Chapter 5

“Priming” with CBD enhances cell death

5. “Priming” with CBD enhances cell death

5.1. An emerging chemotherapy treatment – “priming”

“Priming” is a novel chemotherapy treatment that treats cancers in a two-staged process (Section 1.4.1.) (Sharma *et al.* 2005, Chonghaile *et al.* 2011, Reed 2011, Kumar and Singh 2012). The first stage uses an agent that sensitizes the mitochondria to cell death (Section 1.4.3. and 1.4.4.) (Chonghaile *et al.* 2011, Reed 2011, Sarosiek *et al.* 2013). Apoptosis is controlled through the mitochondria and reducing its membrane potential and increasing its sensitivity to chemotherapeutic agents can increase this cell death potential (Brookes *et al.* 2004, Galluzzi *et al.* 2006, Kuhar *et al.* 2006, Chonghaile *et al.* 2011, Reed 2011, Wallace 2012, Sarosiek *et al.* 2013). The second stage of “priming” is the use of a chemotherapy agent to kill the sensitized cancer cells (Section 1.4.1. and 1.4.5.) (Yang *et al.* 2006, Chonghaile *et al.* 2011, Reed 2011, Sarosiek *et al.* 2013).

5.2. Natural plant products effectiveness in enhancing cell death through “priming”

The mechanism of “priming” has been shown to be effective in many different cancers with various agents as mentioned in section 1.4.. The natural plant products curcumin, quercetin, aspirin and thymoquionone have been shown to enhance the potential of chemotherapy when used as a “priming” agent (Section 1.4.2.) (Sharma *et al.* 2005, Kuhar *et al.* 2007, Kunnumakkara *et al.* 2007, Kumar and Singh 2012, Mu *et al.* 2014). Furthermore, quercetin showed to increase ROS levels following “priming”, curcumin has been noted to interact with the mitochondrial receptor VDAC1 and aspirin increased apoptotic mitochondrial regulators (Kuhar *et al.* 2006, Kuhar *et al.* 2007, Kumar and Singh 2012, Tewari *et al.* 2015).

5.3. The potential of CBD as a “priming” agent

The positive effect of killing cancer cells with CBD treatment for 24 hours as seen in section 3.4.1.1. confirmed its potential as a therapeutic treatment. Chronic treatment with CBD reduced cancer cell viability while the acute treatment had no change. This led to the hypothesis that a 24 hour treatment would be superior to an acute treatment in enhancing chemotherapeutic treatment. As expected, a reduction in cancer cell growth was also observed following cisplatin treatment (section 4.3.). However, when the two therapeutic agents were combined there was no added benefit, if anything cisplatin in the presence of CBD seemed to have reduced efficacy (section 4.5.). The novel idea of “priming”, which has been previously used with other agents, mentioned in section 1.4. and 5.2., appeared as a logical course of investigation with CBD (Ni Chonghaile et al., 2011; Kuhar et al., 2007). “Priming” consists of pre-treating cells prior to treatment with a chemotherapeutic agent (Section 2.6.5.). In this study “priming” was initially tested in MDA-MB231 and MCF10A cells with increasing concentrations of CBD (0.1 μM, 0.5 μM, 1 μM, 2.5 μM, 5 μm) 24 hours prior to 50 μM and 100 μM cisplatin treatment (Section 3.4.1.1. and 4.3.1.). “Priming” was further tested in additional cancer cell lines which included A549 lung cancer cells with and without functioning mitochondria (Rho0).

5.4. Aims of this study

- Does “priming” with CBD enhance the effectiveness of the chemotherapy agent, cisplatin, in cancer cells alone.

- Eluting the mode of action for “priming” with CBD in cancer cells.

- Examining the potential translational effect of “priming” *in vivo*.

5.5. The assessment of cell viability following “priming”

5.5.1. How “priming” in cancer cells effects cell viability

5.5.1.1. The effect of “priming” prior to 50 μM cisplatin on cancer cell viability

MDA-MB231 cells were “primed” with 0.1 μM - 5 μM CBD 24 hours prior to 50 μM cisplatin treatment for 24 hours (Figure 5.1.) (Section 2.6.5.). The cell viability was assessed using an MTT assay (Section 2.7.2.). Cisplatin treatment alone led to ~50% cell death (51.04 ± 2.963 %) ($p<0.001$) as previously reported (section 4.3.1.). “Priming” led to a reduction in cell viability following 0.1 μM CBD (76.00 ± 1.225 %) ($p<0.001$) and 0.5 μM CBD “priming” treatment (64.17 ± 2.522 %) ($p<0.01$). However, this decrease was not as enhanced as cisplatin treatment alone and were increased compared to cisplatin treatment (0.1 μM CBD, $p<0.001$; 0.5 μM CBD, $p<0.01$). There was a reduction in cell viability with 1 μM CBD (55.33 ± 4.910 %) ($p<0.01$), 2.5 μM CBD (41.33 ± 2.404 %) ($p<0.001$) and 5 μM CBD “primed” (16.00 ± 2.082 %) ($p<0.001$). These decreases observed were not significant to cisplatin treatment alone.

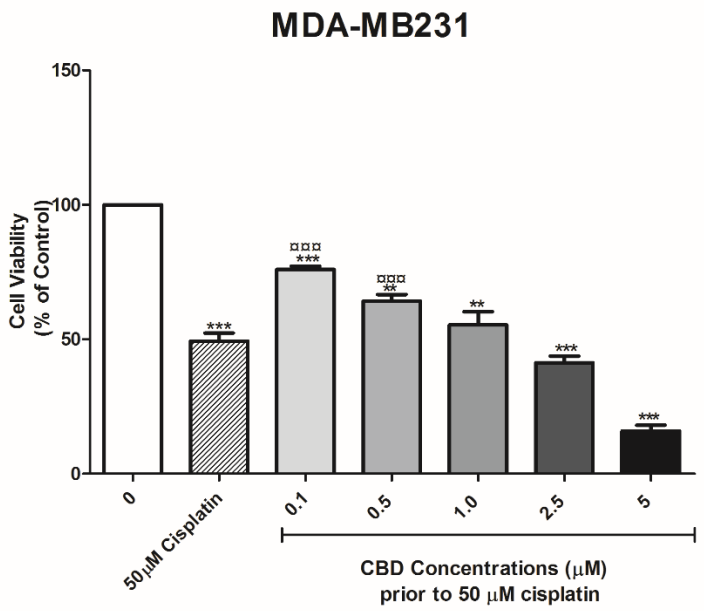

Figure 5.1. The effect of "priming" with CBD in MDA-MB231 cells prior to 50 μM cisplatin treatment.
MDA-MB231 cells following "priming" with 0.1 μM-5 μM CBD 24 hours prior to 50 μM cisplatin treatment for 24 hours. The cells viability was examined using an MTT assay. Data shown is repeated 3 times with 5 technical replicates per plate. Data is represented as mean ± SEM. ** p<0.01, *** p<0.001 *vs* Untreated; ¤¤¤ p<0.001 *vs* Cisplatin.

5.5.1.2. The effect of "priming" with CBD prior to 100 μM cisplatin in cancer cells

MDA-MB231 cells were "primed" prior to 100 μM cisplatin treatment for 24 hours and cell viability was assessed using an MTT assay (Figure 5.2.) (Section 2.7.2.). A significant reduction in cell viability was observed following cisplatin (47.33 ± 5.044 %) (p<0.001) and combination treatment (72.67 ± 1.453 %) (p<0.01). Following "priming" treatment, a significant reduction in cell viability was observed; 0.1 μM CBD (30.11 ± 7.012 %), 0.5 μM CBD (27.33 ± 4.333 %), 1 μM CBD (21.33 ± 0.882 %), 2.5 μM CBD (7.66 ± 1.453 %), 5 μM CBD (8.33 ± 0.881 %)) (p<0.001). A significant reduction in cell viability compared to cisplatin treatment alone was observed following "priming" treatments with 1 μM CBD (p<0.01), 2.5 μM CBD (p<0.001), 5 μM CBD (p<0.01). Comparing "primed" treatments against combination a significant reduction for all

treatments was seen; 0.1 μM CBD (p<0.01), 0.5 μM CBD (p<0.01), 1 μM CBD (p<0.001), 2.5 μM CBD (p<0.001), 5 μM CBD (p<0.01).

Note that further to these viability assessments "priming" will consist of a complete profile of pre-treatment with 0.1 μM CBD, 0.5 μM CBD, 1 μM CBD, 2.5 μM CBD and 5 μM CBD for 24 hours prior to 100 μM cisplatin for 24 hours. Moreover a comparison of 1 μM CBD, 100 μM cisplatin, combination and 1 μM "priming" will be examined for each assessment unless otherwise stated.

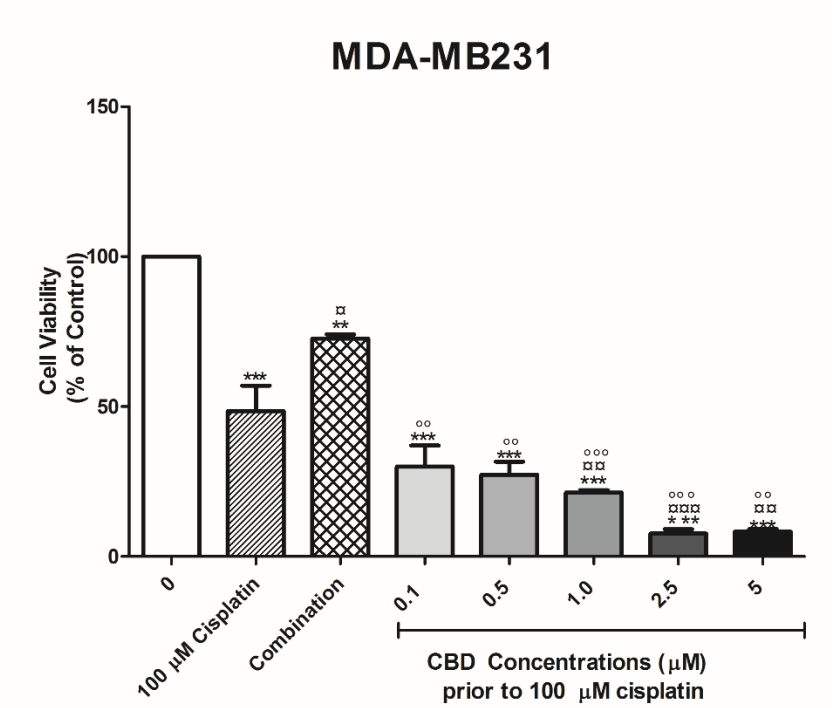

Figure 5.2. The effect of "priming" with CBD prior to 100 μM cisplatin treatment for 24 hours on cancer cell viability.
Cancer cells following "priming" with 0.1 μM-5 μM CBD 24 hours prior to 100 μM cisplatin treatment for 24 hours. The cells viability was examined using an MTT assay. Data shown is repeated 3 times with 5 technical replicates per plate. Data is represented as mean ± SEM. ** p<0.01, *** p<0.001 *vs* Untreated; ¤ p<0.05, ¤¤ p<0.01, ¤¤¤ p<0.001 *vs* Cisplatin; °° p<0.01, °°° p<0.001 *vs* Combination.

To further asses cell viability a lactate dehydrogenase assay was carried out which assess damage and toxicity to the cell (Section 2.7.3.). An increase in lactate dehydrogenase concentrations was observed following "priming" treatment with 0.1 μM CBD (348.8 ± 4.33 %) (p<0.001), 0.5 μM CBD (279.6 ± 44.24%) (p<0.05) and 1 μM CBD (263.5 ± 41.15 %) (p<0.001) (Figure 5.3.). However the increase observed had a dose

dependant decrease as “priming” dose increased. Furthermore, this decrease returned to non-significant levels as the dose increased to 2.5 μM CBD and 5 μM CBD “primed”.

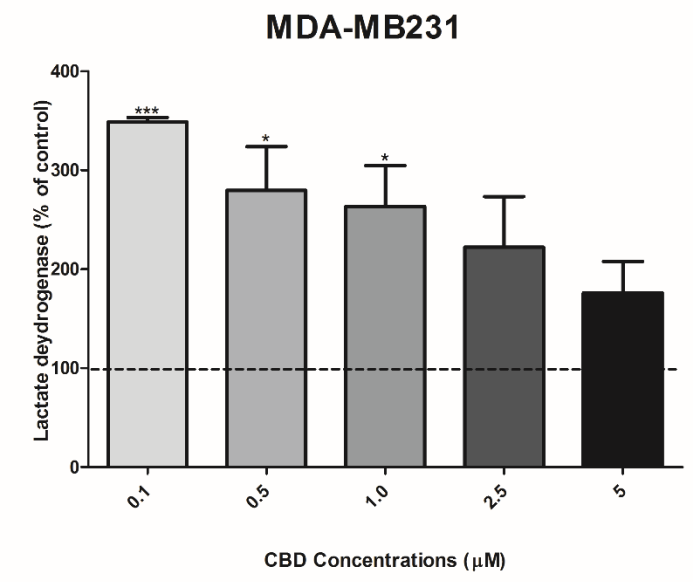

Figure 5.3. The effect of “priming” with CBD on MDA-MB231’s lactate dehydrogenase concentrations.
MDA-MB231 cell’s lactate dehydrogenase were analysed following “priming” treatment. Data shown is repeated 3-5 times with 5 technical replicates per plate. The dashed line represents untreated cells and data was normalised to cell count. Data is represented as mean ± SEM. * $p<0.05$, *** $p<0.001$ *vs* Untreated.

Lactate dehydrogenase concentrations were compared for each treatment carried out: CBD, cisplatin, combination and “priming” (Figure 5.34). Levels of lactate dehydrogenase which indicated cellular damage and toxicity were unchanged with cisplatin and combination treatment however, when the cells were “primed” there was an increase compared to untreated cells (263.5 ± 41.15 %) ($p<0.05$) (Figure 5.4.). This increase was also significantly enhanced compared to CBD ($p<0.05$).

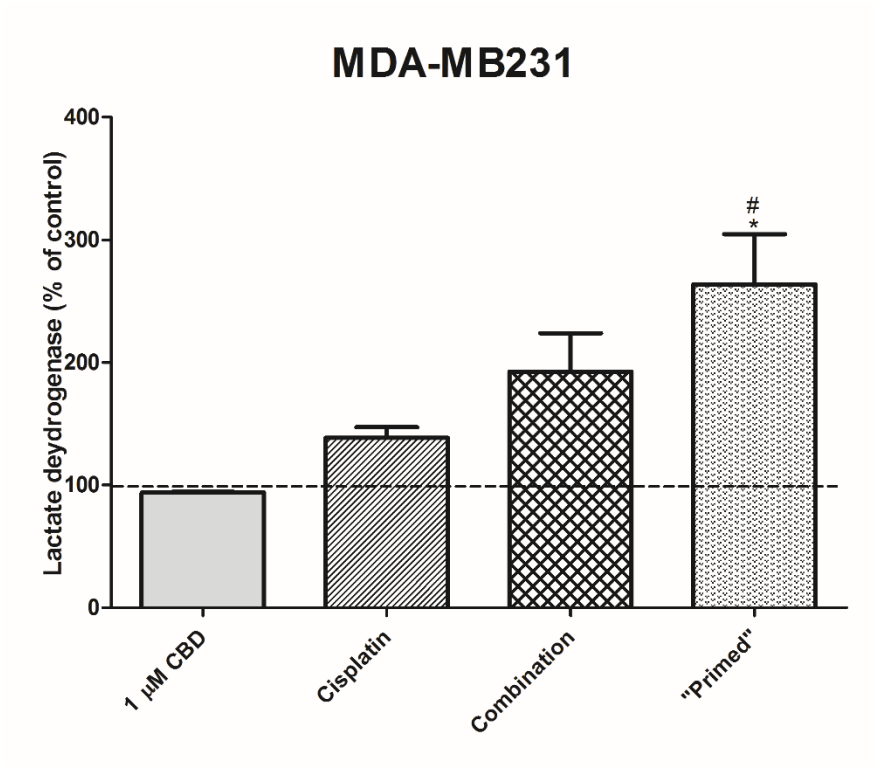

Figure 5.4. MDA-MB231 lactate dehydrogenase concentration following treatment with CBD, cisplatin, combination and "priming" with CBD.
Lactate dehydrogenase concentration was compared against each other. Data shown is repeated 3-5 times with 5 technical replicates per plate. The dashed line represents untreated cells and data was normalised to cell count. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs* Untreated; # $p<0.05$ *vs* CBD.

Assessment of the cancer cells growth and proliferation was carried out using a BrdU cell proliferation assay (Section 2.7.4.). Cancer cell's growth and proliferation showed a significant decrease with 100 μM cisplatin and all "priming" doses ($p<0.001$) (Figure 5.5.) (Table 5.1.).

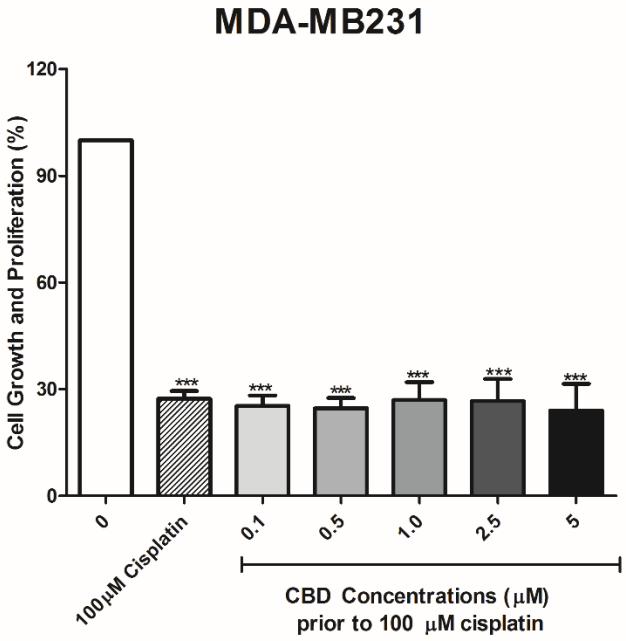

Figure 5.5. The cell growth and proliferation of MDA-MB231 cells following treatment with 100 μM cisplatin and "priming".
MDA-MB231's growth was examined following 100 μM cisplatin and 0.1 μM-5 μM CBD "priming" treatment. Data shown is repeated 3 times with 5 technical replicates per plate. Data is represented as mean ± SEM. *** $p<0.001$ *vs* Untreated.

