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### ► To cite this version:

Olivier Le Bacquer, Phelipe Sanchez, Véronique Patrac, César Rivoirard, Nicolas Saroul, et al.. Cannabidiol protects C2C12 myotubes against cisplatin-induced atrophy by regulating oxidative stress. *American Journal of Physiology - Cell Physiology*, 2024, 326 (4), pp.C1226-C1236. 10.1152/ajp-cell.00622.2023 . hal-04619811

**HAL Id: hal-04619811**

**<https://hal.inrae.fr/hal-04619811>**

Submitted on 21 Jun 2024

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# **Cannabidiol protects C2C12 myotubes against cisplatin-induced atrophy by regulating oxidative stress**

**Running title:** CBD prevents cisplatin-induced muscle atrophy in vitro

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Supplemental data available at :

URL : [https://figshare.com/articles/figure/Supplemental\\_Figure\\_S1-AJPCellPhysiol\\_2024\\_pdf/25196435](https://figshare.com/articles/figure/Supplemental_Figure_S1-AJPCellPhysiol_2024_pdf/25196435)

DOI : <https://doi.org/10.6084/m9.figshare.25196435>

32 **ABSTRACT**

33 Cancer and chemotherapy induce a severe loss of muscle mass (known as cachexia), which  
34 negatively impact cancer treatment and patient survival. The aim of the present study was to  
35 investigate whether CBD administration may potentially antagonize the effects of cisplatin in  
36 inducing muscle atrophy, using a model of myotubes in culture. Cisplatin treatment resulted in  
37 a reduction of myotube diameter ( $15.7\pm 0.3$  vs.  $22.2\pm 0.5$   $\mu\text{m}$ ,  $p<0.01$ ) that was restored to control  
38 level with  $5\mu\text{M}$  CBD ( $20.1\pm 0.4$   $\mu\text{M}$ ,  $p<0.01$ ). Protein homeostasis was severely altered with a  
39  $\approx 70\%$  reduction in protein synthesis ( $p<0.01$ ) and a 2-fold increase in proteolysis ( $p<0.05$ ) in  
40 response to cisplatin. Both parameters were dose dependently restored by CBD co-treatment.  
41 Cisplatin treatment was associated with increased TBARS content ( $0.21\pm 0.03$  to  $0.48\pm 0.03$   
42  $\text{nmol/mg prot}$ ,  $p<0.05$ ), catalase activity ( $0.24\pm 0.01$  vs.  $0.13\pm 0.02$   $\text{nmol/min}/\mu\text{g prot}$ ,  $p<0.01$ ),  
43 whereas CBD co-treatment normalized TBARS content to control values ( $0.22\pm 0.01$   $\text{nmol/mg}$   
44  $\text{prot}$ ,  $p<0.01$ ) and reduced catalase activity ( $0.17\pm 0.01$   $\text{nmol/min}/\mu\text{g prot}$ ,  $p<0.05$ ). These  
45 changes were associated with increased mRNA expression of GPX1, SOD1, SOD2 and CAT  
46 mRNA expression in response to cisplatin ( $p<0.01$ ), which was corrected by CBD co-treatment  
47 ( $p < 0.05$ ). Last, cisplatin treatment increased the mitochondrial protein content of NDUFB8,  
48 UQCRC2, COX4 and VDAC1 (involved in mitochondrial respiration and apoptosis), and CBD  
49 co-treatment restored their expression to control values. Altogether, our results demonstrated that  
50 CBD antagonizes the cisplatin-induced C2C12 myotube atrophy and could be used as an  
51 adjuvant in the treatment of cancer cachexia to help maintain muscle mass and improve patient  
52 quality of life.

53

54 **New and noteworthy:** In an in vitro model, cisplatin treatment led to myotube atrophy  
55 associated with dysregulation of protein homeostasis and increased oxidative stress, resulting  
56 in increased apoptosis. Co-treatment with cannabidiol was able to prevent this phenotype by  
57 promoting protein homeostasis and reducing oxidative stress.

58

59 **KEYWORDS**

60 cachexia, endocannabinoid system, protein homeostasis, oxidative stress, mitochondrial  
61 function

62

## 63 INTRODUCTION

64 Cachexia is a multifactorial wasting syndrome characterized by a severe involuntary loss of  
65 body weight (i.e. more than 5% body weight loss in the last 12 months or less), loss of skeletal  
66 muscle mass (with or without loss of fat mass), anorexia, and dysregulated energy and protein  
67 metabolism (1). Prevalence of cachexia varies with cancer type, from 15% in prostate cancer  
68 up to 60% in pancreatic cancer (2). Several studies have clearly demonstrated that survival  
69 times are shorter in patients who have experienced weight loss than in those who have not.  
70 Weight loss is not only predictive of survival but also of response to chemotherapy (3). One of  
71 the mechanisms that has been advanced to explain why patients with cachexia have poorer  
72 survival is their higher incidence of complications related to surgical, radiotherapeutic and  
73 chemotherapeutic treatments (4). Cachexia is associated with increased fatigue and frailty,  
74 reduced physical activity leading to loss of autonomy, and decreased quality of life (5, 6). In  
75 some cases, the impact of cachexia is severe enough to necessitate chemotherapy dose  
76 reductions, treatment postponements or permanent discontinuation, in which case patients who  
77 lose weight do not get the full potential benefit of their cancer therapy (4, 7). Maintaining  
78 skeletal muscle mass in cancer patients is therefore crucial to their management, improved  
79 response to associated therapies, and improved quality of life. There are no formal guidelines  
80 for the management of cancer-related muscle wasting, but an effective strategy should aim to  
81 reduce muscle wasting in order to promote survival in patients with advanced cancer (8). One  
82 straightforward way to do this is through nutrition (9).

83 Cancer treatment often involves chemotherapy, which itself contributes to the development and  
84 progression of muscle atrophy and weakness in treated patients and preclinical models (10-13).  
85 Chemotherapy has been found to reduce muscle mass index and strength in lung and breast  
86 cancer patients (10), and cisplatin treatment in head and neck cancer patients decreased muscle  
87 strength measured by chair-lift and arm flexion tests (11). This same type of muscle dysfunction  
88 is also observed in preclinical models of cancer and chemotherapy. For example, in mice,  
89 implantation of C26 colonic tumor or lung carcinoma led to muscle atrophy associated with a  
90 decrease in mitochondrial respiration (14) and an alteration of the processes regulating  
91 mitochondrial biogenesis and dynamics (fusion/fission) (15). Similarly, treatments using  
92 cisplatin or doxorubicin, two widely-used chemotherapy agents, increased muscle  
93 mitochondrial dysfunction by altering mitochondrial biogenesis and dynamics (16-18). These  
94 data are consistent with the mitochondrial dysfunction, decreased mitochondrial content, and  
95 alterations in mitochondrial dynamics that are well-documented in cancer patients (19). As a

96 result, damaged mitochondria accumulate in skeletal muscle and, in addition to being less  
97 bioenergetically efficient, promote oxidative stress through increased production of reactive  
98 oxygen species (ROS). In mice, administration of doxorubicin or cisplatin leads to an increase  
99 in ROS production (16-18). Mitochondrial dysfunction and increased oxidative stress disrupt  
100 protein turnover pathways, leading to decreased protein synthesis and increased activity of  
101 muscle proteolytic systems (proteasome, autophagy) (12, 20), ultimately resulting in muscle  
102 fiber atrophy (16, 17).

