pm 0.15 *
ΣPUFA	1588.33 \pm 14.33	1373.67 \pm 29.60	1512.94 \pm 4.59	1318.22 \pm 34.65 ¥	870.35 \pm 39.09 ¥	872.40 \pm 22.61 #	562.57 \pm 0.47 *	628.02 \pm 1.89 *
ΣPUFA (n-3)	638.29 \pm 5.97	573.58 \pm 26.79	610.05 \pm 9.99	392.65 \pm 5.60 ¥	390.04 \pm 17.32 ¥	261.95 \pm 0.95 #	248.25 \pm 8.02 *	266.85 \pm 8.71
ΣPUFA (n-6)	487.70 \pm 5.07	436.96 \pm 1.18	418.35 \pm 1.16	488.78 \pm 43.66 ¥	480.31 \pm 21.77 ¥	307.91 \pm 4.25 #	311.61 \pm 6.01	328.45 \pm 17.76
ΣPUFA n-3/ΣPUFA n-6	1.31 \pm 0.00	1.31 \pm 0.06	1.46 \pm 0.03	0.81 \pm 0.06 ¥	0.81 \pm 0.00 ¥	0.85 \pm 0.01 #	0.80 \pm 0.04	0.82 \pm 0.07
Δ 4 : C22:6 n-3/C22 :5 n-3	1.04 \pm 0.00	0.91 \pm 0.03	1.01 \pm 0.00	1.06 \pm 0.12	1.09 \pm 0.02 ¥	1.12 \pm 0.03 #	0.92 \pm 0.00 *	1.03 \pm 0.05 *
Δ 5: C20:4 n-6 /C20:3 n-6	3.64 \pm 0.37	3.29 \pm 0.12	3.98 \pm 0.01	4.16 \pm 0.67	3.55 \pm 0.22 ¥	3.13 \pm 0.07	2.27 \pm 0.15 *	2.70 \pm 0.06 *
Δ 8: C20:3 n-6 /C20 :2 n-6	1.64 \pm 0.06	1.77 \pm 0.05	1.31 \pm 0.06	2.51 \pm 0.09 ¥	2.53 \pm 0.32 ¥	4.25 \pm 0.73 #	4.67 \pm 0.52	2.10 \pm 0.16 *
Δ 9: C16:1 n-7/ C16:0	0.19 \pm 0.01	0.18 \pm 0.02	0.16 \pm 0.00	0.21 \pm 0.00 ¥	0.24 \pm 0.00 ¥	0.18 \pm 0.00	0.23 \pm 0.00 *	0.21 \pm 0.00 *
Δ 9: C18:1 n-9/ c18:0	1.81 \pm 0.02	1.74 \pm 0.07	1.92 \pm 0.05	2.35 \pm 0.01 ¥	1.86 \pm 0.02	2.73 \pm 0.01 #	2.17 \pm 0.02 *	2.12 \pm 0.01 *
Elongase: 22:5 n-3 /C20:5 n-3	0.20 \pm 0.00	0.23 \pm 0.00	0.18 \pm 0.01	0.47 \pm 0.04 ¥	0.44 \pm 0.01 ¥	0.50 \pm 0.00 #	0.77 \pm 0.07 *	0.65 \pm 0.03 *
Elongase : C18:0/C16:0	0.53 \pm 0.00	0.51 \pm 0.02	0.42 \pm 0.01	0.45 \pm 0.02 ¥	0.58 \pm 0.01 ¥	0.42 \pm 0.02 #	0.49 \pm 0.00 *	0.32 \pm 0.00 *

Concentrations are expressed as ng/million cells. Results are expressed as mean \pm SD of three independent experiments. A two way ANOVA followed by a Student's t-test was realized. Significant difference between vehicle (EtOH 0.6%) and 7 β -OHC-treated cells is indicated by #; $P < 0.05$; significant difference between 7 β -OHC and (7 β -OHC + DMF) or (7 β -OHC + MMF) is indicated by *; $P < 0.05$; significant difference between vehicle (DMSO 0.05%) and DMF or MMF treated cells is indicated by ¥; $P < 0.05$. No significant differences are observed between Control, EtOH and DMSO. Σ PUFA: sum of polyunsaturated fatty acids; Δ 4: delta 4 desaturase; Δ 5: delta 5 desaturase; Δ 8: delta 8 desaturase; Δ 9: delta 9 desaturase; 7 β -hydroxycholesterol: 7 β -OHC.



**Neuroprotective properties
of DMF and MMF
(158N oligodendrocytes)**

- Oxiapoptophagy**
- *Oxidative stress*
 - *Mitochondrial dysfunctions / apoptosis*
 - *Autophagy*

**Modifications of lipid profile
(sterols, fatty acids)**