Table 5.1. Cell growth and proliferation of MDA-MB231 values following "priming".

	0.1 μM CBD	0.5 μM CBD	1 μM CBD	2.5 μM CBD	5 μM CBD	100 μM Cisplatin
Average (%)	25.33	24.67	27.00	26.67	24.00	27.33
S.E.M.	2.906	2.848	5.033	6.173	7.506	2.186

5.5.2. The effect of "priming" on control cell's viability

Control cells were also examined for "priming" to observe whether the "normal" cells would react the same as cancer cells. Cell viability was again assessed by MTT assay (Section 2.7.2.) and lactate concentration (Section 2.7.3). Cell proliferation was assessed using a BrdU proliferation assay (Section 2.7.4.).

5.5.2.1. "Priming" with CBD prior to 50 μM cisplatin treatment's effect on control cell viability

Control cells cell viability was measured using an MTT assay (Section 2.7.2.) and was reduced following 50 μM cisplatin treatment (83.54 ± 2.280 %) ($p<0.05$) (Figure 5.6.). No significant reduction in cell viability was observed following "priming" treatment until 2.5 μM CBD (73.67 ± 4.667 %) ($p<0.05$) and 5 μM CBD treatment (61.04 ± 6.429 %) ($p<0.001$).

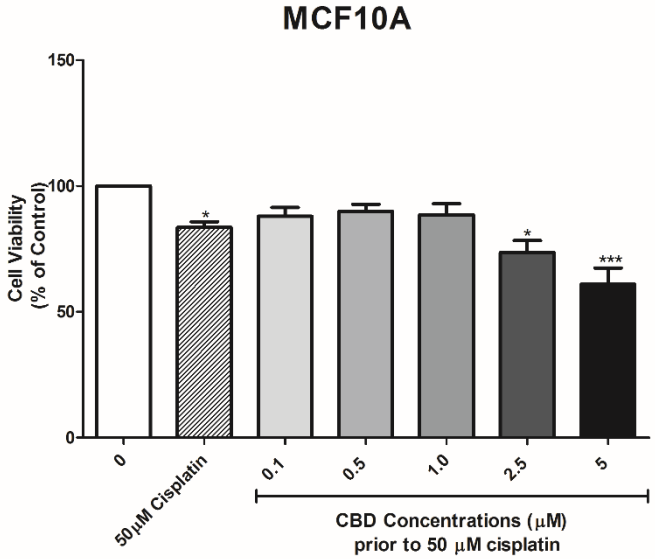

Figure 5.6. The effect of "priming" with CBD prior to 50 μM cisplatin treatment on control cell's viability.
Control cells following "priming" with 0.1 μM- 5 μM CBD 24 hours prior to 50 μM cisplatin treatment for 24 hours. Cell viability was examined using an MTT assay. Data shown is repeated 3 times with 5 technical replicates per plate. Data is represented as mean ± SEM. * $p<0.05$, *** $p<0.001$ *vs* Untreated.

5.5.2.2. The effect of "priming" with CBD prior to 100 μM cisplatin treatment on control cell's viability

To assess cell viability following "priming" with CBD prior to 100 μM cisplatin an MTT assay was carried out (Section 2.7.2.). A significant reduction in cell viability was observed following 100 μM cisplatin (33.46 ± 2.794 %) ($p<0.001$) and combination treatment (70.67 ± 5.457 %) ($p<0.05$) (Figure 5.7.). Control cell viability was reduced

following “priming” treatment; 0.1 μM CBD (45.23 ± 11.382 %) (p<0.01), 0.5 μM CBD, (48.38 ± 15.621 %) (p<0.01), 1.0 μM CBD (41.17 ± 9.914 %) (p<0.001), 2.5 μM CBD, (33.90 ± 14.551 %) (p<0.001), 5 μM CBD (30.61 ± 16.190 %) (p<0.001). However, no difference between “priming” and 100 μM cisplatin alone was observed.

Note that further to these viability assessments “priming” will consist of a complete profile of pre-treatment and a comparison graph as discussed in section 5.2.1.2.

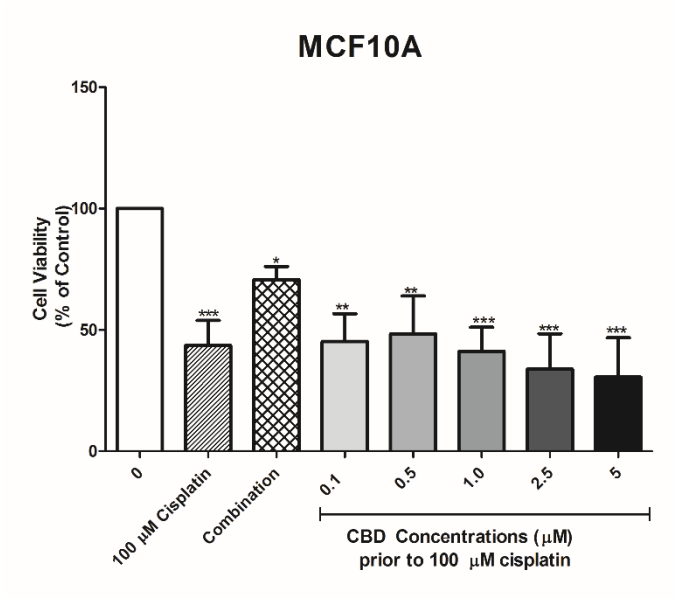

Figure 5.7. Control cell viability following treatment of “priming” with CBD prior to 100 μM cisplatin treatment for 24 hours.
Control cells following “priming” with 0.1 μM- 5 μM CBD 24 hours prior to 100 μM cisplatin treatment for 24 hours. The cells viability was examined using an MTT assay. Data shown is repeated 3 times with 5 technical replicates per plate. Data is represented as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001 *vs* Untreated.

To measure cell viability further a lactate dehydrogenase assay was carried out (Section 2.7.3.). Lactate dehydrogenase concentration showed a significant dose dependant increase following “priming” treatment (Figure 5.8.). The increase was observed following 0.1 μM CBD (305.3 ± 5.33 %) (p<0.001), 0.5 μM CBD (317.1 ± 6.92 %) (p<0.001), 1 μM CBD (326.5 ± 8.76 %) (p<0.001), 2.5 μM CBD (535.1 ± 11.21 %)

(p<0.001) and 5 μM CBD (587.2 ± 84.17 %) (p<0.01) treatment compared to untreated cells.

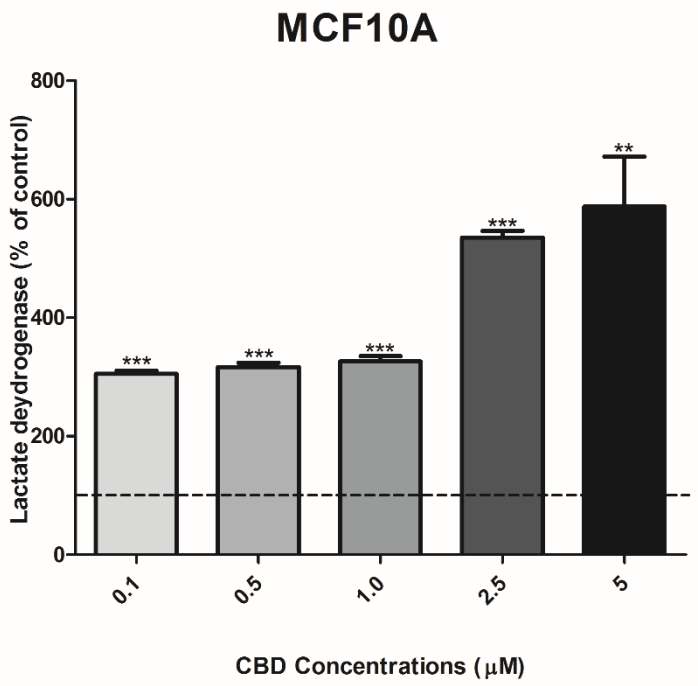

Figure 5.8. Control cells lactate dehydrogenase concentration following "priming" treatment.
Control cell's lactate dehydrogenase concentrations were analysed following "priming" treatment. Data shown is repeated 3-5 times with 5 technical replicates per plate. The dashed line represents untreated cells and data was normalised to cell count. Data is represented as mean ± SEM. ** p<0.01, *** p<0.001 *vs* Untreated.

The lactate dehydrogenase concentration of each previous treatment used: CBD, cisplatin, combination and "priming", was compared to each other (Figure 5.9). Lactate dehydrogenase concentrations were unchanged following CBD treatment. An increase in lactate dehydrogenase concentrations was observed following treatment with cisplatin (232.8 ± 4.85 %) compared to untreated cells (p<0.05), CBD (p<0.001) and combination (p<0.001) treatment. Following treatment with combination a significant increase (147.7 ± 3.33 %) compared to untreated cells (p<0.05) and CBD (p<0.001) was observed. "Priming" treatment showed an increase in lactate dehydrogenase

concentrations (326.5 ± 8.76 %) compared to untreated cells (p<0.01), CBD (p<0.001), cisplatin (p<0.001) and combination (p<0.001).

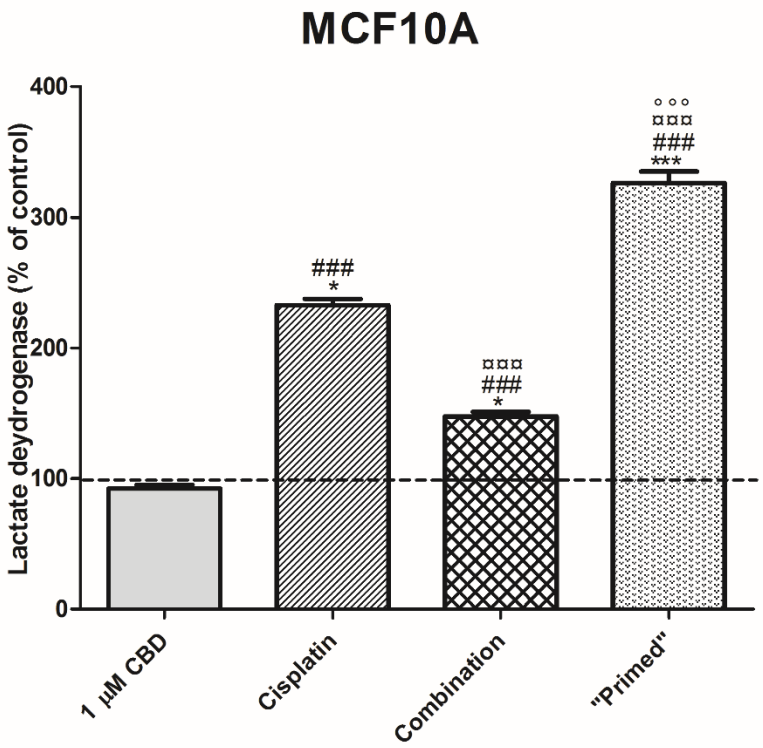

Figure 5.9. Lactate dehydrogenase concentration in control cells for all treatments compared to each other.
Control cell's lactate dehydrogenase concentrations were compared against each other. Data shown is repeated 3-5 times with 5 technical replicates per plate. The dashed line represents untreated cells and data was normalised to cell count. Data is represented as mean ± SEM. * p<0.05, *** p<0.001 *vs* Untreated; ### p<0.001 *vs* CBD; ¤¤¤ p<0.001 *vs* Cisplatin; °°° p<0.001 *vs* Combination.

Control cell growth and proliferation was investigated using a BrdU proliferation assay (Section 2.7.4.). Control cell growth was inhibited following treatment with 100 µM cisplatin (p<0.001) (Figure 5.10.) (Table 5.2.). There was a similar response following "priming" treatment. No difference was observed between cell growth of 100 µM cisplatin and "primed" cells.

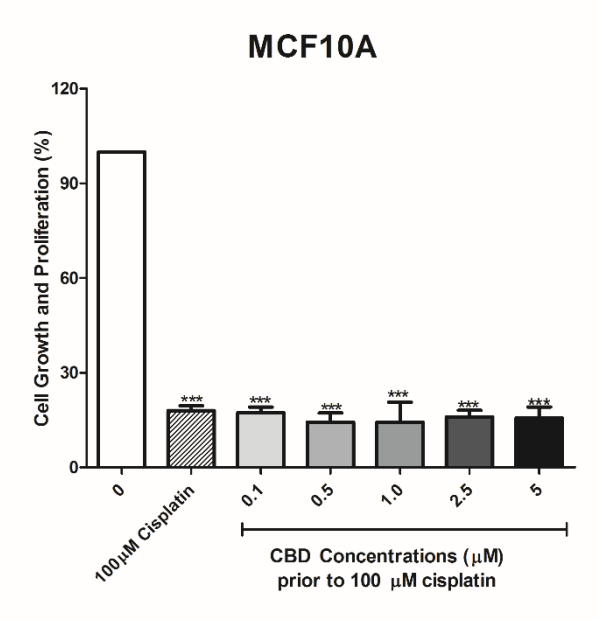

Figure 5.10. The effect of control cell growth and differentiation following treatment with 100 μM cisplatin and "priming" with CBD.
Normal cell's growth was examined after 100 μM cisplatin and 0.1 μM-5 μM CBD "priming". Data shown is repeated 3 times with 5 technical replicates per plate. Data is represented as mean ± SEM. *** $p<0.001$ *vs* Control.

Table 5.2. Control cell growth and differentiation absolute values following "priming".

	0.1 μM CBD	0.5 μM CBD	1 μM CBD	2.5 μM CBD	5 μM CBD	100 μM Cisplatin
Average (%)	17.33	14.33	14.33	16.00	15.67	18.00
S.E.M.	1.764	2.963	6.360	2.082	3.480	1.528

5.5.3. The effect of "priming" with CBD on additional cancer cell's viability

"Priming" with 1 μM CBD and 100 μM cisplatin treatment on MDA-MB231 breast cancer cells was shown to enhance cancer cell death. Several additional cancer cell lines were examined following "priming" with CBD. Cell viability was assessed using an MTT assay (Section 2.7.2.).

5.5.3.1. The effect of "priming" with CBD in liver cancer cells (HepG2) cell viability

Liver cancer cells (HepG2) showed a significant reduction in cell viability following 100 μM cisplatin (57.33 ± 0.882 %) ($p<0.01$) and combination treatment (58.33 ± 5.608 %) ($p<0.05$) (Figure 5.11.). "Priming" showed a significant reduction in viable cells with all treatments; 0.1 μM CBD (67.40 ± 5.644 %) ($p<0.01$), 0.5 μM CBD (64.80 ± 4.352 %) ($p<0.01$), 1 μM CBD (54.50 ± 2.723 %) ($p<0.001$), 2.5 μM CBD (52.50 ± 1.258 %) ($p<0.001$), 5 μM CBD (39.13 ± 1.958 %) ($p<0.001$). Furthermore, 5 μM CBD "primed" showed a significant decrease compared to 100 μM cisplatin treatment alone ($p<0.05$).

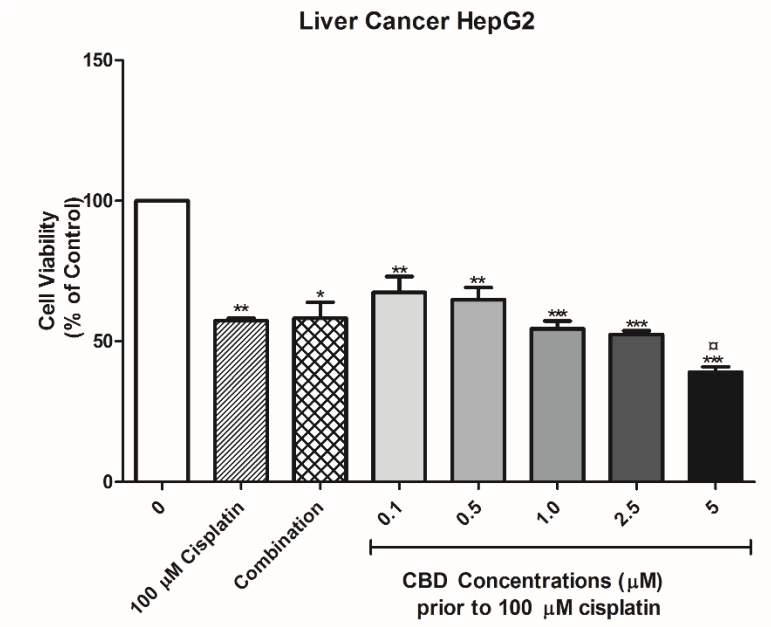

Figure 5.11. Liver cancer cell's viability following "priming" with CBD prior to 100 μM cisplatin treatment, cisplatin and combination treatments.
Liver cancer cells were examined following "priming" with 0.1 μM-5 μM CBD for 24 hours prior to 100 μM cisplatin treatment for 24 hours. Data shown is repeated 3 times with 5 technical replicates per plate. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs* Untreated; ¤ $p<0.05$ *vs* Cisplatin.

5.5.3.2. Cell viability's effect following "priming" with CBD in colon cancer cells (HT29)

HT29, colon cancer cell lines showed a significant reduction in cell viability with 100 μM cisplatin treatment (81.50 ± 2.742 %) (p<0.001) (Figure 5.12.). No difference between combination and control treatments was observed. However, "priming" showed a significant reduction in cell viability; 0.1 μM CBD (72.11 ± 2.646 %) (p<0.01), 0.5 μM CBD (71.25 ± 6.460 %) (p<0.01), 1 μM CBD (65.04 ± 3.215 %) (p<0.001), 2.5 μM CBD (56.23 ± 2.646 %) (p<0.001), 5 μM CBD (55.33 ± 2.667 %) (p<0.001). A significant decrease was observed compared to cisplatin following 1 μM CBD "primed" (p<0.01), 2.5 μM CBD "primed" (p<0.001) and 5 μM CBD "primed" treatment (p<0.001). Furthermore, a significant decrease was observed compared to combination treatment following 1 μM CBD "primed" (p<0.01), 2.5 μM CBD "primed" (p<0.001) and 5 μM CBD "primed" treatment (p<0.001).