103 The endocannabinoid system is a major molecular system responsible for controlling  
104 metabolism throughout the body, and is becoming an increasingly popular target for  
105 pharmacotherapy. Endocannabinoids (EC) and phytocannabinoids are the two main subclasses  
106 of cannabinoids. EC are produced by mammals, whereas phytocannabinoids, including  
107 cannabidiol (CBD), are produced by plants such as *Cannabis sativa* (21). EC exert their  
108 pharmacological effects via the endogenous endocannabinoid system, mainly by interacting  
109 with various receptors, primarily CB1 and CB2 (21). CBD is a weak agonist of CB1 and CB2,  
110 and can activate multiple cellular targets (e.g. TRPV1, PPAR $\gamma$ ) or inhibit (e.g. GPR55) (22).  
111 The EC system is involved in numerous physiological processes, such as memory, appetite, and  
112 the regulation of metabolic energy balance (21). EC exert a central effect by stimulating food  
113 intake but also by modulating lipid and carbohydrate metabolism in the liver, adipose tissue  
114 and skeletal muscle to favor energy accumulation (21). There is also growing evidence that the  
115 EC system and CBD plays an important role in regulating mitochondrial biogenesis, membrane  
116 integrity and oxidative capacity (23). Our laboratory and other teams have demonstrated that  
117 the EC system also controls muscle development (24, 25) and that alterations in the EC system  
118 are associated with muscle dysfunction (26-29). The use of CBD could therefore hold benefit  
119 for improving the treatment of cancer and cancer-induced cachexia, in particular by protecting  
120 skeletal muscle mass.

121 Recent studies have shown that CBD has antineoplastic and anti-inflammatory properties in  
122 numerous in vitro models (30). Recent evidence also indicates that CBD regulates oxidative  
123 activity and mitochondrial content in the myocardium by modulating the expression of several  
124 markers of mitochondrial biogenesis that had been severely reduced by doxorubicin treatment  
125 (13). In addition to its effects on mitochondria, CBD also has beneficial effects on skeletal  
126 muscle. In mdx mice, i.e. a model of Duchenne muscular dystrophy, Iannotti *et al.* reported that  
127 CBD was able to prevent loss of motor activity by promoting myotube formation and reducing

128 inflammation (27). CBD also reduces the production of ceramides (deleterious lipid derivatives  
129 responsible for mitochondrial dysfunction) in high-fat diet-induced obesity (31).

130 Taken together, these data show that there is currently no treatment to prevent or reduce  
131 cachexia, and that CBD could be a promising candidate compound for use as an adjuvant in  
132 cancer treatment, due to its demonstrated positive effects on mitochondrial function, oxidative  
133 stress, and skeletal muscle development, metabolism and. Here, we used a model of myotubes  
134 in culture to investigate whether CBD treatment was able to counteract the muscle atrophy  
135 induced by chemotherapy (cisplatin).

136

137 **MATERIAL AND METHODS**

138 **Chemicals and reagents.** Dulbecco's modified Eagle medium (DMEM) and phosphatase  
139 inhibitor cocktail were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Fetal  
140 bovine serum, horse serum, trypsin-EDTA, PBS, and penicillin-streptomycin were purchased  
141 from PAA (Pasching, Austria). Primary antibodies were obtained from the following sources.  
142 Thr389-phosphorylated S6K (#34475, 1/1000) total S6K (#9202, 1/1000), Ser473-  
143 phosphorylated Akt (#9271, 1/1000), total Akt (#9272, 1/1000), Ser51- phosphorylated  
144 eIF2 $\alpha$  (#3398, 1/1000), total eIF2 $\alpha$  (#5324, 1/1000), Thr172-phosphorylated AMPK (#2535,  
145 1/1000), total AMPK (#2532, 1/1000), caspase 3 (#9662, 1/1000), and VDAC (#4866, 1/1000)  
146 antibodies were from Cell Signaling Technology (distributed by Ozyme, Saint-Quentin-en-  
147 Yvelines, France). Mouse anti-puromycin mAb (clone 12D10) (#MABE343, 1/1000) was from  
148 Sigma-Aldrich (Saint-Quentin-Fallavier, France). Horseradish peroxidase-conjugated  
149 secondary antibodies were from DAKO (Trappes, France). GAPDH antibody (#9545, 1/5000)  
150 was from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Polyubiquitin antibody (#ENZ-  
151 ABS840-0100, 1/1000) was purchased from Enzo Life Sciences (Villeurbanne, France). Total  
152 OXPHOS antibody (#MS604, 1/1000) was purchased from Mitosciences (Eugene, Oregon,  
153 USA). CoxIV antibody (#MA5-31470, 1/1000) was from Thermo Fisher Scientific  
154 (Courtaboeuf, France). Cisplatin and cannabidiol and the TBARS and catalase assay kits were  
155 purchased from Cayman Chemicals (distributed by INTERCHIM, Montluçon, France).

156 **Cell culture and differentiation.** Mouse C2C12 myoblasts cells were purchased from the  
157 ATCC (#CRL-1772, American Type Culture Collection; Manassas, VA). Myoblasts were  
158 cultured in a growth medium composed of DMEM containing 4.5 g/L glucose, 2.4 g/L sodium  
159 bicarbonate, 10% fetal bovine serum, 100 UI/mL penicillin, and 0.1 mg/mL streptomycin, and  
160 incubated at 37°C in humidified air with 5% CO<sub>2</sub>. The medium was changed every other day  
161 to ensure growth until 90% confluence. Myotube formation was induced by changing the  
162 growth medium to a differentiation medium consisting of DMEM supplemented with 2% horse  
163 serum, 100 UI/mL penicillin, and 0.1 mg/mL streptomycin for 5 days before cell treatment.  
164 Passages between 4 and 10 were used for the experiments.

165 **Cell treatments.** For all experiments, C2C12 cells were incubated in differentiation medium.  
166 Cisplatin was prepared extemporaneously in PBS to obtain a 0.5 mg/mL stock solution, then  
167 administered to the cells at a final concentration of 50  $\mu$ M for 24–48 h. A stock solution of CBD  
168 was prepared in ethanol and stored at -80°C. For each experiment, cells were pre-treated for 2  
169 h prior to cisplatin treatment with CBD concentrations corresponding to those used in

170 subsequent cisplatin conditions (ranging from 1 to 5 $\mu$ M of CBD). After this pre-treatment, cells  
171 were incubated with cisplatin with or without a CBD concentration corresponding to the pre-  
172 treatment CBD concentration. Control experiments were conducted with the equivalent amount  
173 of PBS and ethanol used in the cisplatin and CBD settings. C2C12 viability was estimated using  
174 the CellTiter-Glo® luminescent assay (Promega, France).

175 **C2C12 myotube morphology analysis.** Myotubes were photographed directly in the culture  
176 plates without fixation, using an AxioCam ERc5s digital camera coupled to an AxioVert.A1  
177 microscope and ZEN 2.3 software (Zeiss, Germany). Myotube diameter was measured from  
178 three independent experiments on myotubes in each condition. Three random measurements  
179 were performed along the length of each myotube (n=3 measurements/myotube) using the ZEN  
180 2.3 software, and the average of the three measures was considered as a single value.