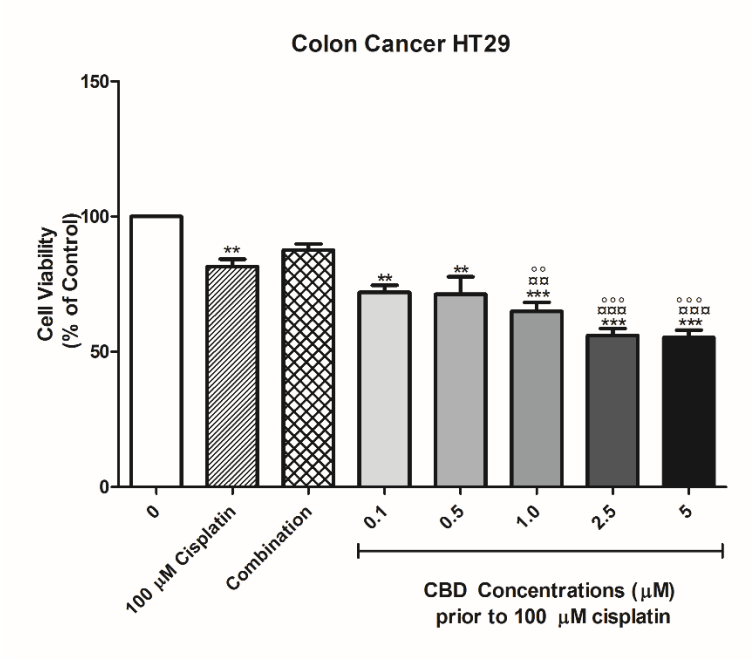

Figure 5.12. The effect of cisplatin, combination and "priming" treatment on colon cancer cells viability.
Colon cancer cells were examined following "priming" with 0.1 μM-5 μM CBD for 24 hours prior to 100 μM cisplatin treatment for 24 hours. Data shown is repeated 3 times with 5 technical replicates per plate. Data is represented as mean ± SEM. ** p<0.01, *** p<0.001 *vs* Untreated; ¤¤ p<0.01, ¤¤¤ p<0.001 *vs* Cisplatin; °° p<0.01, °°° p<0.001 *vs* Combination.

5.5.3.3. The effect of "priming" with CBD in breast cancer cells (MCF7) cell viability

MCF7, breast cancer cells were assessed as a negative "priming" control due to cisplatin having no effect on MCF7 cell viability. No change was also observed with combination treatments (Figure 5.13.). Following "priming", a significant increase in viable cells was observed; 0.1 μM CBD (136.41 ± 5.913 %) ($p<0.001$), 0.5 μM CBD (135.63 ± 7.760 %) ($p<0.001$), 1 μM CBD (123.42 ± 9.147 %) ($p<0.05$). This increase had a tendency for reduction as the dose increased resulting in no difference between untreated cells and 2.5 μM CBD and 5 μM CBD "primed".

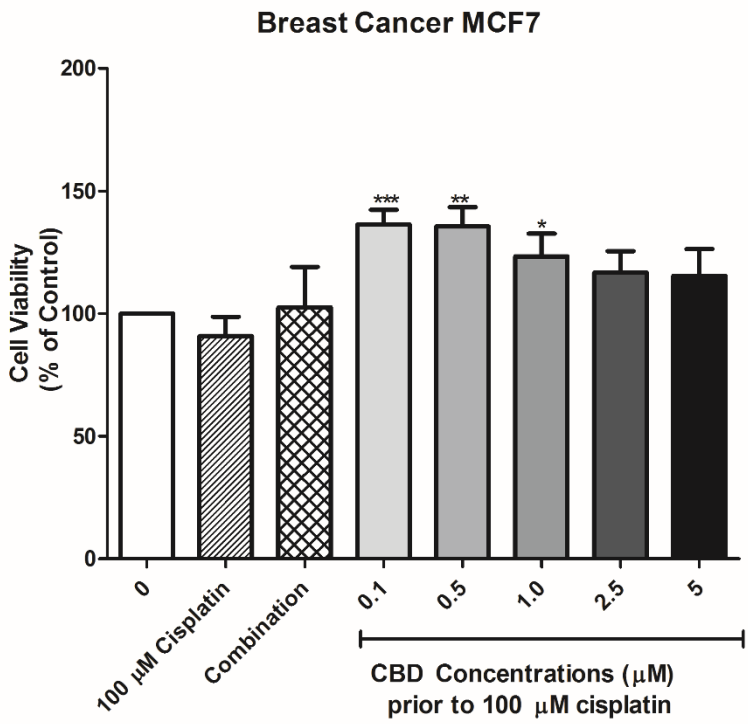

Figure 5.13. Breast cancer (MCF7) cells cell viability following treatment with cisplatin, combination and "priming" with CBD prior to 100 μM cisplatin.
Breast cancer (MCF7) cells following "priming" with 0.1 μM-5 μM CBD for 24 hours prior to 100 μM cisplatin treatment for 24 hours. Data shown is repeated 3 times with 5 technical replicates per plate. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs* Untreated.

5.5.3.4. The effect of "priming" with CBD in lung cancer cells (A549) cell viability

Lung cancer, A549, cells showed no difference following 100 μM cisplatin and combination treatments (Figure 5.14.). As another negative control to "priming" a trend to increase in cell viability was observed as doses of CBD "priming" increased from 1 μM CBD (138.53 ± 11.273 %) ($p<0.05$), 2.5 μM CBD (164.82 ± 15.221 %) ($p<0.05$) to 5 μM CBD (135.21 ± 9.394 %) ($p<0.05$). Compared to cisplatin a significant increased difference was observed with 0.5 μM CBD ($p<0.05$), 1 μM CBD ($p<0.05$), 2.5 μM CBD ($p<0.001$) and 5 μM CBD ($p<0.05$). A significant increase compared to combination treatment was observed with 2.5 μM CBD ($p<0.01$).

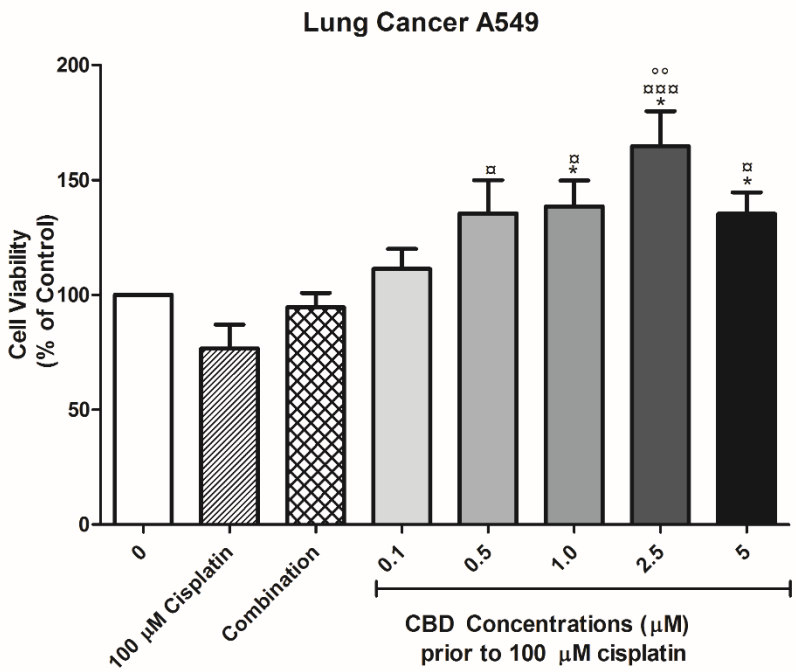

Figure 5.14. The effect of cisplatin, combination and "priming" on the cell viability of lung cancer cells.
Lung cancer cells following treatment with "priming" 0.1 μM-5 μM CBD for 24 hours prior to 100 μM cisplatin treatment. Data shown is repeated 3 times with 5 technical replicates per plate. Data is represented as mean ± SEM. * $p<0.05$ *vs* Untreated; ¤ $p<0.05$, ¤¤¤ $p<0.001$ *vs* Cisplatin; °° $p<0.01$ *vs* Combination.

5.5.3.5. "Priming" with CBD's effect on the cell viability of mitochondria altered cells (Rho0)

Complex I and IV in the mitochondria were altered in A549 cells to create a mitochondria "free" cell line, Rho0. Rho0 cell viability was significantly reduced compared to the control with 100 μM cisplatin (77.67 ± 8.762 %) ($p<0.01$) and combination treatment (67.67 ± 3.480 %) ($p<0.001$) (Figure 5.15.). A trend to decrease in cell viability was observed following "priming" from 0.1 μM CBD (59.60 ± 3.280 %), 0.5 μM CBD (54.13 ± 2.302 %), 1 μM CBD (51.40 ± 2.874 %), 2.5 μM CBD (46.40 ± 2.227 %), 5 μM CBD (36.20 ± 3.184 %) ($p<0.001$). A significant difference compared to cisplatin treatment was observed with 0.1 μM CBD ($p<0.05$), 0.5 μM CBD ($p<0.05$), 1 μM CBD ($p<0.001$), 2.5 μM CBD ($p<0.001$) and 5 μM CBD ($p<0.001$). A significant decrease compared with combination was observed following 2.5 μM CBD "priming" ($p<0.01$), 5 μM CBD "priming" ($p<0.001$) treatment.

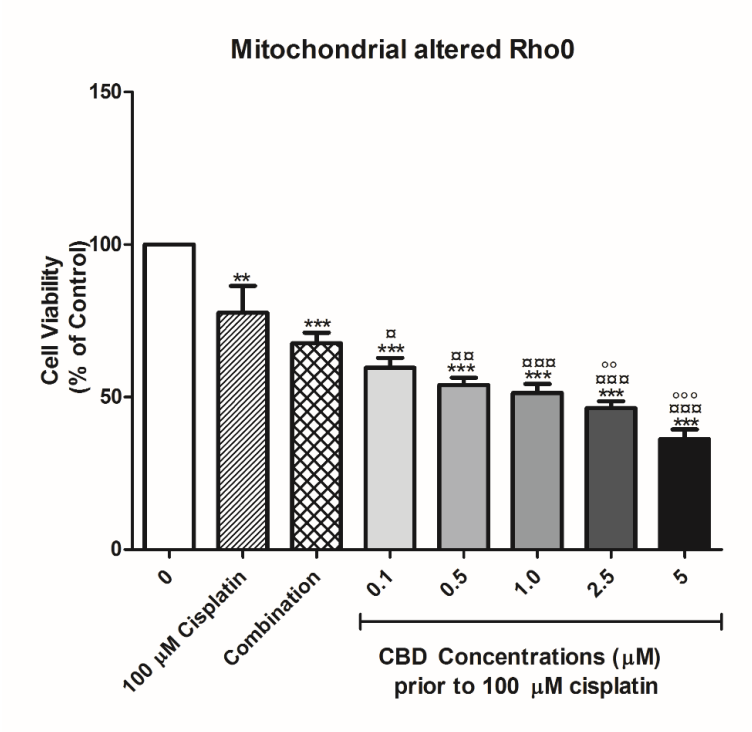

Figure 5.15. The effect of cell viability in mitochondria altered cells following treatment with cisplatin, combination and "priming" with CBD.
Mitochondria altered cells following "priming" treatment with 0.1 μM-5 μM CBD for 24 hours prior to 100 μM cisplatin treatment for 24 hours. Data shown is repeated 3 times with 5 technical replicates per plate. Data is represented as mean ± SEM. ** $p<0.01$, *** $p<0.001$ *vs* Untreated; ¤ $p<0.05$, ¤¤ $p<0.01$, ¤¤¤ $p<0.001$ *vs* Cisplatin; °° $p<0.01$, °°° $p<0.001$ *vs* Combination.

5.5.4. The effect of cell viability following "priming" with other molecules

5.5.4.1. "Priming" with quercetin's effect on cell vaibility

The effect of "priming" with quercetin treatment has already been pursued in cancer cells (Sharma *et al.* 2005, Kuhar *et al.* 2007). This work was re-evaluated here using MDA-MB231 cancer cells and MCF10A control cells with the same "priming" dose cited.

Cancer cells showed no difference in cell viability following treatment with 40 μM quercetin alone but a significant reduction with cisplatin treatment was observed (44.25 ± 2.529 %) ($p<0.01$) (Figure 5.16.A.). A significant reduction in cell viability was shown following "priming" with quercetin (42.28 ± 8.064 %) however, it was only significantly different to untreated ($p<0.01$) and 40 μM quercetin alone treatment ($p<0.05$).

Control cells showed no difference when treated with 40 μM quercetin (Figure 5.16.B.). A significant reduction in cell viability was observed following "priming" (45.94 ± 8.148 %) however, again this was only significantly different to untreated cells ($p<0.01$) and 40 μM quercetin treatment ($p<0.05$).

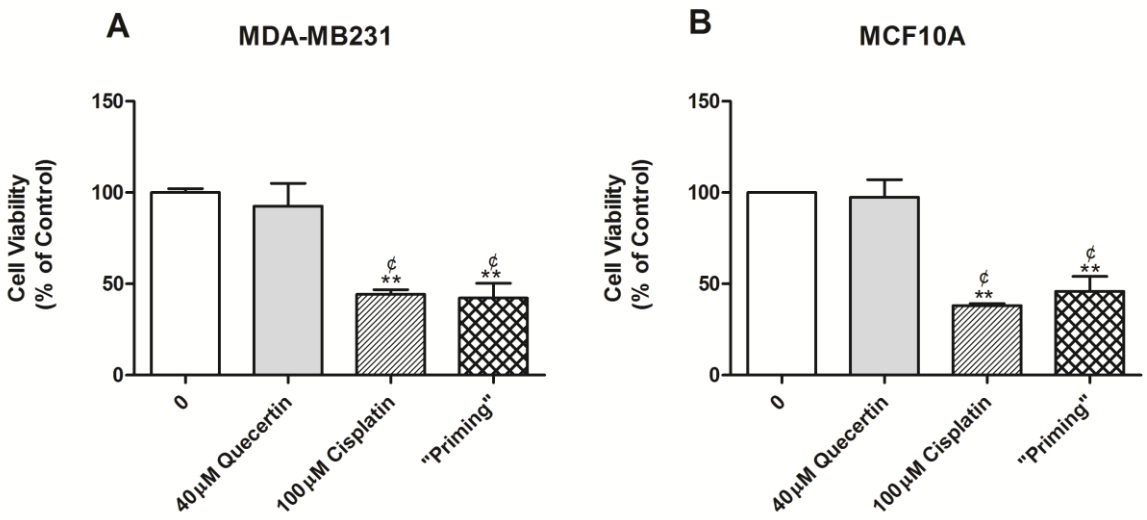

Figure 5.16. The effect of cell viability following treatment with 40 µM quercetin, cisplatin and "priming" with quercetin in MDA-MB231 and MCF10A cells.
"Priming" with 40 µM quercetin for 24 hours prior to 100 µM cisplatin treatment for 24 hours was assessed. **(A)** MDA-MB231 cells and **(B)** MCF10A cells were assessed using an MTT assay. Data shown is repeated 3 times with 5 technical replicates per plate. Data is represented as mean ± SEM. ** $p<0.01$ *vs* Untreated; ¢ $p<0.05$ *vs* Quercetin.

5.5.4.2. The effect of cell viability following "priming" with acetate

Acetate was also examined as a potential "priming" model for both the MDA-MB231 cancer cells and control cells.

Treatment with 1mM acetate showed no change in cell viability (98.33 ± 1.453 %) (Figure 5.17.A.). "Priming" with acetate showed a significant reduction in cell viability (58.11 ± 0.577 %) ($p<0.001$). This reduction was also significate to 1 mM acetate alone ($p<0.001$) and 50 µM cisplatin ($p<0.01$).

The control cells showed no change in cell viability following 1 mM acetate treatment (112.11 ± 11.551 %) (Figure 5.17.B.). However, when the cells were "primed" a significant increase (194.71 ± 0.882 %) in viable cells was observed compared to untreated cells ($p<0.001$), 1 mM acetate ($p<0.001$) and 50 µM cisplatin ($p<0.001$).