181 **Western blotting and measurement of protein synthesis rate.** Protein synthesis was assessed  
182 according to the SUNSET method. The SUNSET technique uses puromycin, which incorporates  
183 into nascent polypeptide chains and terminates the elongation, resulting in an accumulation of  
184 puromycin-conjugated peptides that reflects the rate of protein synthesis. Briefly, C2C12 cells  
185 were incubated with 1  $\mu$ M puromycin for the last 30 min of experimental treatments, then  
186 washed twice with ice-cold PBS and homogenized in ice-cold buffer (50 mM HEPES pH 7.4,  
187 150 mM NaCl, 10 mM EDTA, 10 mM NaPPi, 25 mM  $\beta$ -glycerophosphate, 100 mM NaF, 2  
188 mM Na orthovanadate, 10% glycerol, 1% Triton X-100) containing 1% protease inhibitor  
189 cocktail (Sigma-Aldrich, Saint-Quentin-Fallavier, France). Homogenates were centrifuged at  
190 13,000  $\times g$  for 10 min at 4°C. Denatured proteins were separated by SDS-PAGE and transferred  
191 to a PVDF membrane (Millipore, Molsheim, France). Immunoblots were blocked with 0.1%  
192 TBS-Tween-20 containing 5% dry milk, and then probed overnight at 4°C with primary  
193 antibodies. After several washes with 0.1% TBS-Tween-20, the immunoblots were incubated  
194 with a horseradish peroxidase-conjugated secondary antibody (DAKO, Trappes, France) for  
195 one hour at room temperature. Immune-reactive bands or whole lanes were visualized by  
196 chemiluminescence (ECL Western blotting substrate, Thermo Fisher Scientific, Courtaboeuf,  
197 France). escent secondary antibodies were visualized using an MF-ChemiBIS 2.0 camera  
198 (Fusion Solo, Vilber Lourmat, France). Band densities were quantified using MultiGauge 3.2  
199 software (Fujifilm Corporation, FSVT, Courbevoie, France). An internal control was used on  
200 each gel to normalize signal intensities between gels.

201 **RNA extraction and quantitative real-time PCR.** Total RNA was extracted using Trizol  
202 reagent (Invitrogen) according to the manufacturer's instructions. RNA was quantified by



203 measuring optical density at 260 nm. The concentrations of the mRNAs corresponding to genes  
204 of interest were measured by reverse transcription followed by real-time PCR using an AriaMX  
205 Real-Time PCR System (Agilent, Les-ULis, France). One microgram of total RNA was reverse-  
206 transcribed using SuperScript® III reverse transcriptase and a combination of random hexamer  
207 and oligo-dT primers (Invitrogen). PCR amplification was performed in a 20 µL total reaction  
208 volume. The real-time-PCR mixture contained 5 µL of diluted cDNA template, 10 µL of 2x  
209 ONE Green® Fast qPCR premix (Ozyme, Saint-Cyr-l'École, France), and 0.5 µM of forward  
210 and reverse primers. The amplification profile was initiated by 3 min incubation at 95°C to  
211 activate the hot-start Taq DNA Polymerase, followed by 40 cycles of two steps: 95°C for 5 sec  
212 (denaturation step) and 60°C for 30 sec (annealing/extension step). Relative mRNA  
213 concentrations were analyzed using the AriaMX software. Relative mRNA abundance was  
214 calculated using the 2- $\Delta\Delta$ CT method with 18S as housekeeping gene. Mitochondrial DNA  
215 (mtDNA) was quantified by measuring the ratio between the expression of mitochondrial ND1  
216 DNA and nuclear actin DNA as reference. Details of the primers used in the PCR can be found  
217 in table 1.

218 **Statistical analysis.** Data are expressed as mean  $\pm$  SE. Between group differences were  
219 analyzed using one-way ANOVA and Tukey's test for post hoc comparisons. Statistical  
220 significance was set at  $P < 0.05$  for all analyses.

221

## 222 RESULTS

223 **CBD prevents cisplatin-induced C2C12 myotube atrophy and death.** In a first set of  
224 experiments, we studied whether CBD was able to prevent the atrophy of C2C12 myotubes in  
225 response to cisplatin treatment. In absence of CBD, cisplatin induced myotube atrophy, as  
226 exemplified by a  $\approx 30\%$  reduction ( $p < 0.01$ ) in myotube diameter (Fig. 1A,B). However, co-  
227 treatment with CBD prevented myotube atrophy in a dose-dependent manner (Fig. 1A, B). As  
228 muscle morphology analysis revealed a high degree of cell death in response to cisplatin (Fig.  
229 1A), we measured ATP content as an index of cell viability. We observed that myotube viability  
230 was marginally affected by cisplatin and CBD after 24 h of treatment (data not shown) but was  
231 reduced by  $\approx 30\%$  after 48 h of treatment (Fig. 1C). CBD restored myotube viability in a dose-  
232 dependent manner, reaching full protection at a 3  $\mu\text{M}$  concentration (Fig. 1C). Western blot  
233 analysis revealed that the reduction in cell viability observed in response to cisplatin was  
234 associated with the appearance of the cleaved form of caspase 3 indicating induction of  
235 apoptosis (Fig. 1D,E). Similar results were observed in myoblast (supplemental figure). As  
236 expected, CBD treatment prevented the apoptotic process, as evidenced by the dose-dependent  
237 reduction in protein expression of the cleaved-caspase 3 (Fig. 1D,E).

238

239 **CBD restores protein homeostasis in cisplatin-treated C2C12 myotubes.** To investigate  
240 whether cisplatin alters protein synthesis and proteolysis and whether CBD is able to counteract  
241 this effect, we incubated C2C12 myotubes for 24 h in differentiation media in the presence of  
242 cisplatin and increasing concentrations of CBD, and then measured protein synthesis using the  
243 SUnSET technique. As shown in Fig. 2A and B, puromycin incorporation was reduced by  
244  $\approx 75\%$  ( $p < 0.01$ ) in response to cisplatin treatment. Protein synthesis is mainly controlled by the  
245 Akt/mTOR/S6K and eIF2 $\alpha$  signaling pathways, where anabolic conditions lead to increased  
246 phosphorylation of Akt on Ser473 and S6K on Thr389 and decreased phosphorylation of  
247 eIF2 $\alpha$  on Ser51 residue. As shown in Fig. 2A, C-E, cisplatin treatment was associated with  
248 decreased phosphorylation of both Akt and S6K and increased phosphorylation of eIF2 $\alpha$ , in  
249 agreement with the observed reduced protein synthesis. Co-treatment with CBD was able to  
250 restore protein synthesis (Fig. 2A, B), which was associated with a dose-dependent increase in  
251 Akt phosphorylation on Ser473 and S6K phosphorylation on Thr389, and a dose-dependent  
252 decrease of Ser51 phosphorylation on eIF2 $\alpha$  (Fig. 2A, C-E). AMPK kinase is activated by an  
253 elevated AMP/ATP ratio due to cellular and environmental stress. We observed that cisplatin  
254 treatment was associated with an increase in AMPK phosphorylation on Thr172 that was

255 corrected by CBD co-treatment (Fig. 2A). To investigate how cisplatin and cannabidiol impact  
256 proteolysis, we evaluated the level of protein poly-ubiquitination and the mRNA expression of  
257 MAFBx and MuRF1. As shown in Fig. 3A,B, cisplatin treatment increased protein  
258 polyubiquitination by  $\approx 70\%$  ( $p < 0.01$ ) and induced a  $\approx 2$ -fold increase in mRNA expression of  
259 Atrogin1/MAFBx (Fig. 3C) and MuRF1 (Fig. 3D). CBD reduced protein polyubiquitination  
260 levels in a dose-dependent manner (Fig. 3A,B), and CBD treatment at a 5  $\mu$ M concentration  
261 restored the mRNA expression of both Atrogin1/MAFBx and MuRF1 to control levels (Fig.  
262 3C,D).