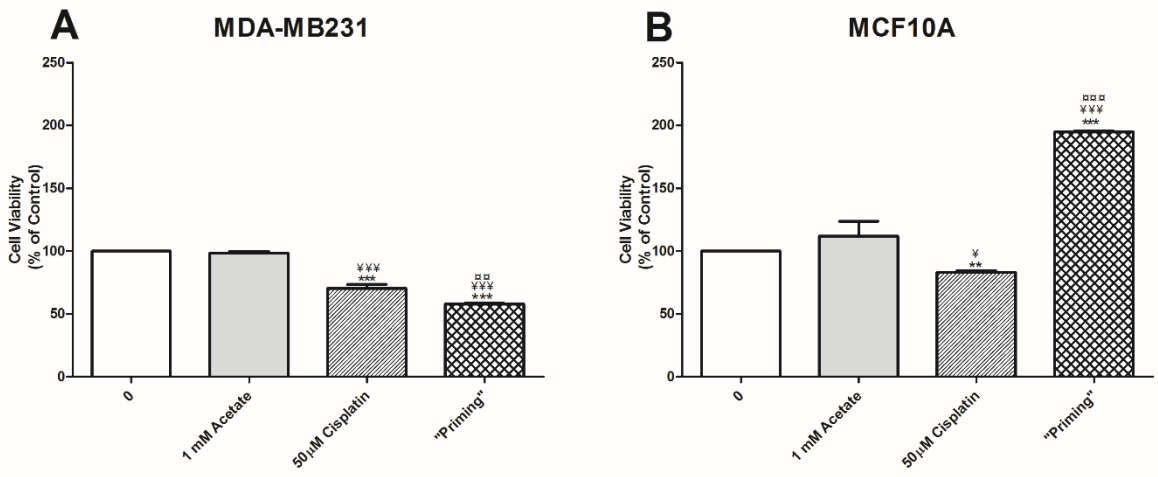

Figure 5.17. The effect of 1 mM acetate, 50 µM cisplatin and "priming" with acetate prior to 50 µM cisplatin in cancer and control cell viability.
Acetate (1 mM) was used as a "priming" treatment 24 hours prior to 50 µM cisplatin treatment for 24 hours. **(A)** MDA-MB231 cells were treated with 1 mM acetate, 50 µM cisplatin and acetate "priming". **(B)** MCF10A cells were treated with the same conditions prior to MTT assay. Data shown is repeated 3 times with 5 technical replicates per plate. Data is represented as mean ± SEM. ** $p<0.01$, *** $p<0.001$ *vs* Untreated; ¥¥¥ $p<0.001$ *vs* Acetate; ¤ $p<0.05$, ¤¤ $p<0.01$, ¤¤¤ $p<0.001$ *vs* Cisplatin.

"Priming" with 1 mM acetate and 100 µM cisplatin treatment showed no change in cell viability following 1mM acetate treatment (Figure 5.18.A.). However, acetate "priming" showed a significant decrease in cell viability (56.14 ± 2.646 %) ($p<0.01$). This was not only significant to untreated levels but also when compared with 1 mM acetate alone ($p<0.001$). However, no added enhancement was observed compared to cisplatin alone.

Control cells showed no change following 1 mM acetate treatment. Interestingly a significant decrease in cell viability was observed with "priming" (80.13 ± 4.564 %) ($p<0.05$) (Figure 5.18.B.). No added effect was observed when compared to acetate or cisplatin treatment alone.

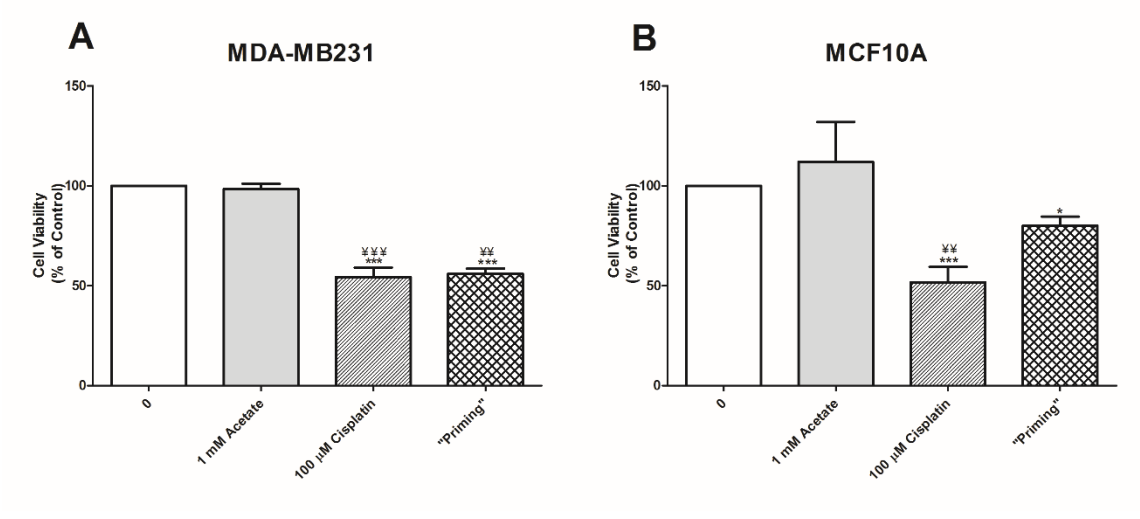

Figure 5.18. The effect of 1 mM acetate, 100 µM cisplatin and "priming" with acetate prior to 100 µM cisplatin treatment on cancer and control cell viability.
Acetate (1 mM) was used as a "priming" treatment 24 hours prior to 100 µM cisplatin treatment for 24 hours. **(A)** MDA-MB231 cells were treated with 1 mM acetate, 100 µM cisplatin and acetate "priming". **(B)** MCF10A cells were treated with the same conditions prior to MTT assay. Data shown is repeated 3 times with 5 technical replicates per plate. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs* Untreated; ¥¥¥ $p<0.001$ *vs* Acetate.

5.6. "Priming" with CBD's effect on AMPK activity in the cell

AMPK activity was measured as it has been previously mentioned to increase tumourogensis when AMPK itself is increased (Section 3.5.). Following CBD treatment there was a trend to decreased AMPK activity in cancer cells (3.5.1.). Investigating AMPK's role in "priming" with CBD could highlight the molecular mechanism by which "priming" is working.

5.6.1. Cancer cell's AMPK activity following "priming" with CBD

5.6.1.1. The effect of "priming" with CBD in cancer cell's AMPK activity

AMPK activity was measured following "priming" with CBD in cancer cells (Section 2.8.). A significant decrease in AMPK activity was observed following treatment with 0.1 μM CBD (65.80 ± 7.742 %), 0.5 μM CBD (62.80 ± 10.032 %), 1 μM CBD (69.13 ± 9.051 %), 2.5 μM CBD (66.20 ± 7.832 %) and 5 μM CBD (67.85 ± 5.967 %) ($p<0.05$) (Figure 5.19.). This decrease remained constant for all doses of CBD "priming".

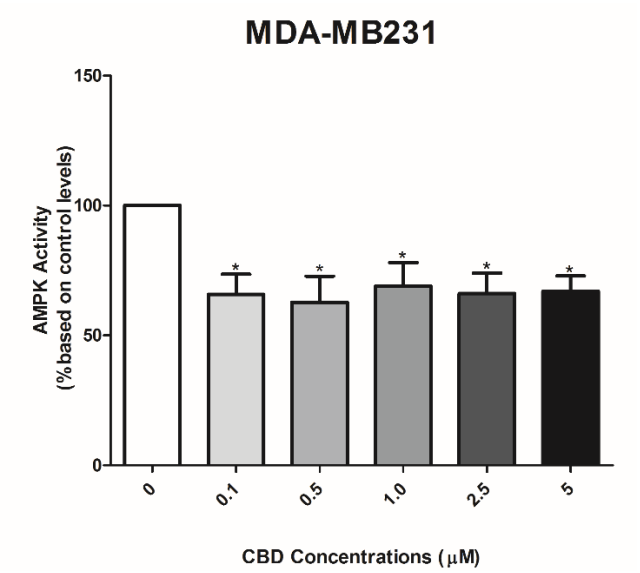

Figure 5.19. AMPK activity was assayed following treatment with "priming" in cancer cells. MDA-MB231 cells were analysed for any alteration to AMPK activity following "priming" treatment. Data shown is repeated 3-4 times. Data is represented as mean ± SEM. * $p<0.05$, *vs* Untreated.

5.6.1.2. The comparison of "priming" with CBD with all other previous treatments in cancer cells

AMPK activity of all the treatments were compared against each other and showed no added difference between treatments (Figure 5.20.).

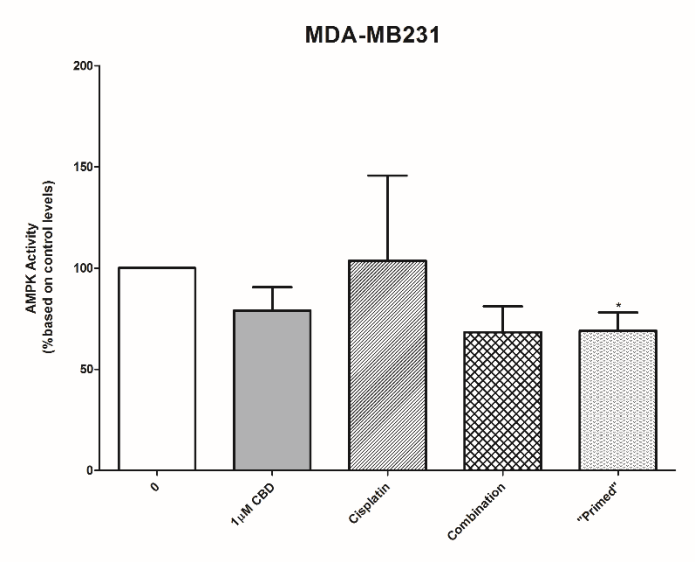

Figure 5.20. The effect of CBD, cisplatin, combination and "priming" on MDA-MB231's AMPK activity.
MDA-MB231 cell treatments were compared against each other for AMPK activity comparison. Data shown is repeated 3-4 times. Data is represented as mean ± SEM. * $p<0.05$, *vs* Untreated.

5.6.2. The effect of "priming" with CBD on the AMPK activity of control cells

5.6.2.1. "Priming" with CBD's effect on AMPK activity in control cells

Control cell's AMPK activity showed no difference to untreated levels with all treatment doses of "priming" (Figure 5.21.). "Priming" with CBD was similar to untreated cell levels.

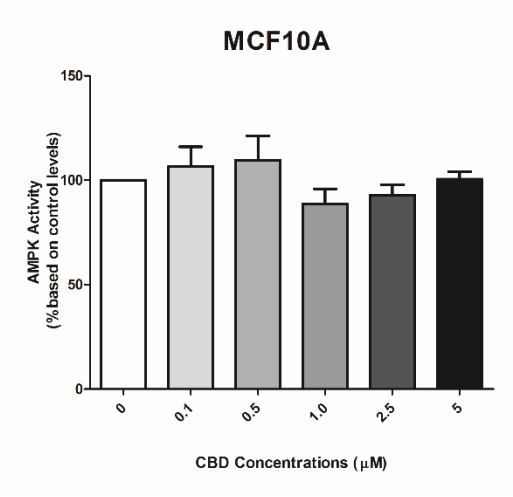

Figure 5.21. Control cell's AMPK activity following "priming" treatment.
Control cells were analysed for any alteration to AMPK activity following "priming" treatment. Data shown is repeated 3-4 times. Data is represented as mean ± SEM.

5.6.2.2. Comparing "priming" with CBD with all other previously examined treatments

Comparing all treatments performed on the control cells showed no further difference between treatments (Figure 5.22.).

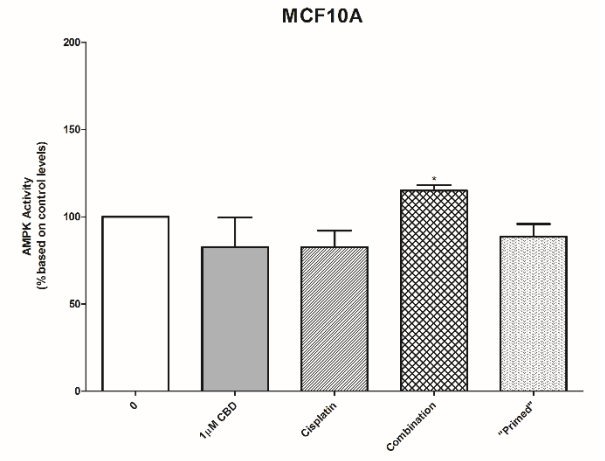

Figure 5.22. Control cell's AMPK activity treatments compared to each other.
The treatments performed on control cell's AMPK activity was compared against each other. Data shown is repeated 3-4 times. Data is represented as mean ± SEM. * $p<0.05$, *vs* Untreated.

5.7. The effect of "priming" with CBD on the ROS levels in the cell

There are several key regulators of cellular metabolism which include ROS (Section 1.1.3.4. and 3.6.). ROS levels can control a cell's fate whereby increased ROS leads to increased cell death (Brookes *et al*. 2004, Sena and Chandel 2012). Increased levels of ROS were observed in cancer cells following CBD treatment (Section 3.6.1.) and cisplatin treatment (4.5.1.).

5.7.1. Cancer cell's ROS levels following "priming" with CBD

5.7.1.1. The effect of "priming" with CBD on cancer cell's ROS levels

ROS levels were measured using a DCFDA assay (Section 2.9.) following "priming" treatment (Section 2.6.5.). There was a significant increase in ROS following "priming" treatment with 0.1 μM CBD (8.95 ± 0.256 A.U.) ($p<0.01$), 0.5 μM CBD (8.88 ± 0.253 A.U.) ($p<0.01$), 1 μM CBD (9.87 ± 0.338 A.U.) ($p<0.01$), 2.5 μM CBD (15.39 ± 0.629 A.U.) ($p<0.01$) and 5 μM CBD (18.32 ± 1.898 A.U.) ($p<0.05$) (Figure 5.23.). A dose dependant increase was observed with "priming".

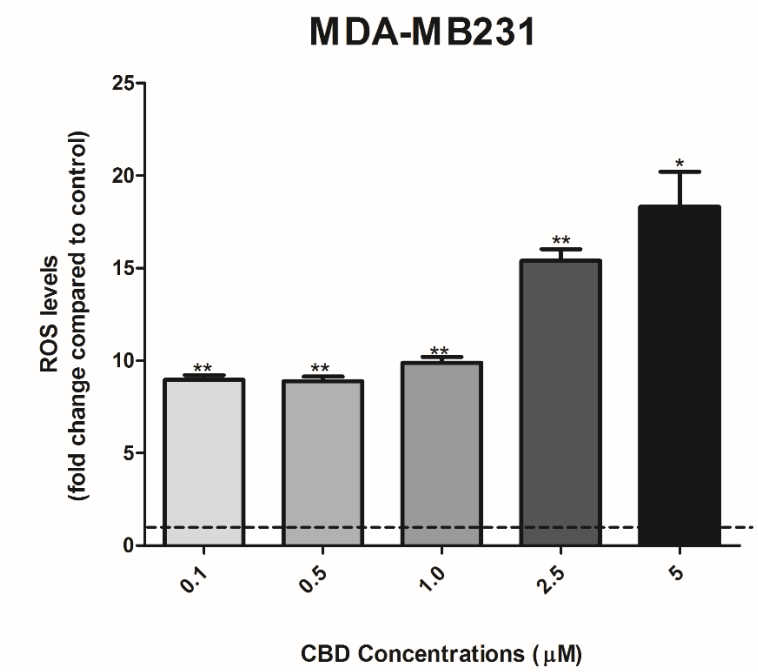

Figure 5.23. MDA-MB231 ROS levels following "priming" treatment.
Cancer cells (MDA-MB231) ROS levels were analysed following "priming" treatment. Data shown was repeated 3-5 times with 5 technical replicates per plate. The dashed line represents untreated cells and data was normalised to cell count. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs* Untreated.

5.7.1.2. The effect of "priming" with CBD compared to all other previously examined treatments

Following cisplatin treatment ROS levels increased (7.11 ± 0.150 A.U.) compared to untreated cells ($p<0.001$), CBD ($p<0.001$) and combination treatment ($p<0.001$) (Figure 5.24.). The increase observed with "priming" was not only significant to untreated cells ($p<0.05$) but also to CBD ($p<0.001$), cisplatin ($p<0.01$) and combination ($p<0.001$) treatments.

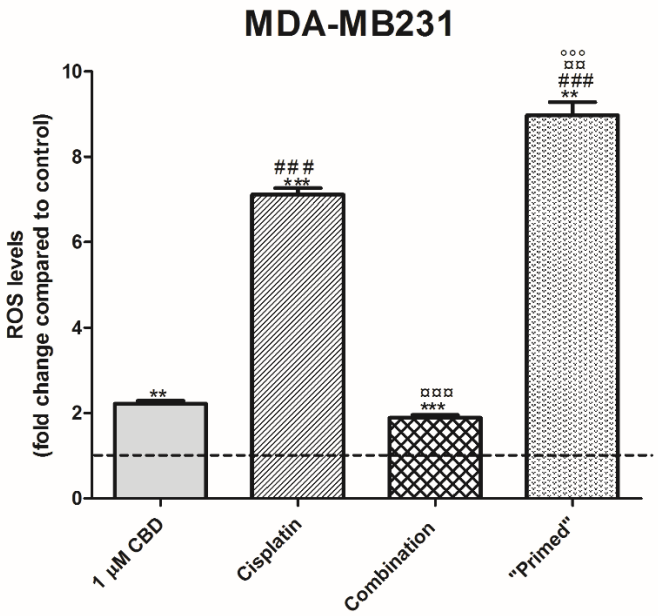

Figure 5.24. MDA-MB231 cells ROS levels were compared to all other treatments.
MDA-MB231 cell's ROS levels were compared against each other. Data shown is repeated 3-5 times with 5 technical replicates per plate. The dashed line represents untreated cells and data is normalised to cell count. Data is represented as mean ± SEM. ** p<0.01, *** p<0.001 *vs* Untreated; ### p<0.001 *vs* 1 µM CBD; ¤¤ p<0.01, ¤¤¤ p<0.001 *vs* Cisplatin; °°° p<0.001 *vs* Combination.

5.7.2. The effect of "priming" with CBD in control cell's ROS levels

5.7.2.1. "Priming" with CBD's effect on control cell's ROS levels

ROS showed a trend to decrease following "priming" compared to untreated cells (Figure 5.25.). A significant reduction was observed following treatment with 0.5 μM CBD (0.81 ± 0.044 A.U.) ($p<0.05$), 1 μM CBD (0.69 ± 0.047 A.U.) ($p<0.05$) and 2.5 μM CBD "primed" (0.58 ± 0.047 A.U.) ($p<0.05$).