263

264 **CBD prevents cisplatin-induced oxidative stress in C2C12 myotubes.** The toxicity of  
265 cisplatin has been described as a function of DNA binding followed by single-stranded DNA  
266 breaks. More recently, cisplatin has been shown to generate oxidative stress, which can also  
267 contribute to its anti-tumor effects (32). To determine whether cisplatin-induced C2C12  
268 myotube atrophy was associated with oxidative stress, we measured the level of TBARS, a  
269 marker of lipid peroxidation, in C2C12 myotubes in response to cisplatin and CBD treatments.  
270 We found a two-fold increase in TBARS content in response to 24 h cisplatin treatment that  
271 was prevented by co-treatment with 5  $\mu$ M CBD (Fig. 4A). Catalase is one of the main enzymes  
272 responsible for the detoxification of hydrogen peroxide, a reactive oxygen species. We therefore  
273 measured catalase activity in response to cisplatin and CBD treatments (Fig. 4B). A 24 h  
274 cisplatin treatment induced a  $\approx 90\%$  increase in catalase activity, indicating severe oxidative  
275 stress ( $0.236 \pm 0.020$  vs  $0.125 \pm 0.013$  nmol/min/ $\mu$ g prot,  $p < 0.01$ ) that was partially restored by  
276 co-treatment with CBD ( $0.172 \pm 0.010$  vs  $0.236 \pm 0.020$  nmol/min/ $\mu$ g prot,  $p < 0.05$ ). We then  
277 measured the mRNA expression levels of several anti-oxidant systems. As shown in Fig. 4C,  
278 mRNA expression of GPX1, SOD1, SOD2 and CAT were all increased in response to 24 h  
279 cisplatin treatment. Co-treatment with 5  $\mu$ M CBD was unable to correct the mRNA expression  
280 levels of SOD2 and CAT (Fig. 4C) but decreased GPX1 and SOD1 mRNA expression levels  
281 compared to cisplatin treatment (Fig. 4C).

282

283 **Effect of cisplatin and CBD treatment on mitochondria.** In order to analyze the early effects  
284 of cisplatin and CBD on mitochondrial biogenesis and quality control, we estimated  
285 mitochondrial density by measuring mtDNA (mitochondrial DNA) content and we measured  
286 the mRNA expression levels of genes involved in mitochondrial biogenesis (PGC1 $\alpha$ ), fission  
287 (DRP1, FIS1), fusion (OPA1), and mitophagy (PRKN) in response to 24 h cisplatin and CBD  
288 treatments. In these conditions, there were no significant changes in mtDNA content (Fig. 5A)

289 or mRNA expression levels of PGC1 $\alpha$ , FIS1, DRP1 and OPA1 (Fig. 5B-E), but we observed a  
290 dramatic  $\approx$ 95% reduction in PRKN mRNA expression compared to controls (Fig. 5F). Adding  
291 5 $\mu$ M CBD to the cisplatin treatment did not result in any changes in mtDNA content or mRNA  
292 expression of PGC1 $\alpha$ , OPA1, DRP1, FIS1 and PRKN compared to cisplatin treatment alone  
293 (Fig. 5A-F). In a second step, we studied the effect of cisplatin and CBD treatments on the  
294 expression of the different mitochondrial respiratory chain complexes. As shown in Figure 6A-  
295 B, western blot quantification showed a marked increase in the content of several mitochondrial  
296 proteins in C2C12 myotubes treated with 50 $\mu$ M cisplatin, with significant increases in  
297 NDUFB8 (complex I,  $p$ <0.01), UQCRC2 (complex III,  $p$ <0.05) and COX4 (complex IV,  
298  $p$ <0.05) that were restored to control levels by co-treatment with CBD. Finally, we studied the  
299 expression of VDAC1, which is a major mitochondrial transporter that plays a key role in ATP  
300 production and is recognized as a key protein in mitochondria-mediated apoptosis (33).  
301 Cisplatin treatment of C2C12 myotubes led to a 5-fold increase in VDAC1 protein level, which  
302 was partly corrected by CBD treatment (Fig. 6C-D). In a final step, we measured citrate  
303 synthase and COX activities. Treatment of C2C12 myotubes with 50 $\mu$ M cisplatin resulted in a  
304 significant  $\approx$ 15% increase in citrate synthase activity (Fig. 6E,  $p$ <0.05) and a  $\approx$ 50% increase in  
305 COX activity (Fig. 6F,  $p$ =0.07) that were not corrected by CBD co-treatment.

306

## 307 **DISCUSSION**

308 The objective of this study was to investigate whether treatment with CBD was able to prevent  
309 chemotherapy-induced skeletal muscle atrophy in a cisplatin-treated model of myotubes in  
310 culture. In a first set of experiments, we demonstrated that CBD was able to prevent cisplatin-  
311 induced apoptosis and myotube atrophy. The increased protein synthesis and decreased  
312 proteolysis induced by CBD in cisplatin-treated myotubes explain the anti-atrophic effects of  
313 CBD. In a second set of experiments, we showed that cisplatin-induced atrophy was associated  
314 with the induction of oxidative stress that was prevented by CBD co-treatment. Finally, we  
315 observed early cisplatin-induced alterations in the expression of several mitochondrial proteins  
316 involved in mitochondrial respiration and control of apoptosis, and these alterations were also  
317 prevented by CBD co-treatment.

318

319 Mitochondria plays an important role in regulating many cellular functions, including ATP  
320 production, generation of reactive oxygen species (ROS), and induction of apoptosis (34). Cells  
321 maintain optimal mitochondrial health through several pathways, including mitochondrial  
322 biogenesis, mitochondrial dynamics (fusion and fission processes shaping mitochondrial

323 morphology), and mitophagy (the process that removes defective mitochondria through  
324 autophagy) (35). Alterations in mitochondrial distribution, morphology and function have been  
325 reported in many conditions that lead to skeletal muscle wasting, including cachexia and  
326 sarcopenia (36, 37). Here, we analyzed the mRNA expression levels of several markers of  
327 mitochondrial dynamics in response to cisplatin and CBD treatments in myotubes in culture.  
328 We observed no major effect of cisplatin or CBD on the mRNA expression levels of genes  
329 involved in mitochondrial biogenesis (PGC1 $\alpha$ ), fusion (Opa1), and fission (DRP1, FIS1).  
330 However, there was a drastic reduction in the expression of the PRKN gene encoding the Parkin  
331 protein. This could reflect inhibition of PARKIN-dependent mitophagy, leading to an  
332 accumulation of dysfunctional mitochondria and, ultimately, muscle wasting (38). Previous  
333 studies have shown that cisplatin (and chemotherapy in general) causes a decrease in  
334 mitochondrial respiration and alterations in mitochondrial biogenesis and fusion/fission  
335 processes in models of cancer or chemotherapy, but also in cancer patients (13, 16, 18, 19). Our  
336 findings therefore seem to contradict the literature, but there are several possible explanations  
337 for this divergence. The majority of studies characterizing the effect of cisplatin on muscle  
338 atrophy were carried out *in vivo*, either in rodents or in cancer patients, and treatments were  
339 carried out over periods of several days, sometimes with tumor implantation. One limitation of  
340 our *in vitro* myotube model is that it does not represent the cellular heterogeneity found in  
341 skeletal muscle, which consists of satellite cells, myoblasts, fibro-adipogenic progenitor cells,  
342 and immune cells. Further research is needed to investigate the impact of CBD on this  
343 heterogeneity and the coordinated functioning of different cell types.

344

345 In the present study, we also documented alterations in the expression of several proteins of the  
346 mitochondrial electron transport chain (ETC). Cisplatin treatment was associated with  
347 increased protein expression of NDUFB8 (complex I), UQCRC2 (complex III), and COX4  
348 (complex IV) that were corrected by CBD, while protein expression of SDHB (complex II) and  
349 ATP5A (complex V) was unaffected. In sarcopenia, deficient ETC activity is associated with  
350 loss of muscle mass (39). Given that normal ETC function requires proportionally balanced  
351 activities of these different complexes (40), the increased expression of NDUFB8, UQCRC2  
352 and COX4 could reflect imbalanced ETC activity and reduced energy production. The reduced  
353 ATP content observed in cisplatin-treated myotubes and the increased level of AMPK  
354 phosphorylation (indicating energy depletion) supports this hypothesis, but further  
355 investigation is needed to fully understand the effects of cisplatin and CBD treatments on the  
356 activity of the different mitochondrial complexes and on mitochondrial respiration. One of the

357 key proteins that control mitochondrial function is VDAC1, which plays a crucial role in the  
358 release of ROS and in regulating apoptosis through the release of mitochondrial pro-apoptotic  
359 factors such as cytochrome C and subsequent cleavage of caspase 3 (41). Several studies have  
360 shown that the cytotoxic effect of cisplatin on cancer cell lines is associated with upregulation  
361 of VDAC1 and excessive production of ROS (16, 42). To our knowledge, our study is the first  
362 to demonstrate increased expression of VDAC1 in response to cisplatin in a model of skeletal  
363 muscle in vitro, and its correction by CBD. It is still unclear how CBD counters these  
364 mitochondrial alterations, but the answer may lie in its antioxidative properties.

365

366 Cisplatin is widely used to combat multiple types of cancers, but it has side effects such as  
367 oxidative stress in muscle cells (12, 16). Antioxidative strategies are expected to be useful in  
368 limiting oxidative stress-induced skeletal muscle damage (43). As expected, cisplatin treatment  
369 resulted in increased TBARS levels (index of lipid peroxidation) together with increased  
370 catalase activity and mRNA expression levels of several antioxidant systems (GPX1, SOD1/2,  
371 CAT) aimed at detoxifying excessive ROS production. To our knowledge, our study is the first  
372 to demonstrate the antioxidative properties of CBD in cisplatin-treated myotubes in vitro.  
373 Previous studies have shown that CBD has an antioxidant function (22) that comes from its  
374 ability to capture free radicals or transform them into less active forms. For example, CBD was  
375 shown to directly prevent the formation of superoxide radicals in a renal nephropathy model  
376 using cisplatin-treated mice (22), to reduce nitric oxide (NO) levels in the liver of doxorubicin-  
377 treated mice (22), and to suppress ROS production by chelating transition metal ions involved  
378 in the Fenton reaction (22). Note too that CBD modifies cellular redox status by modulating  
379 both the expression and enzymatic activity of antioxidant systems (22). Our results  
380 demonstrated that CBD was able to prevent the cisplatin-induced accumulation of TBARS. The  
381 fact that CBD reduced the mRNA expression of antioxidant systems (GPX1, SOD1/2, CAT)  
382 and catalase activity in response to cisplatin strongly suggests a direct effect of CBD by  
383 capturing ROS and reducing NO production in response to cisplatin, or by reducing their  
384 production by increasing the levels of antioxidants such as glutathione (22).

385 On top of these direct antioxidant effects, CBD also indirectly modulates redox state by  
386 interacting with several molecular targets, including the EC receptors CB1 and CB2, and  
387 PPAR $\gamma$  which controls the expression of antioxidant systems such as catalase and SOD2 (22).  
388 CBD is not only a PPAR $\gamma$  receptor agonist, but can also increase enzymatic antioxidant  
389 activities (22). CBD could also improve oxidative state by modulating the activity of the CB1  
390 and CB2 receptors. Indeed, CB1 activation increases ROS production whereas CB2 activation

391 decreases ROS production (44), and it has been shown that CBD is a negative allosteric  
392 modulator of the CB1 receptor (45). Further studies are needed to fully understand which of  
393 these targets mediate the protective effect of CBD in our *in vitro* model.

394 Oxidative stress is one of the primary causes of skeletal muscle atrophy in several patho-  
395 physiological conditions, including muscle inactivity, muscular dystrophy, sarcopenia, and  
396 cachexia (43). Regulation of skeletal muscle mass depends on the balance between protein  
397 synthesis and degradation. Protein synthesis is mainly controlled by the Akt/mTOR  
398 (mammalian target of rapamycin) and GCN2/eIF2 $\alpha$  pathways (46, 47). Excessive production  
399 of ROS is known to activate PERK, a key endoplasmic reticulum stress transducer of the  
400 unfolded protein response pathway (47) that activates eIF2 $\alpha$  and inhibits protein synthesis. In  
401 skeletal muscle, excessive oxidative stress is also known to impair insulin signaling and Akt  
402 activation upstream of mTOR, which is important for controlling both protein synthesis and  
403 degradation (46). Given the drastic reduction of lipid peroxidation observed here with CBD in  
404 cisplatin-treated myotubes, it is highly conceivable that CBD helps maintain protein  
405 homeostasis by preventing excessive oxidative stress.

406

407 In summary, using a model of cultured C2C12 myotubes, we demonstrated that CBD prevented  
408 cisplatin-induced atrophy by maintaining protein homeostasis (i.e. promoting protein synthesis  
409 and limiting proteolysis) by reducing oxidative stress. In cancer patients, muscle mass is  
410 predictive of survival but also of response to chemotherapy, which makes it crucial to develop  
411 strategies for maintaining muscle mass in these patients. Cisplatin is an antineoplastic agent  
412 that is commonly used in the treatment of solid tumors such as ovarian carcinoma and in head  
413 and neck squamous cell carcinoma (HNSCC) (48). The toxicity of cisplatin is due to its DNA  
414 binding followed by single-stranded DNA breaks, but also its ability to generate oxidative stress  
415 in tumor cells. Consequently, some may consider that using CBD for its antioxidant activity  
416 could undermine the efficacy of cisplatin treatment. However, rather than reducing cisplatin  
417 toxicity, CBD was recently found to potentiate the antineoplastic effect of cisplatin in a model  
418 of HNSCC (48). Taken together, the evidence suggests that CBD could be used as an adjuvant  
419 in the treatment of cancer cachexia to help maintain muscle mass and improve patient quality  
420 of life.

421

## 422 **GRANTS**

423 This study was supported by the INRAE AlimH (Alimentation Humaine) Department.

424 **CONFLICT OF INTEREST**

425 The authors declare they have no conflict of interest.

426

427 **FIGURE LEGENDS**

428 **Figure 1.** CBD prevents cisplatin-induced atrophy and apoptosis in C2C12 myotubes. **a)**  
429 Representative pictures of myotube morphology at 24 h of incubation. **b)** Myotube diameter of  
430 cisplatin and CBD treated cells. **c)** 48 h Myotube viability. **d)** Representative western blot  
431 showing level of cleaved caspase 3 in response to 24 h treatments. **e)** Quantification of cleaved  
432 caspase 3 level from (d). Results are expressed as mean  $\pm$  sem. \*\*  $p < 0.01$  vs. CTL,  $\blacklozenge$   $p < 0.05$   
433 vs. CIS,  $\blacklozenge$   $p < 0.01$  vs. CIS, ##  $p < 0.01$  between CBD conditions.