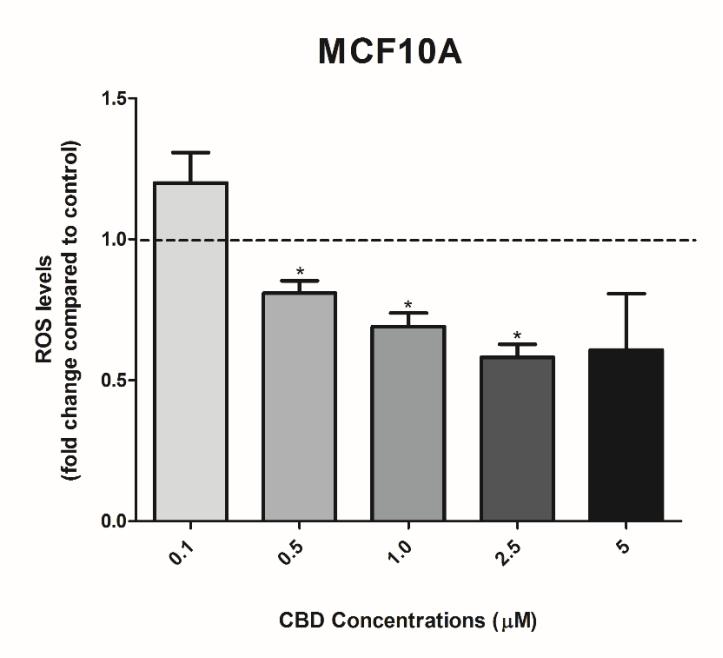

Figure 5.25. The effect of "priming" with CBD on control cell's ROS levels.
Control cell's ROS levels were analysed following "priming". Data shown is repeated 3-5 times with 5 technical replicates per plate. The dashed line represents untreated cells and data is normalised to cell count. Data is represented as mean ± SEM. * $p<0.05$, *vs* Untreated.

5.7.2.2. The effect of "priming" with CBD compared to all other previously examined treatments

Following cisplatin treatment ROS levels were significantly increased compared to untreated levels (7.15 ± 0.927 A.U.) ($p<0.01$), CBD ($p<0.05$), combination ($p<0.01$) and "priming" ($p<0.05$) treatment (Figure 5.26.).

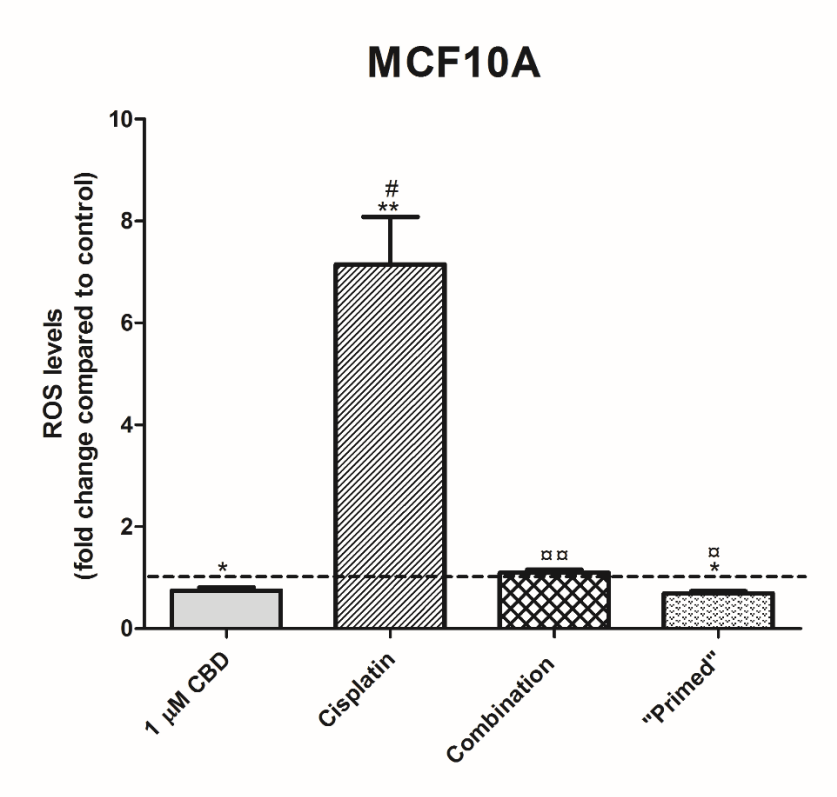

Figure 5.26. Control cell's ROS levels compared with all treatments.
Control cell's ROS levels were compared against each other. Data shown is repeated 3-5 times with 5 technical replicates per plate. The dashed line represents untreated cells and data is normalised to cell count. Data is represented as mean ± SEM. * $p<0.05$, *vs* Untreated; # $p<0.05$ *vs* 1 µM CBD; ¤ $p<0.05$, ¤¤ $p<0.01$ *vs* Cisplatin.

5.8. The cellular Ca^{2+} level's following "priming" with CBD

As mentioned previously another important regulator of cellular metabolism and cell viability is Ca^{2+} levels (Section 1.1.3.3. and 3.7.). Following both CBD (Section 3.7.1.) and cisplatin (Section 4.6.1.) treatment an increase in Ca^{2+} levels was observed.

5.8.1. The effect of "priming" with CBD in the Ca^{2+} levels of cancer cells

5.8.1.1. "Priming" treatment's effect on the Ca^{2+} levels of cancer cells

Measuring Ca^{2+} levels was carried out using a Fluo-4 Direct™ Calcium Assay (Section 2.10.). Following "priming" treatment a significant dose dependant increase in calcium levels was observed compared to untreated cells (Figure 5.27.). This increase was observed with all doses of "priming" ($p<0.001$) (Table 5.3.).

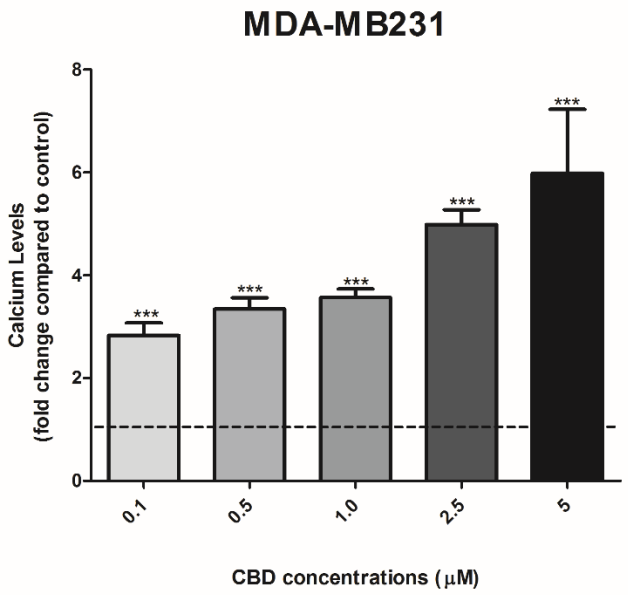

Figure 5.27. MDA-MB231 calcium levels following "priming" treatment.
MDA-MB231 cell's calcium levels following "priming" treatment. Data shown is repeated 3-5 times with 5 technical replicates per plate. The dashed line represents untreated cells and data is normalised to cell count. Data is represented as mean ± SEM. *** $p<0.001$ *vs* Untreated.

Table 5.3. MDA-MB231 calcium levels absolute values following "priming".

	0.1 μM CBD	0.5 μM CBD	1 μM CBD	2.5 μM CBD	5 μM CBD
Average (A.U.)	2.83	3.35	3.57	4.99	5.98
S.E.M.	0.244	0.218	0.168	0.286	1.24

5.8.1.2. The effect of "priming" with CBD compared against the previously examined treatments

A significant increase in calcium levels was observed following cisplatin treatment (1.52 ± 0.157 A.U.) which was significant to untreated cells ($p<0.05$), CBD ($p<0.05$), combination ($p<0.001$) and "priming" ($p<0.001$) treatment (Figure 5.28.). Combination treatment (0.72 ± 0.086 A.U.) showed a significant decrease in calcium levels compared to untreated cells ($p<0.05$) and CBD (1.19 ± 0.038 A.U.) ($p<0.001$). Finally, a significant increase in calcium levels was observed with "priming" (3.57 ± 0.168 A.U.) compared to untreated cells ($p<0.001$), CBD ($p<0.001$), cisplatin ($p<0.001$) and combination ($p<0.001$).

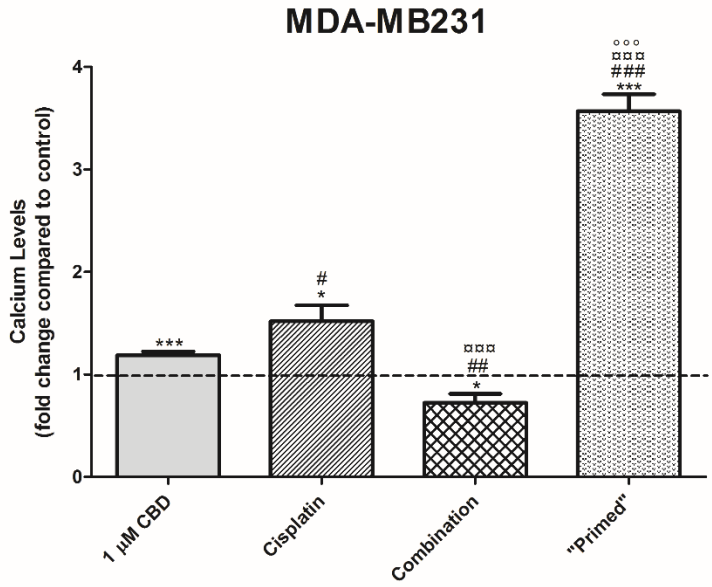

Figure 5.28. MDA-MB231 calcium levels for all treatments compared against each other. MDA-MB231 cell's calcium levels were compared against each other. Data shown is repeated 3-5 times with 5 technical replicates per plate. The dashed line represents untreated cells and data is normalised to cell count. Data is represented as mean ± SEM. * $p<0.05$, *** $p<0.001$ *vs* Untreated; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ *vs* 1 μM CBD; ¤¤¤ $p<0.001$ *vs* Cisplatin; °°° $p<0.001$ *vs* Combination.

5.8.2. The effect of Ca^{2+} levels in control cells following "priming" with CBD

5.8.2.1. "Priming" with CBD's effect on Ca^{2+} levels in control cells

A significant increase in calcium levels was observed following "priming" with 0.1 µM, 0.5 µM, 1 µM, 2.5 µM and 5 µM CBD compared to untreated cells ($p<0.001$) (Figure 5.29.) (Table 5.4.). The increase observed was at a constant level for all doses of "priming".

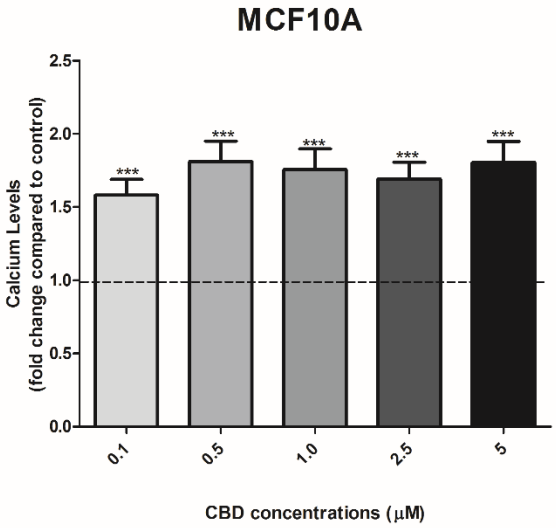

Figure 5.29. Control cells calcium levels following "priming" treatment.
Control cell's calcium levels were analysed following "priming". Data shown is repeated 3-5 times with 5 technical replicates per plate. The dashed line represents untreated cells and data is normalised to cell count. Data is represented as mean ± SEM. *** $p<0.001$ *vs* Untreated.

Table 5.4. Control cells calcium levels after "priming" values.

	0.1 µM CBD	0.5 µM CBD	1 µM CBD	2.5 µM CBD	5 µM CBD
Average (A.U.)	1.58	1.81	1.75	1.69	1.80
S.E.M.	0.109	0.139	0.143	0.115	0.145

5.8.2.2. The effect of "priming" on control cell's Ca^{2+} levels when compared to all other previously examined treatments

Calcium levels following cisplatin treatment (4.91 ± 0.457 A.U.) showed a significant increase compared to untreated cells (p<0.01), CBD (p<0.001) and combination treatment (p<0.001) (Figure 5.30.). An increase was also observed following combination treatment (3.25 ± 0.303 A.U.) compared to untreated cells (p<0.01) and CBD (p<0.001). Following treatment with "priming" (1.75 ± 0.143 A.U.) a significant increase was observed compared to untreated levels (p<0.001) and CBD (p<0.05). However, a significant decrease was observed compared to cisplatin (p<0.001).

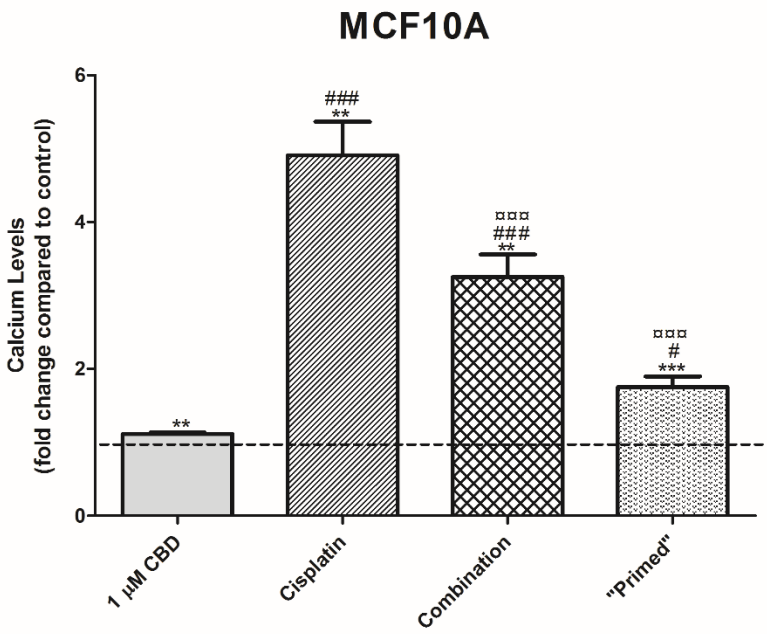

Figure 5.30. The effect of CBD, cisplatin, combination and "priming" treatment in control cells calcium levels.
Control cell's calcium levels were compared against each other. Data shown is repeated 3-5 times with 5 technical replicates per plate. The dashed line represents untreated cells and data is normalised to cell count. Data is represented as mean ± SEM. ** p<0.01, *** p<0.001 *vs* Untreated; # p<0.05, ### p<0.001 *vs* 1 μM CBD; ¤¤¤ p<0.001 *vs* Cisplatin.

5.9. "Priming" with CBD's effect on the mitochondrial function in the cell

Mitochondrial sensitization has been shown to be important for "priming" and understanding any alterations within mitochondrial function is key (Section 1.4.1., 1.4.3. and 1.4.4.) (Chonghaile *et al.* 2011, Reed 2011, Sarosiek *et al.* 2013). As seen earlier, treatment with CBD for 24 hours increased in mitochondrial dysfunction and respiration (Section 3.8.).

5.9.1. The cancer cell's mitochondrial function following "priming" with CBD

5.9.1.1. The effect of "priming" with CBD compared to previously examined treatments

Mitochondrial respiration was analysed using a Seahorse Bioanalyser Mitostress test (Section 2.11.). An increase in basal mitochondrial respiration was observed following CBD treatment (411.0 ± 28.19 pMoles/min) ($p<0.05$). However, following treatment with "priming" (226.3 ± 18.63 pMoles/min) a decrease was observed compared to untreated cells (313.1 ± 41.95 pMoles/min) ($p<0.01$) (Figure 5.31.A). No further significance was observed between the treatments.

Non-mitochondrial respiration was reduced following treatment with CBD (279.6 ± 31.92 pMoles/min) ($p<0.05$), cisplatin (175.1 ± 25.29 pMoles/min) ($p<0.001$), combination (229.9 ± 61.53 pMoles/min) ($p<0.01$) and "priming" (193.9 ± 64.06 pMoles/min) ($p<0.01$) compared to untreated cells (499.1 ± 99.19 pMoles/min) (Figure 5.31.B). There was no additional significant alterations between treatments.

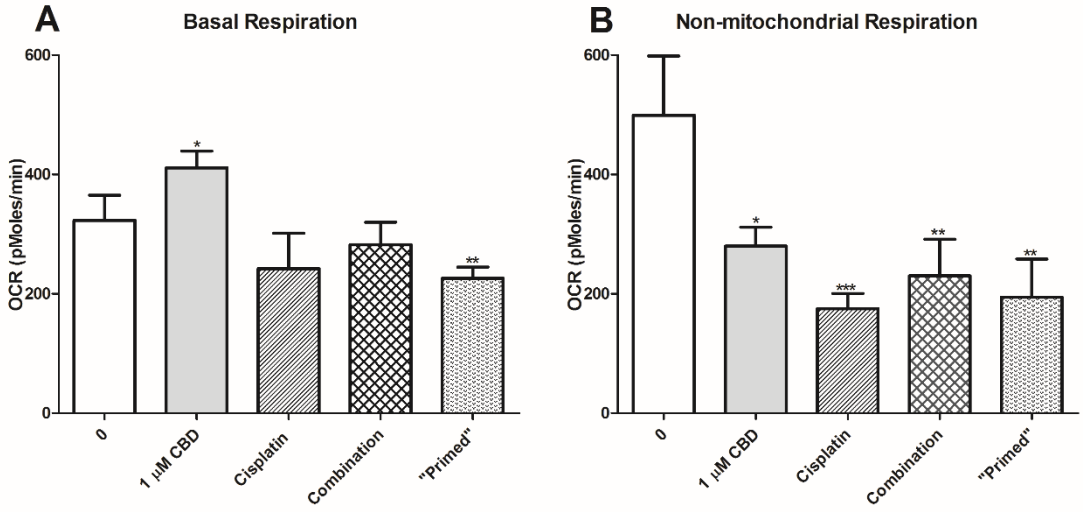

Figure 5.31. Mitochondrial and non-mitochondrial respiration of MDA-MB231 cells with all treatments compared to each other.
MDA-MB231 cell's **(A)** basal mitochondrial and **(B)** non-mitochondrial respiration was compared against each other. Data shown is repeated 3-5 times with 4 technical replicates per plate. Data is normalised to protein concentration. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs* Untreated.