434 **Figure 2.** CBD prevents cisplatin-induced decrease in protein synthesis in C2C12 myotubes.  
435 **a)** Representative western blot of puromycin incorporation and the phosphorylation state of Akt  
436 (Ser473-phospho-Akt), S6K (Thr389-phospho-S6K) and eIF2 $\alpha$  (Ser51-phospho- eIF2 $\alpha$ ) in  
437 response to 24 h treatment with cisplatin and CBD. Quantification of **(b)** puromycin  
438 incorporation signal, **(c)** phospho-S6K, **(d)** phospho-Akt, and **(e)** phospho- eIF2 $\alpha$  levels.  
439 Results are expressed as mean  $\pm$  sem. \*\*  $p < 0.01$  vs. CTL, \*  $p < 0.05$  vs. CTL,  $\blacklozenge$   $p < 0.05$  vs. CIS,  
440  $\blacklozenge$   $p < 0.01$  vs. CIS, ##  $p < 0.01$  between CBD conditions.

441 **Figure 3.** CBD prevents cisplatin-induced proteolysis and atrogene expression. **a)**  
442 Representative western blot showing the level of protein poly-ubiquitination in response to 24  
443 h treatment with cisplatin and CBD. **b)** Quantification of total protein polyubiquitination from  
444 (a). **c)** Real-time PCR quantification of *Atrogin/MAFbx* mRNA expression. **d)** Real-time PCR  
445 quantification of *MuRF1* mRNA expression. Results are expressed as mean  $\pm$  sem. \*\*  $p < 0.01$   
446 vs. CTL, \*  $p < 0.05$  vs. CTL,  $\blacklozenge$   $p < 0.05$  vs. CIS.

447 **Figure 4.** CBD prevents cisplatin-induced oxidative stress. **a)** TBARS content in C2C12  
448 myotubes treated for 24 h with cisplatin and CBD. **b)** Catalase activity. **c)** Real-time PCR  
449 quantification of *Gpx1*, *Sod1*, *Sod2* and *Cat* mRNA expression. Results are expressed as mean  
450  $\pm$  sem. \*\*  $p < 0.01$  vs. CTL, \*  $p < 0.05$  vs. CTL,  $\blacklozenge$   $p < 0.05$  vs. CIS.  $\blacklozenge$   $p < 0.01$  vs. CIS.

451 **Figure 5.** Effect of cisplatin and CBD co-treatment on C2C12 myotube mitochondrial  
452 dynamics. **a)** Real-time PCR quantification of mtDNA expression in C2C12 myotubes treated  
453 for 24 h with cisplatin and CBD. Real-time PCR quantification of *Pgc1alpha* **(b)**, *Fis1* **(c)**,



454 *Drp1* (d), *Opal* (e), and *Prkn* (f) mRNA expression. Results are expressed as mean  $\pm$  sem. \*\*  
 455  $p < 0.01$  vs. CTL.

456 **Figure 6.** Effect of cisplatin and CBD on C2C12 myotube mitochondrial protein content and  
 457 activity. **a)** Representative western blot showing the expression levels of proteins related to  
 458 mitochondrial oxidative phosphorylation (OXPHOS) in C2C12 myotubes treated for 24 h with  
 459 cisplatin and CBD. **b)** Quantification of several subunits of each mitochondrial complex from  
 460 (a). **c)** Representative western blot showing the expression levels of VDAC1. **d)** Quantification  
 461 of VDAC1 protein content from (c). **(e)** Citrate synthase activity and **(f)** COX activity. Results  
 462 are expressed as mean  $\pm$  sem. \*\*  $p < 0.01$  vs. CTL, \*  $p < 0.05$  vs. CTL,  $\blacklozenge$   $p < 0.05$  vs. CIS.  $\blacklozenge$   
 463  $p < 0.01$  vs. CIS.

464 **Table 1.** List of primers used for real-time qPCR

Gene name	5'- Sense primer -3'	5'- Antisense primer -3'
Actin (DNA primer)	TACAGCTTCACCACCACAGC	AAGGAAGGCTGGAAAAGAGC
Atrogin/MAFBx	AAGCTTGTGCGATGTTACCCA	CACGGATGGTCAGTGCCCTT
Cat	CCTTCAAGTTGGTTAATGCAGA	CAAGTTTTTGATGCCCTGGT
Drp1	TGCTCAGATCGTCGTAGTG	TGACCACACCAGTTCCTCTG
Fis1	GCCTGGTTCGAAGCAAATAC	CACGGCCAGGTAGAAGACAT
Gpx1	GTGAGCCTGGGCTCCCTGCG	ACTTGAGGGAATTCAGAATC
MuRF1	AGGTGTCAGCGCAAAGCAGT	CCTCCTTTGTCCTCTTGCTG
Nd1 (DNA primer)	GGCCCCCTTCGACCTGACAGA	TAACGCGAATGGGCCGGCTG
Opal	GATGACACGCTCTCCAGTGAAG	CTCGGGGCTAACAGTACAACC
Ppargc1a	GAAGTGGTGTAGCGACCAATC	AATGAGGGCAATCCGTCTTCA
Prkn	ATTCCAAACCGGATGAGTGG	TTGTCTGAGGTTGGGTGTGC
Sod1	CAGGACCTCATTTTAATCCTCAC	TGCCCAGGTCTCCAACAT
Sod2	GACCTGCCTTACGACTAT	TACTTCTCCTCGGTGACG
18S	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT

465

466

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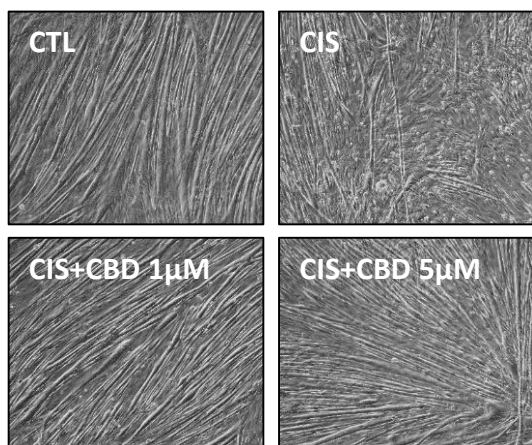
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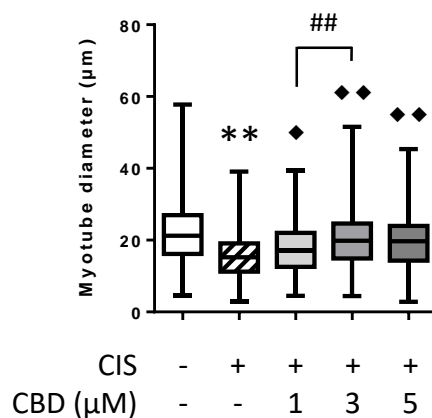
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Figure 1