A significant increase in relative ATP production levels was observed following CBD treatment (358.1 ± 17.32 pMoles/min) ($p<0.01$), which was also significant compared to cisplatin ($p<0.05$) and "priming" treatment ($p<0.05$) (Untreated cells; 243.5 ± 36.93 pMoles/min) (Figure 5.32.A). "Priming" showed a significant reduction in ATP production (136.6 ± 6.87 pMoles/min) compared to untreated cells ($p<0.001$). Proton leak levels had no additional difference compared to each other (Figure 5.32.B). However, proton leak was increased compared to untreated cells following treatment with "priming" (85.5 ± 2.85 pMoles/min) ($p<0.01$) (Untreated cells; 70.4 ± 8.65 pMoles/min). Maximal respiration was increased following CBD treatment compared to untreated cells (625.5 ± 70.17 pMoles/min) ($p<0.001$), cisplatin ($p<0.01$), combination ($p<0.01$) and "priming" ($p<0.05$) (Untreated cells; 406.2 ± 45.56 pMoles/min) (Figure 5.32.C.). Following combination treatment a decrease in maximal respiration was observed compared to untreated cells (319.8 ± 34.27 pMoles/min) ($p<0.05$). A significant reduction in spare respiration capacity was observed following cisplatin treatment (58.9

± 22.82 pMoles/min) (p<0.01) compared to untreated cells (149.5 ± 34.56 pMoles/min) however, there was no added difference to any other treatments (Figure 5.32.D.).

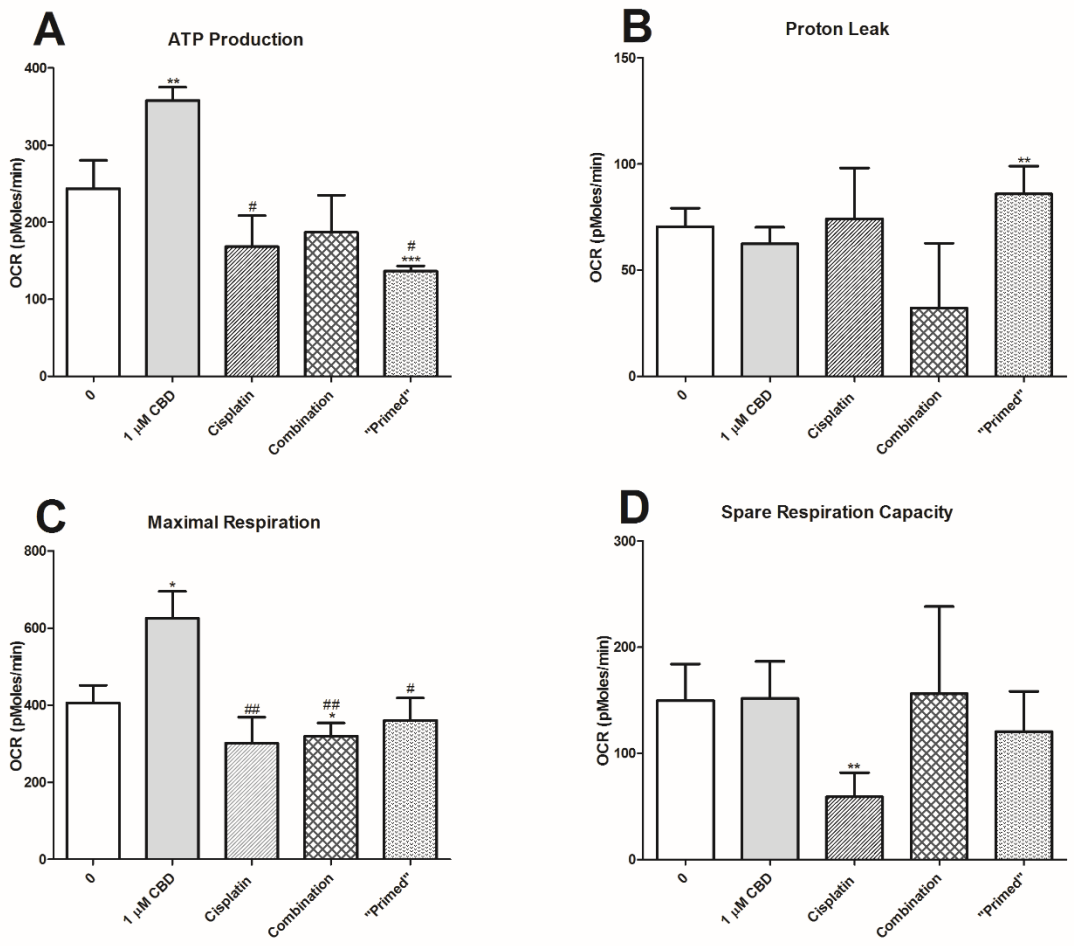

Figure 5.32. MDA-MB231 mitochondrial function analysis for all treatments compared to each other.
MDA-MB231 cell's mitochondrial **(A)** ATP levels, **(B)** Proton leak, **(C)** maximal respiration and **(D)** spare respiration capacity were compared against each other. Data shown is repeated 3-5 times with 4 technical replicates per plate. Data was normalised to protein concentration. Data is represented as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001 *vs* Untreated; # p<0.05 *vs* 1 µM CBD.

5.9.2. The effect of "priming" with CBD on control cell's mitochondrial function

5.9.2.1. "Priming" with CBD's effect on mitochondrial function in control cells compared to previously examined treatments

Basal mitochondria respiration was decreased following treatment with cisplatin (208.1 ± 31.48 pMoles/min) ($p<0.001$) and "priming" (248.2 ± 48.62 pMoles/min) ($p<0.001$) compared to untreated cells (508.4 ± 56.75 pMoles/min) (Figure 5.33.A.). The decrease observed following "priming" treatment was also significant to CBD ($p<0.05$) and combination ($p<0.05$).

A significant decrease in non-mitochondrial respiration was observed following cisplatin treatment (208.1 ± 31.48 pMoles/min) compared to untreated ($p<0.01$) and CBD treatment ($p<0.05$) (Untreated; 348.1 ± 64.52 pMoles/min) (Figure 5.33.B.). "Priming" treatment showed a significant decrease (159.9 ± 19.43 pMoles/min) compared to untreated cells ($p<0.001$) and CBD ($p<0.01$).

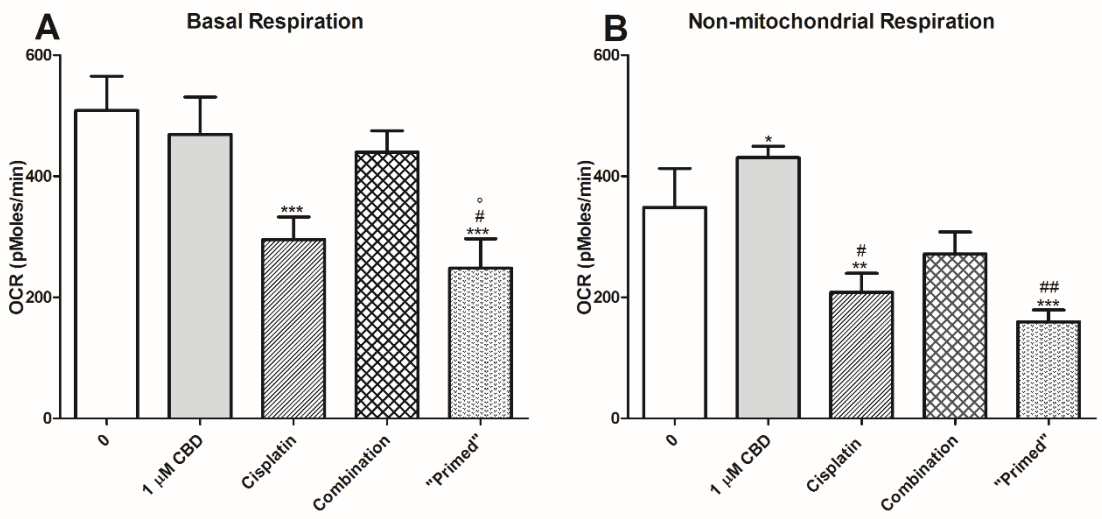

Figure 5.33. Control cells mitochondrial and non-mitochondrial respiration following "priming" treatment.
Control cell's **(A)** basal mitochondrial respiration and **(B)** non-mitochondrial respiration were compared against each other. Data shown is repeated 3-5 times with 4 technical replicates per plate. Data was normalised to cell count. Data is represented as mean ± SEM. ** $p<0.01$, *** $p<0.001$ *vs* Untreated; # $p<0.05$, ## $p<0.01$ *vs* 1 μM CBD; ° $p<0.05$ *vs* Combination.

Relative ATP production levels were unchanged for all treatments compared to the untreated and compared to each other (Figure 5.34.A.). A significant reduction in proton leak levels were observed following treatment with CBD (26.7 ± 4.26 pMoles/min), cisplatin (64.4 ± 11.14 pMoles/min), combination (91.9 ± 16.72 pMoles/min) and “priming” (60.1 ± 19.66 pMoles/min) ($p<0.001$) when compared to untreated levels (184.8 ± 23.21 pMoles/min) (Figure 5.34.B.). Furthermore, combination was significantly increased compared to ($p<0.05$). The maximal respiration capacity of the cell was decreased following cisplatin (499.7 ± 101.11 pMoles/min) ($p<0.05$) and “priming” treatment (423.1 ± 104.62 pMoles/min) ($p<0.01$) (Figure 5.34.C.). However, there was no difference to each other or any other treatments. No change in the cell’s spare respiration capacity was observed for all treatments compared to untreated cells and to each other (Figure 5.34.D.).

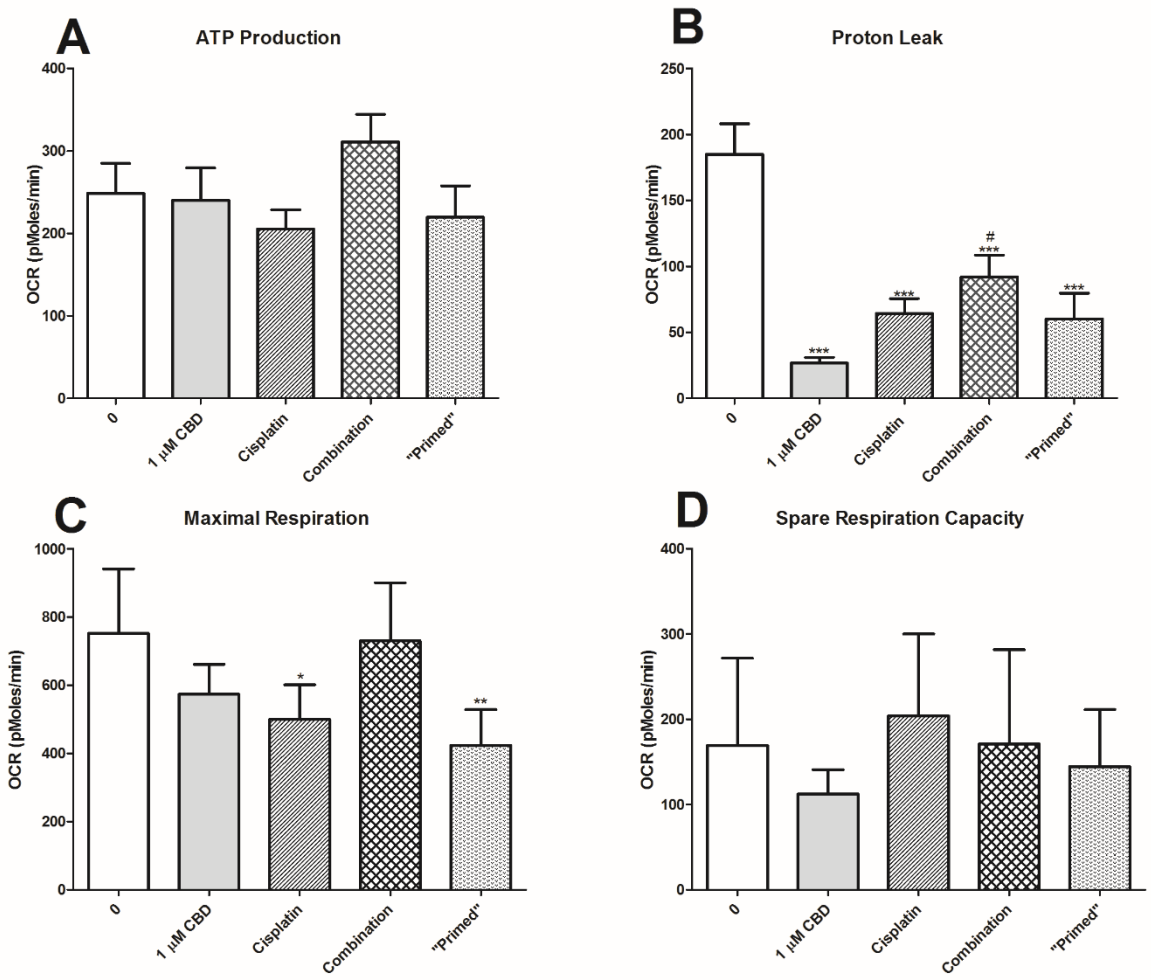

Figure 5.34. Control cells mitochondrial functional analysis compared to all other treatments.
Control cell's mitochondrial **(A)** ATP levels, **(B)** proton leak, **(C)** maximal respiration and **(D)** spare respiration capacity were compared against each other. Data shown is repeated 3-5 times with 4 technical replicates per plate. Data was normalised to cell count. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs* Untreated; # $p<0.05$ *vs* 1 µM CBD.

5.10. The effect of "priming" with CBD on cellular glycolysis

As mentioned previously, cancer cells primarily carry out respiration through glycolysis known as the "Warburg Effect" (Section 1.1.3.). However, following CBD treatment for 24 hours there was a shift from the cancerous glycolytic phenotype towards increased mitochondrial respiration (Section 3.8. and 3.9.).

5.10.1. Cancer cell's glycolysis following "priming" with CBD

5.10.1.1. "Priming" with CBD's effect on glycolysis in cancer cells

The cells glycolysis was measured following "priming" with CBD using a Seahorse Bioanalyser glycotest (Section 2.11.). A decrease in glycolysis was observed following "priming" treatment for all doses of CBD compared to untreated cells (38.2 ± 3.35 mpH/min); 0.1 μM CBD (24.3 ± 2.63 mpH/min), 0.5 μM CBD (23.2 ± 1.89 mpH/min), 1 μM CBD (23.7 ± 2.55 mpH/min), 2.5 μM CBD (18.9 ± 1.79 mpH/min), 5 μM CBD (21.9 ± 2.20 mpH/min) ($p<0.001$) (Figure 5.35.A.). This decrease was at a consistent level for all treatments of "priming".

The glycolytic capacity was also decreased following "priming" treatment for all doses compared to untreated cells (67.0 ± 4.49 mpH/min); 0.1 μM CBD (35.0 ± 3.82 mpH/min), 0.5 μM CBD (32.6 ± 2.60 mpH/min), 1 μM CBD (39.6 ± 4.95 mpH/min), 2.5 μM CBD (27.0 ± 3.02 mpH/min), 5 μM CBD (33.9 ± 3.77 mpH/min) ($p<0.001$) (Figure 5.35.B.). The doses of "priming" were also constant and similar for all doses.

Following "priming" treatment all doses showed a reduction in the glycolytic reserve of the cell compared to untreated cells (28.7 ± 2.79 mpH/min); 0.1 μM CBD (12.3 ± 1.43 mpH/min), 0.5 μM CBD (9.1 ± 1.28 mpH/min), 1 μM CBD (13.1 ± 1.28 mpH/min), 2.5 μM CBD (8.8 ± 1.06 mpH/min), 5 μM CBD (10.2 ± 1.77 mpH/min) ($p<0.001$) (Figure 5.35.C.).

A reduction in non-glycolytic capacity was observed following “priming” treatment compared to untreated cells (2.9 ± 1.96 mpH/min); 0.1 μM CBD (6.4 ± 1.19 mpH/min) ($p<0.01$), 0.5 μM CBD (8.3 ± 1.81 mpH/min) ($p<0.05$), 1 μM CBD (6.5 ± 1.3 mpH/min) ($p<0.001$), 2.5 μM CBD (6.7 ± 1.97 mpH/min) ($p<0.05$), 5 μM CBD (5.4 ± 0.695 mpH/min) ($p<0.001$) (Figure 5.35.D.).