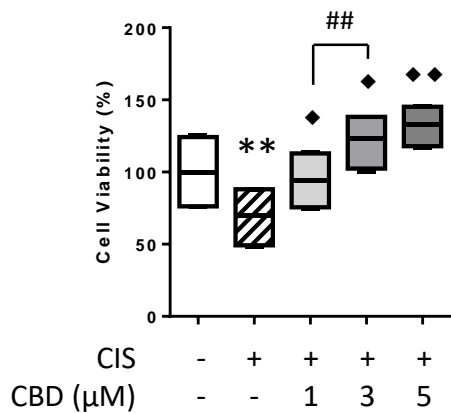
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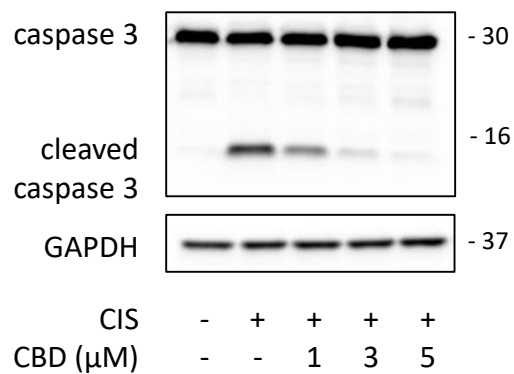
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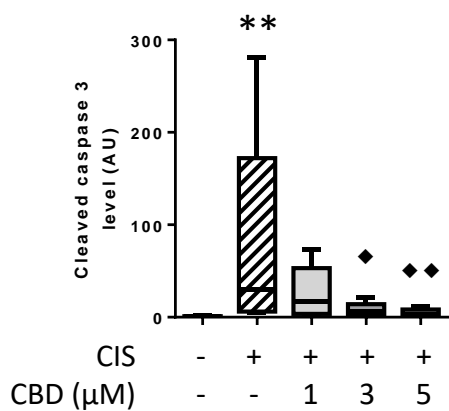
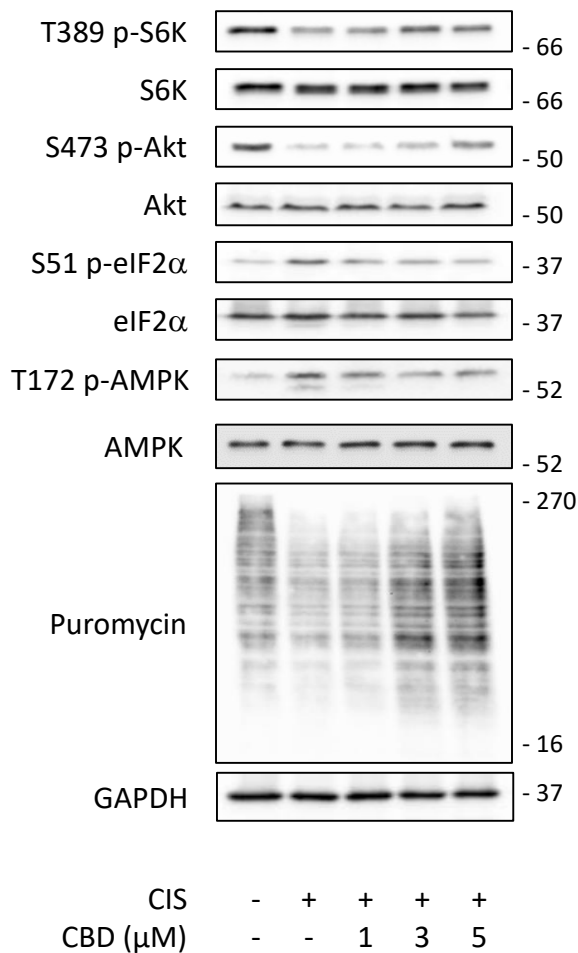
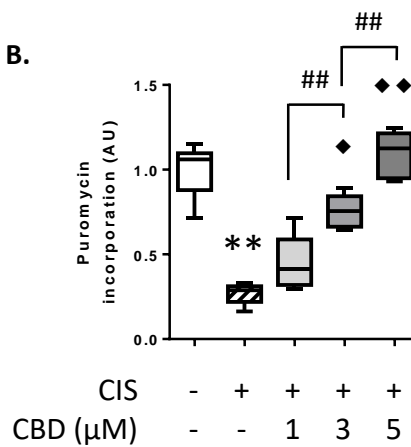


Figure 2

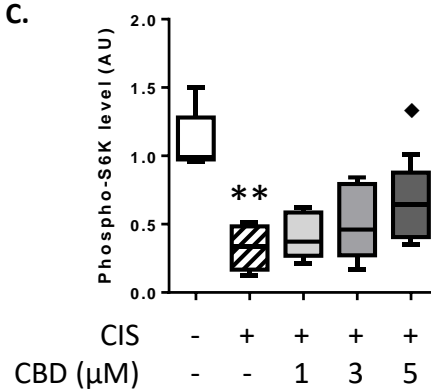
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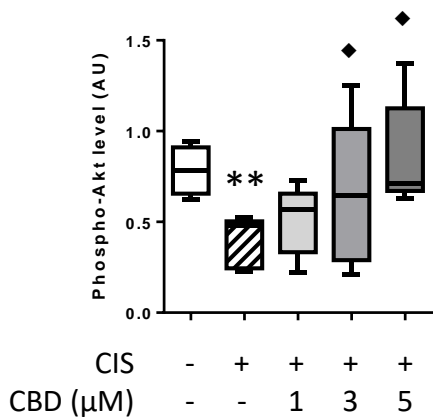
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D.



E.

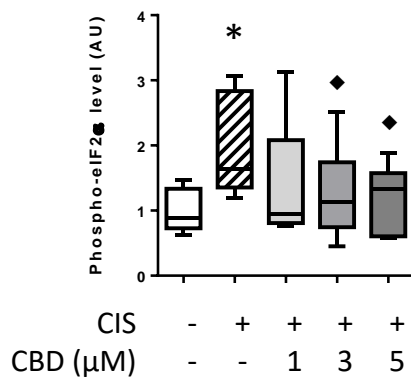
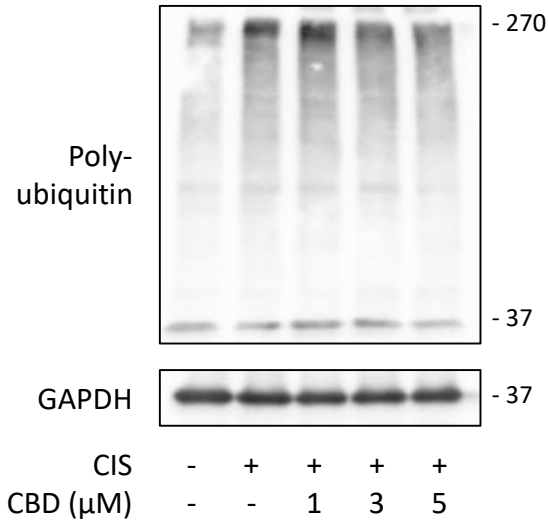
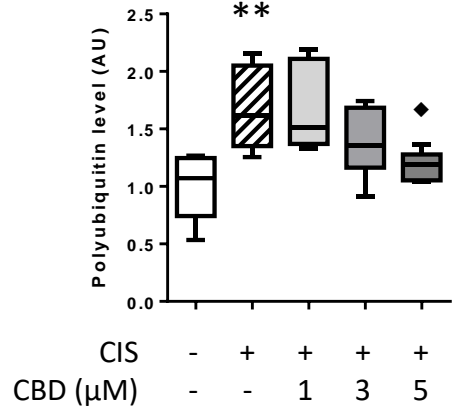


Figure 3

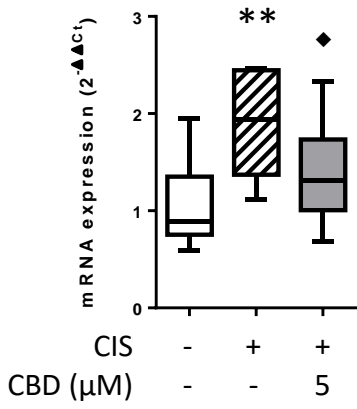
A.



B.

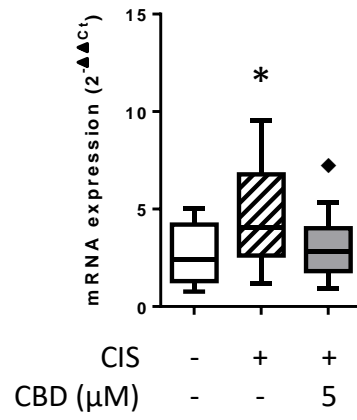


C.



MAFBx

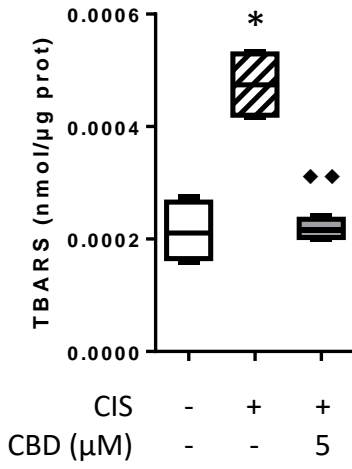
D.



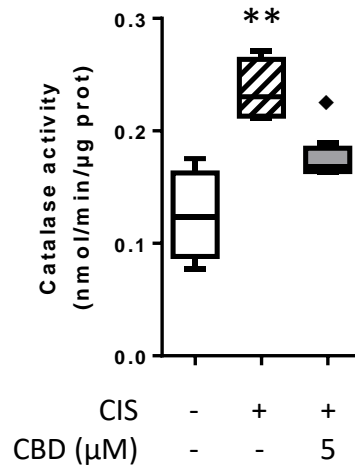
MuRF1

Figure 4

A.



B.



C.

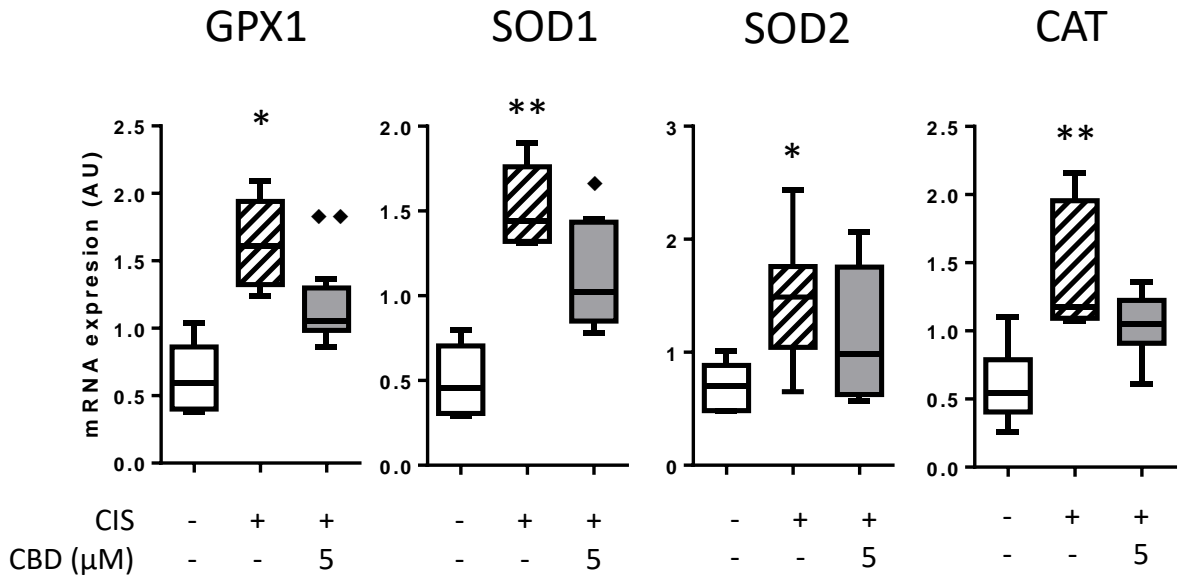




Figure 5

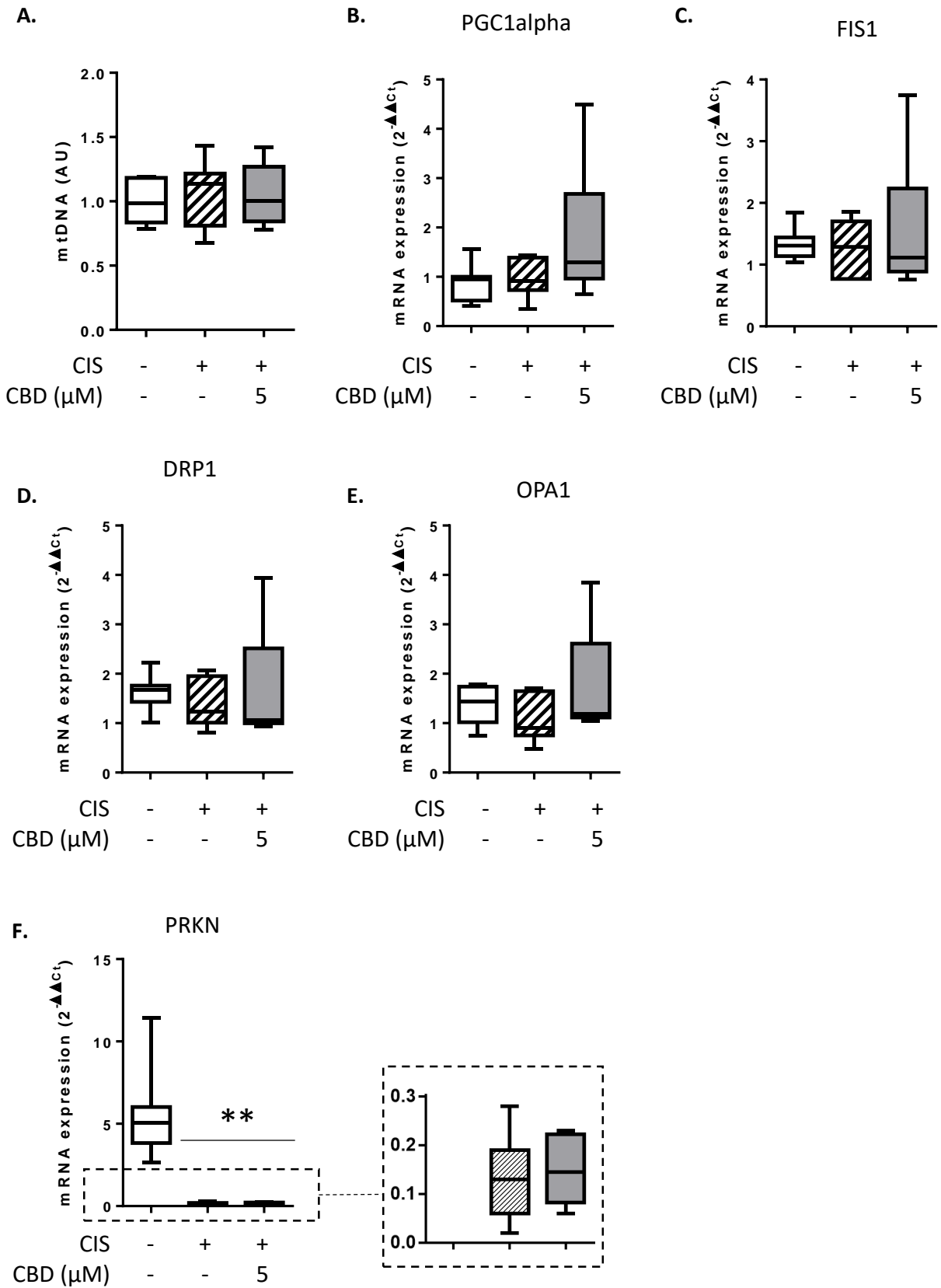


Figure 6