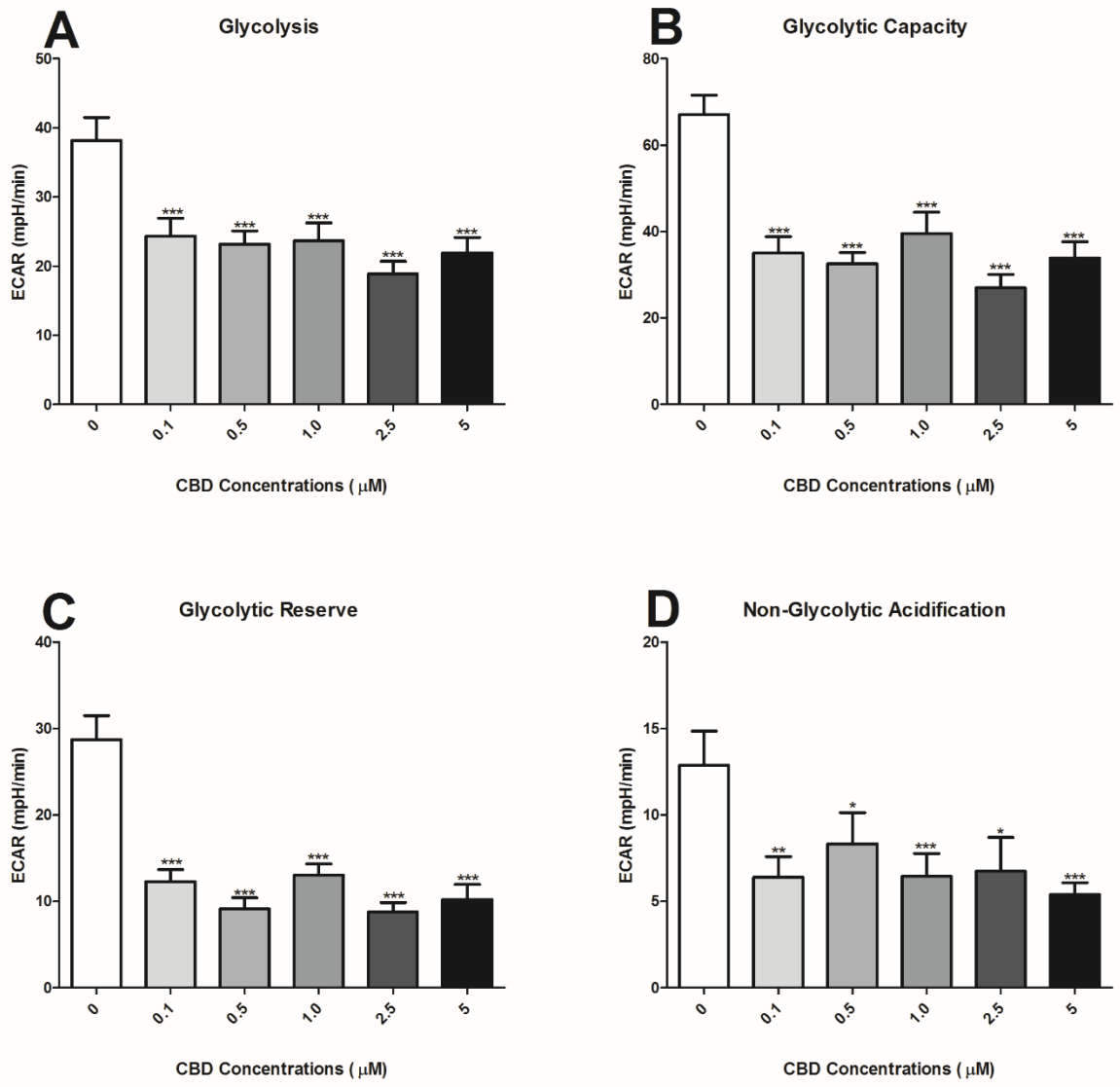

Figure 5.35. The effect of “priming” with CBD in MDA-MB231’s glycolytic function. MDA-MB231 cell’s activity of **(A)** glycolysis, **(B)** glycolytic capacity, **(C)** glycolytic reserve and **(D)** non-glycolytic acidification were analysed following “priming” treatment. Data shown is repeated 3-5 times with 4 technical replicates per plate. Data was normalised to protein concentration. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs* Untreated.

5.10.1.2. The effect of "priming" with CBD compared to other treatments in effecting glycolytic function

An increase in glycolysis following CBD treatment (56.7 ± 3.11 mpH/min.) was observed compared to untreated levels (35.4 ± 2.47 mpH/min) ($p<0.001$), cisplatin ($p<0.001$), combination ($p<0.001$) and "priming" ($p<0.001$) (Figure 5.36.A.). A significant decrease was observed compared to untreated cells following cisplatin (19.9 ± 1.30 mpH/min) ($p<0.001$), combination (17.2 ± 3.55 mpH/min) ($p<0.01$) and "priming" (23.7 ± 2.55 mpH/min) ($p<0.001$) treatment.

No change was observed in glycolytic capacity following CBD treatment compared to untreated cells (73.4 ± 6.45 mpH/min) (Figure 5.36.B.). However, a significant increase was observed compared to cisplatin, combination and "priming" ($p<0.001$). A significant decrease in glycolytic capacity was observed following cisplatin (23.4 ± 2.34 mpH/min) ($p<0.001$), combination (25.1 ± 5.06 mpH/min) ($p<0.001$) and "priming" (43.3 ± 5.41 mpH/min) ($p<0.001$) compared to untreated cells.

A significant reduction in glycolytic reserve was observed following treatment with cisplatin (23.4 ± 2.34 mpH/min) ($p<0.001$) compared to untreated cells (73.4 ± 6.45 mpH/min) and CBD (32.5 ± 5.09 mpH/min) ($p<0.05$) (Figure 5.36.C.). Furthermore, a significant reduction was observed following combination (8.5 ± 2.24 mpH/min) ($p<0.001$) and "priming" (19.3 ± 1.89 mpH/min) ($p<0.001$) compared to untreated cells.

A significant decrease in non-glycolytic acidification was observed following treatment with CBD (7.8 ± 1.24 mpH/min) compared to untreated cells (16.4 ± 1.81 mpH/min) ($p<0.001$) and cisplatin treatment ($p<0.5$) (Figure 5.36.D.). "Priming" also showed a significant decrease (8.2 ± 1.66 mpH/min) compared to untreated levels ($p<0.001$) and cisplatin ($p<0.05$).

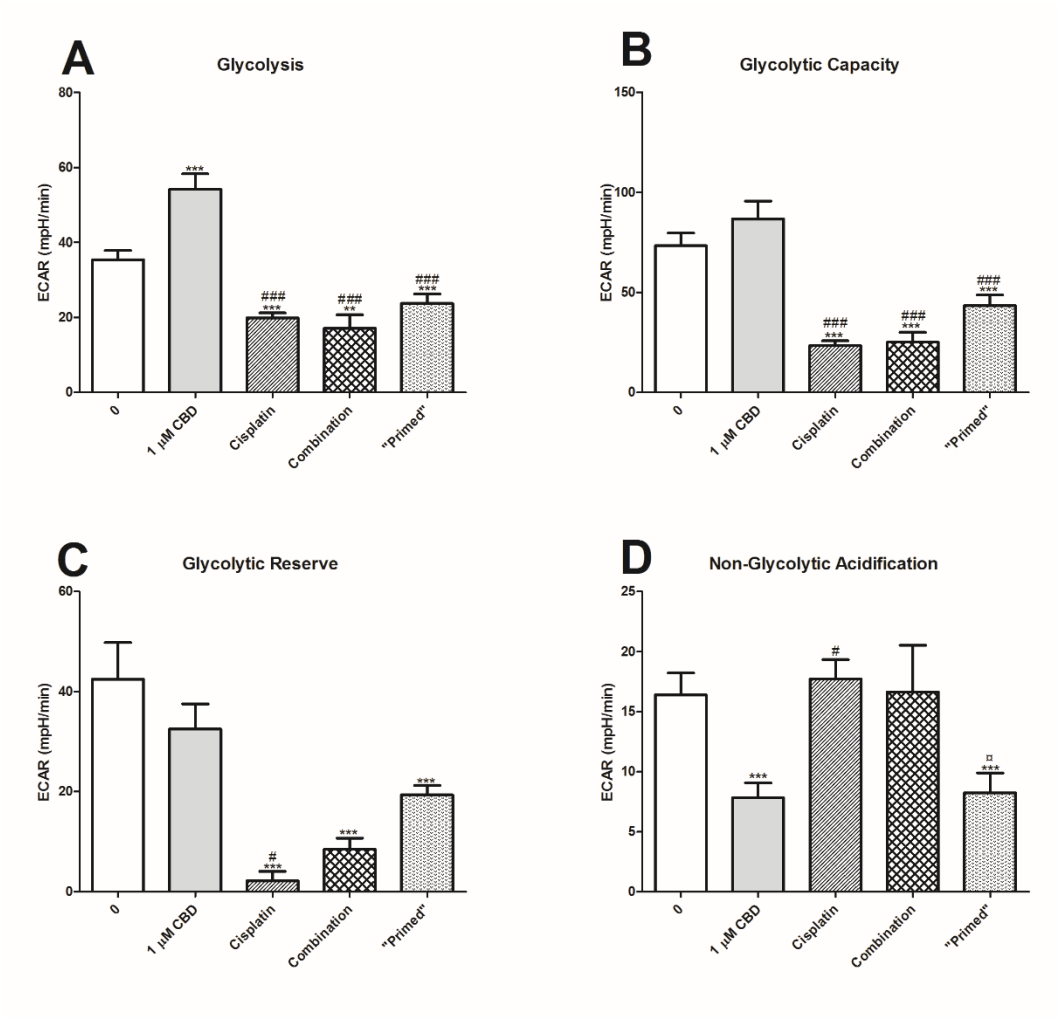

Figure 5.36. MDA-MB231 glycolysis alterations compared to all other treatments to each other.

MDA-MB231 cell's **(A)** glycolysis, **(B)** glycolytic capacity, **(C)** glycolytic reserve and **(D)** non-glycolytic acidification were compared against each other. Data shown is repeated 3-5 times with 5 technical replicates per plate. Data was normalised to protein concentration. Data is represented as mean ± SEM. ** $p<0.01$, *** $p<0.001$ *vs* Untreated; # $p<0.05$, ### $p<0.001$ *vs* CBD; ¤ $p<0.05$ *vs* Cisplatin.

5.10.2. The effect of "priming" with CBD on the glycolysis of control cells

5.10.2.1. "Priming" treatment's effect on control cell's glycolysis

Following "priming" treatment, glycolysis was significantly reduced compared to untreated cells (44.3 ± 4.56 mpH/min) for all treatments; 0.1 μM CBD (18.1 ± 1.88 mpH/min), 0.5 μM CBD (21.0 ± 2.43 mpH/min), 1 μM CBD (17.8 ± 2.62 mpH/min), 2.5 μM CBD (13.0 ± 1.22 mpH/min), 5 μM CBD (14.3 ± 2.36 mpH/min) ($p<0.001$) (Figure 5.37.A.). The reduction observed was consistent across all doses of "primed" CBD.

The glycolytic capacity was also reduced following "priming" treatments compared to untreated cells (89.4 ± 6.68 mpH/min) for all treatments; 0.1 μM CBD (47.6 ± 6.21 mpH/min), 0.5 μM CBD (38.7 ± 3.44 mpH/min), 1 μM CBD (42.9 ± 6.38 mpH/min), 2.5 μM CBD (36.2 ± 2.68 mpH/min), 5 μM CBD (38.3 ± 3.92 mpH/min) ($p<0.001$) (Figure 5.37.B.). Again, the levels of decrease were consistently similar with all "primed" doses.

Glycolytic reserves was decreased following "priming" treatment compared to untreated cells (60.0 ± 4.90 mpH/min) for all doses; 0.1 μM CBD (34.7 ± 4.07 mpH/min), 0.5 μM CBD (30.3 ± 2.46 mpH/min), 1 μ M CBD (37.7 ± 2.69 mpH/min), 2.5 μM CBD (33.7 ± 1.67 mpH/min), 5 μM CBD (38.1 ± 3.08 mpH/min) ($p<0.001$) (Figure 5.37.C.). The decrease observed was similar for all doses of "priming".

Following "priming" treatment, non-glycolytic acidification was reduced in a dose dependant manner showing a u-shaped curve compared to untreated cells (21.2 ± 1.63 mpH/min) (Figure 5.37.D.). A decrease was observed from 0.1 μM CBD (14.9 ± 2.40 mpH/min) ($p<0.05$), 0.5 μM CBD (13.8 ± 1.11 mpH/min) ($p<0.01$) and 1 μM CBD (13.5 ± 2.34 mpH/min). This decrease began to increase with 2.5 μM CBD (17.8 ± 1.19 mpH/min) ($p<0.05$) and 5 μM CBD (15.1 ± 2.45 mpH/min) ($p<0.05$).

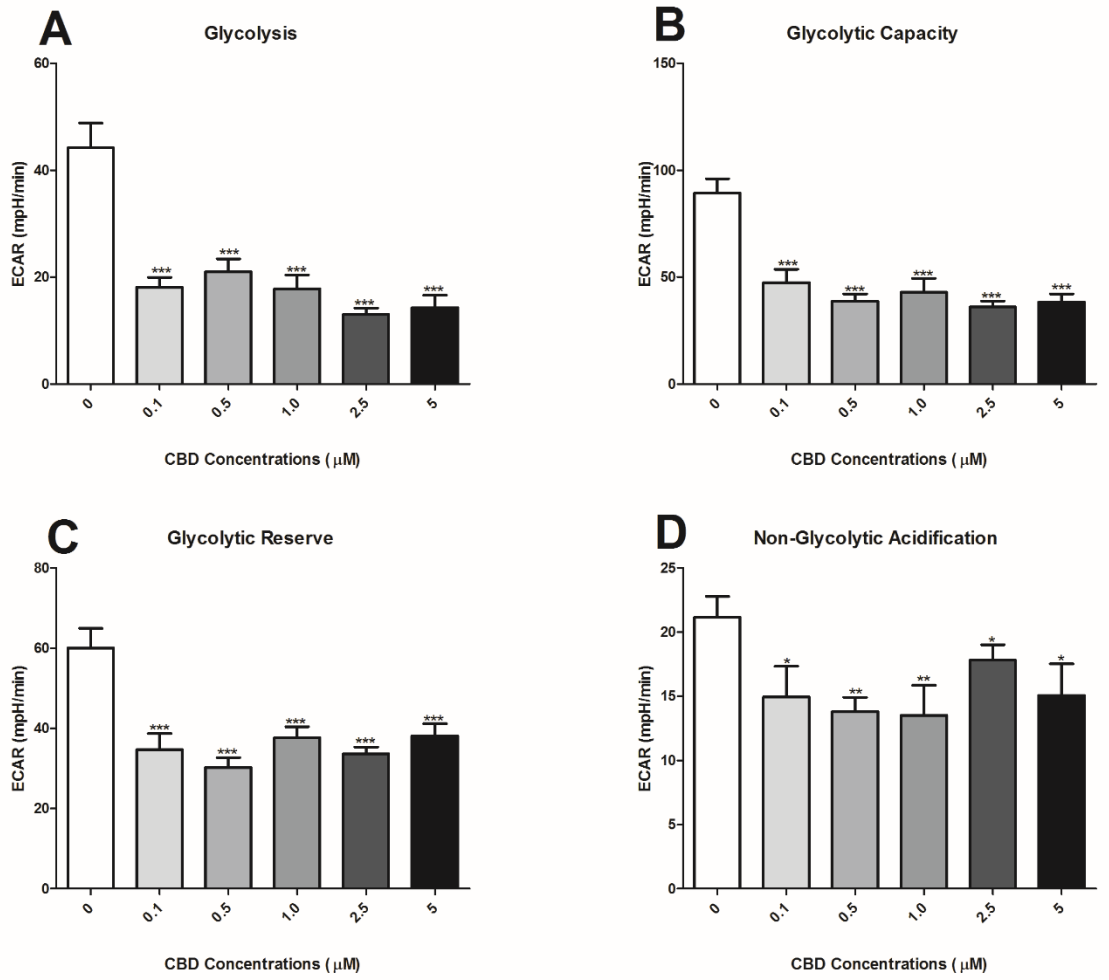

Figure 5.37. Control cells glycolysis activity following "priming" treatment.
Control cell's **(A)** glycolysis, **(B)** glycolytic capacity, **(C)** glycolytic reserve and **(D)** non-glycolytic acidification were analysed following "priming" treatment. Data shown is repeated 3-5 times with 4 technical replicates per plate. Data was normalised to protein concentration. Data is represented as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001 *vs* Untreated.

5.10.2.2. "Priming" with CBD compared to previously examined treatments and their effect on glycolytic functions in control cells

A significant increase in glycolysis was observed following CBD treatment (56.7 ± 3.11 mpH/min) compared to untreated cells (40.4 ± 2.99 mpH/min) (p<0.01), cisplatin (p<0.001), combination (p<0.001) and "priming" (p<0.01) (Figure 5.38.A.). A significant decrease was observed compared to untreated cells following cisplatin (5.2 ± 0.42 mpH/min) (p<0.001), combination (12.2 ± 1.62 mpH/min) (p<0.001) and "priming"

treatment (25.7 ± 3.31 mpH/min) ($p<0.001$). The decrease observed following cisplatin treatment was significantly reduced compared to “priming” ($p<0.001$). Moreover, combination treatment was also significantly reduced compared to “priming” ($p<0.05$).

A reduction in the glycolytic capacity was observed following CBD treatment (72.4 ± 7.35 mpH/min) ($p<0.01$) compared to untreated cells (123.5 ± 6.34 mpH/min), cisplatin ($p<0.001$), combination ($p<0.05$) and “priming” ($p<0.001$) (Figure 5.38.B.). A reduction was also observed following cisplatin (46.5 ± 3.34 mpH/min) ($p<0.001$), combination (57.3 ± 4.04 mpH/min) ($p<0.001$) and “priming” (49.8 ± 7.16 mpH/min) ($p<0.001$) compared to untreated cells.

Glycolytic reserve was reduced compared to untreated cells (83.1 ± 7.19 mpH/min) following CBD (48.5 ± 5.53 mpH/min) ($p<0.01$), cisplatin (42.1 ± 3.23 mpH/min) ($p<0.001$), combination (44.2 ± 2.267 mpH/min) ($p<0.001$) and “priming” (46.1 ± 3.29 mpH/min) ($p<0.001$) treatment (Figure 5.38.C.). The difference was similar to each other and showed no further significant difference.

Non-glycolytic acidification showed a significant reduction following CBD treatment (7.0 ± 0.59 mpH/min) compared to untreated cells (13.2 ± 0.05 mpH/min) ($p<0.001$) and cisplatin ($p<0.05$) (Figure 5.38.D.). A significant reduction was observed following combination (9.6 ± 0.85 mpH/min) ($p<0.01$) and “priming” (9.2 ± 0.96 mpH/min) ($p<0.01$) treatment compared to untreated cells however, no significant difference was seen compared to any other treatment.

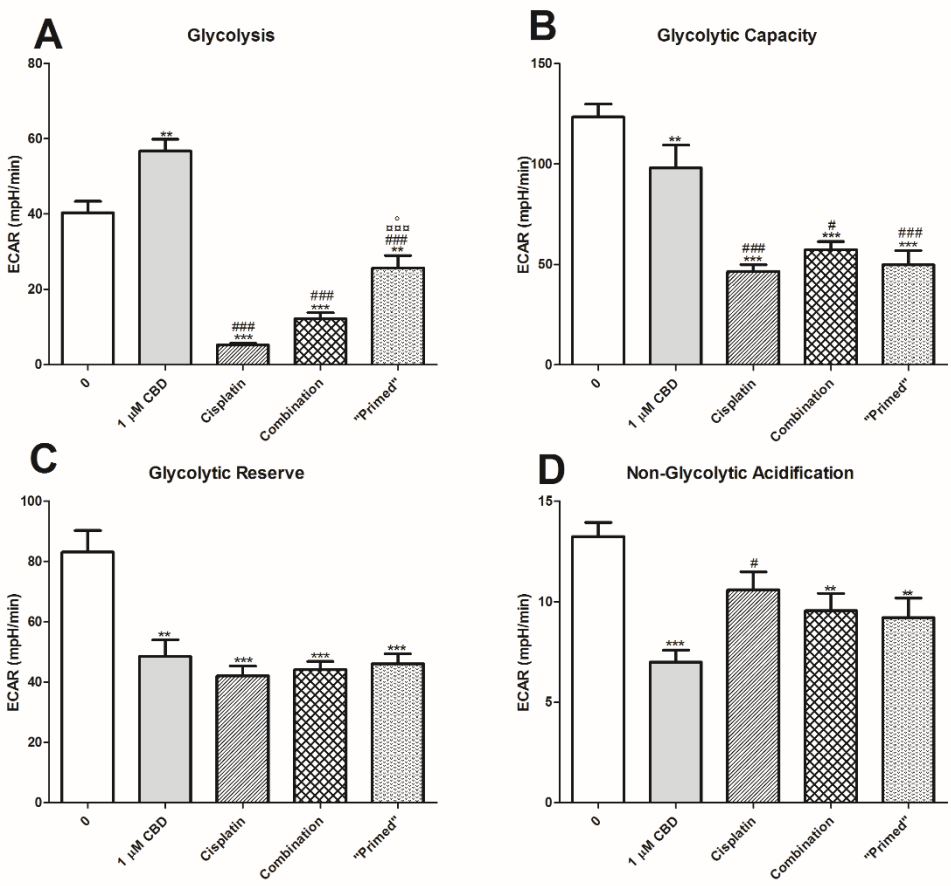

Figure 5.38. Control cell glycolysis following treatment with CBD, cisplatin, combination and "priming" with CBD.
Control cell's **(A)** glycolysis, **(B)** glycolytic capacity, **(C)** glycolytic reserve and **(D)** non-glycolytic acidification were compared against each other. Data shown is repeated 3-5 times with 4 technical replicates per plate. Data was normalised to protein concentration. Data is represented as mean ± SEM. ** p<0.01, *** p<0.001 *vs* Untreated; # p<0.05, ### p<0.001 *vs* CBD; ¤¤¤ p<0.001 *vs* Cisplatin; ° p<0.05 *vs* Combination.

5.11. “Priming” with CBD’s effect on the mitochondrial receptor, VDAC1

As mentioned previously the main suggested mechanism of “priming” is through mitochondrial sensitization (Section 1.4.1., 1.4.3. and 1.4.4.). The VDAC1 channel has been linked to increased mitochondrial apoptosis (Section 1.4.4. and 3.10.). Both CBD and cisplatin showed an increase in VDAC1 activity following treatment (Section 3.10. and 4.9.). However, combination treatment showed a significant decrease in VDAC1 activity (Section 4.9.). VDAC1 levels will be measured using western blot (Section 2.14.).

5.11.1. The effects of “priming” with CBD on cancer cell’s VDAC1 receptor levels

5.11.1.1. “Priming” with CBD’s effect on VDAC1 levels in cancer cell

A dose dependant increase of VDAC1 expression was observed following “priming” treatment (Figure 5.39.). A significant increase was shown following 1 μM CBD (1.32 ± 0.119 R.I.) ($p<0.05$), 2.5 μM CBD (1.59 ± 0.192 R.I.) ($p<0.05$) and 5 μM CBD (1.84 ± 0.157 R.I.) treatment ($p<0.01$).

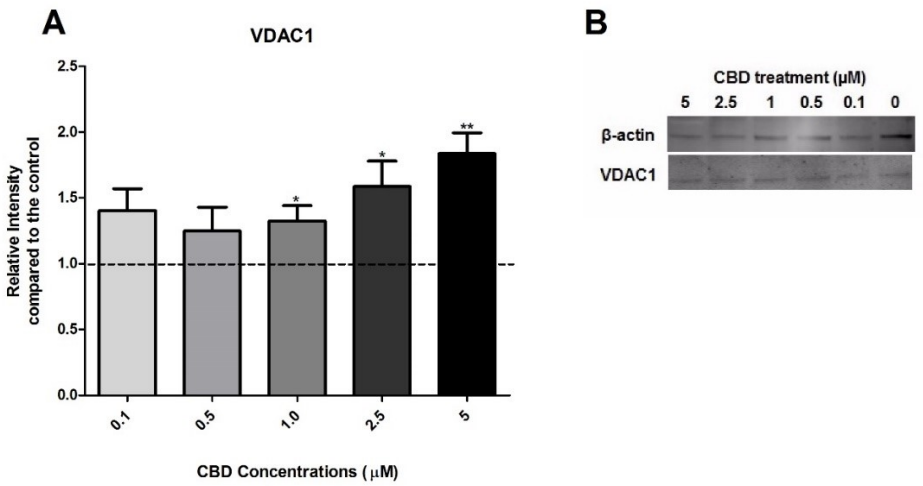

Figure 5.39. MDA-MB231's VDAC1 expression following "priming" treatment.
(A) MDA-MB231 cells protein was extracted following "priming" treatment and VDAC1 expression was analysed. Data shown is repeated 6 times. The dashed line represents untreated cells. Data is normalised to the constitutively expressed house-keeper β-actin. **(B)** Western blot of relevant graphs untreated (0) and protein control of β-actin. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs* Untreated.

5.11.1.2. "Priming" with CBD compared to previously examined treatments and their effects on VDAC1 levels in cancer cells

No change in VDAC1 expression was observed following CBD and cisplatin treatment (Figure 5.40.). A significant decrease was observed following combination treatment (0.28 ± 0.039 R.I.) compared to untreated cells ($p<0.001$), CBD ($p<0.05$) and cisplatin ($p<0.05$). A significant increase of VDAC1 expression was observed following "priming" treatment (1.32 ± 0.119 R.I.) compared to untreated cells ($p<0.05$) and combination treatment ($p<0.001$).

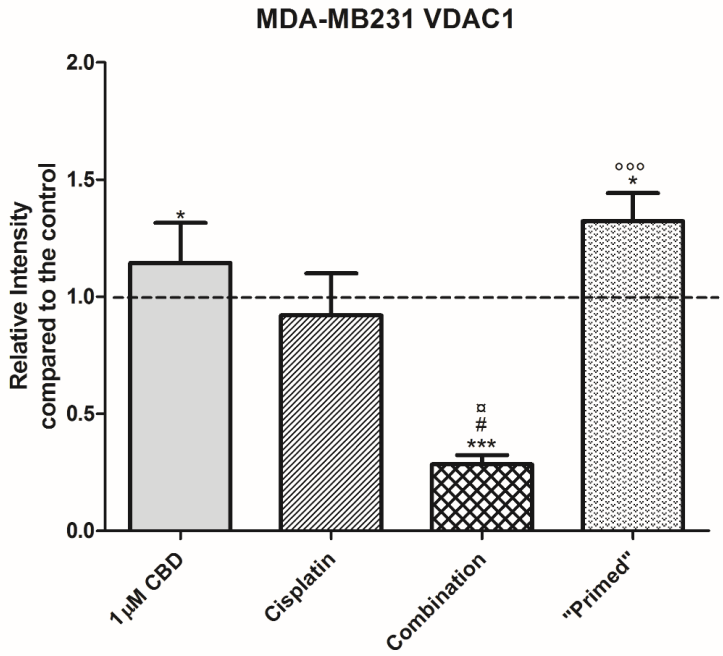

Figure 5.40. VDAC1 expression levels in cancer cells following CBD, cisplatin, combination and "priming" with CBD treatments.
MDA-MB231 cells VDAC1 expression was compared against each other. Data shown is repeated 6 times. The dashed line represents untreated cells. Data is normalised to the constitutively expressed house-keeper β-actin. Data is represented as mean ± SEM. * $p<0.05$, *** $p<0.001$ *vs* Untreated; # $p<0.05$ *vs* CBD; ¤ $p<0.05$ *vs* Cisplatin; °°° $p<0.001$ *vs* Combination.

5.11.2. The effect of "priming" with CBD on VDAC1 levels in control cells

5.11.2.1. "Priming" with CBD treatment's effect on VDAC1 receptor levels in control cells

VDAC1 expression showed a dose dependant increase following "priming" treatment compared to untreated cells (Figure 5.41.). A significant increase was observed following 1 μM CBD (1.56 ± 0.195 R.I.) ($p<0.05$), 2.5 μM CBD (1.57 ± 0.212 R.I.) ($p>0.05$) and 5 μM CBD (1.55 ± 0.087 R.I.) treatment ($p<0.01$).

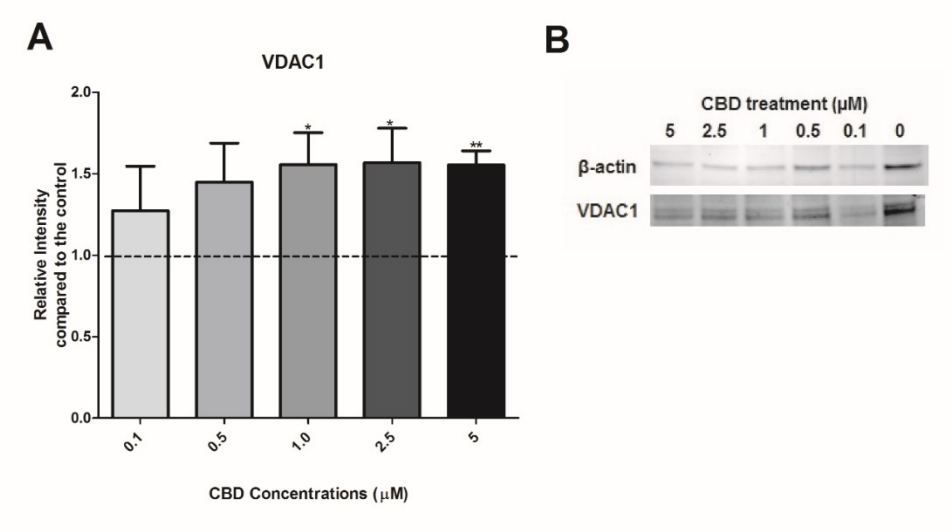

Figure 5.41. Control cells VDAC1 expression following "priming" treatment
(A) Control cells VDAC1 expression following "priming" treatment. Data shown is repeated 7 times. The dashed line represents untreated cells. Data is normalised to the constitutively expressed house-keeper β-actin. **(B)** Western blot of relevant graphs untreated (0) and protein control of β-actin.. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs* Untreated.

5.11.2.2. VDAC1's response to "priming" with CBD compared to previously examined treatments in control cells

A decrease in VDAC1 expression was observed following CBD (0.79 ± 0.021 R.I.) ($p<0.001$), cisplatin (0.37 ± 0.048 R.I.) ($p<0.001$) and combination (0.38 ± 0.083 R.I.) ($p<0.01$) treatment (Figure 5.42.). An increase in VDAC1 expression levels was observed following "priming" treatment (1.56 ± 0.195 R.I.) compared to untreated cells ($p<0.001$), CBD ($p<0.001$), cisplatin ($p<0.001$) and combination ($p<0.001$).

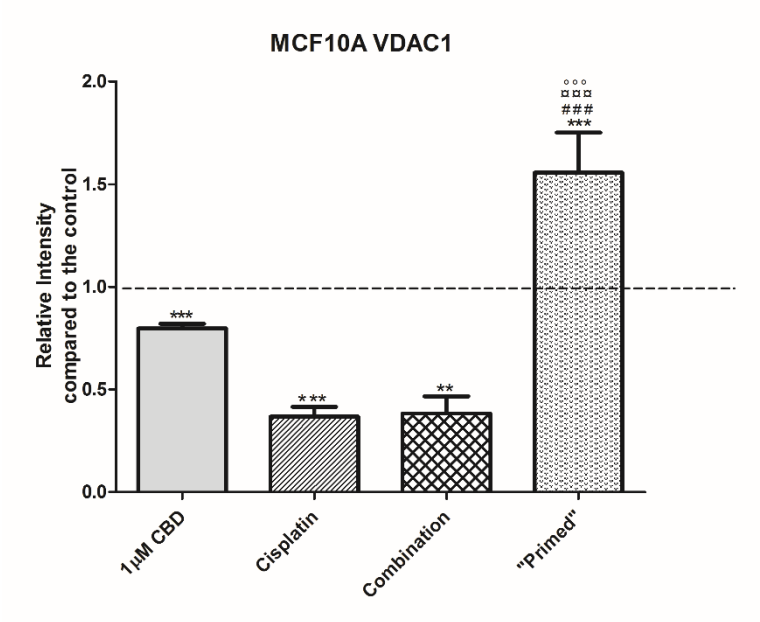

Figure 5.42. Control cells VDAC1 expression levels compared to all treatments.
Control cells (MCF10A) VDAC1 expression was compared to each other. Data shown is repeated 5-7 times. The dashed line represents untreated cells. Data is normalised to the constitutively expressed house-keeper β-actin. Data is represented as mean ± SEM. ** $p<0.01$, *** $p<0.001$ *vs* Untreated; ### $p<0.001$ *vs* CBD; ¤¤¤ $p<0.001$ *vs* Cisplatin; °°° $p<0.001$ *vs* Combination.

5.12. “Priming” with CBD’s effect on the cell’s genetic control

The changes observed in cellular metabolism and cell viability lead to an investigation into pathways that alter cell growth and death. The investigation was carried out as in section 3.11. and 4.10.. The pro-apoptotic pathway p53 was investigated along with the stress response pathway Nrf2. In parallel the cell growth regulator AKT was examined along with its regulators. The genetic analysis was carried out using qPCR with data being normalised to the relevant housekeeping gene (Section 2.12.).

5.12.1. Cancer cell’s genetic expression following “priming” with CBD

5.12.1.1. The effect of “priming” with CBD in the gene expression of cancer cell’s p53 pathway

As previously described in section 3.11.1.1. the pro-apoptotic p53 pathway was analysed for alterations in its regulation. HDAC1 and Sirt1 have been shown to interact with p53 activating its expression and hence increased cell death (Section 1.4.3.).

HDAC1 gene expression showed a dose dependant trend to increase following “priming” treatment (Figure 5.43.A.). However, no significant change to untreated cell levels was observed.

A dose dependant trend to increase was also observed in SirT1 gene expression following “priming” treatment (Figure 5.43.B.). However, no significant change was again observed compared to untreated cells.

Following “priming” treatment p53 expression showed a u-shaped response curve compared to untreated cells (Figure 5.43.C.). An increase in p53 expression was observed following 0.1 μM CBD treatment (3.79 ± 0.542 R.I.) ($p<0.05$). As “priming” increased the expression was reduced similar to the untreated levels. However, as

"priming" further increased a significant increase was observed with 2.5 μM CBD (1.80 ± 0.291 R.I.) ($p<0.05$) and 5 μM CBD (3.82 ± 0.569 R.I.) ($p<0.01$).

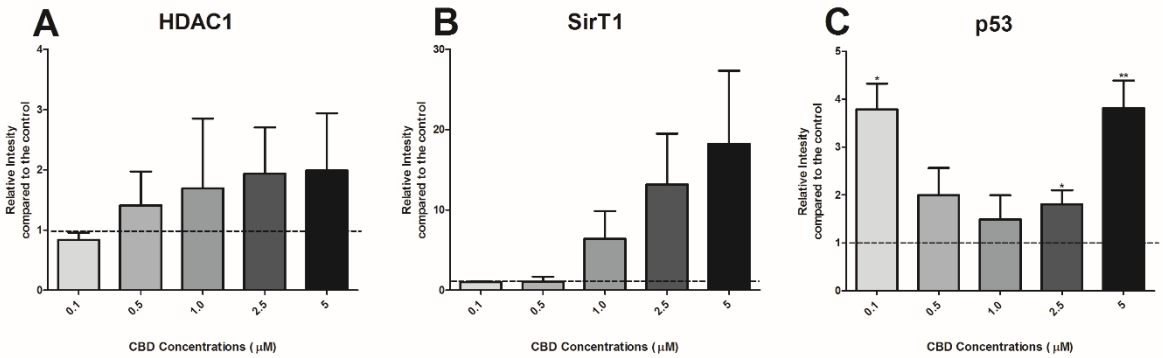

Figure 5.43. MDA-MB231's gene expression of the p53 pathway following "priming" treatment.
MDA-MB231 cell's gene expression of **(A)** HDAC1, **(B)** SirT1 and **(C)** p53 were analysed following "priming" treatments. Data shown is repeated 3-5 times with technical replicates in triplicate per plate. The dashed line represents untreated cells and data was normalised to constitutively expression housekeepers α-tubulin or β-actin. Data is represented as mean ± SEM. ** $p<0.01$ *vs* Untreated.

5.12.1.2. "Priming" with CBD's effect on the gene expression of the Nrf2 pathway in cancer cells

The stress response pathway Nrf2 is activated in response to increased ROS levels (Kensler *et al*. 2007) (Section 1.1.3.4.1.). As previously shown Nrf2 interacts with MAPк and can activate an inflammatory response from IL-6 (Section 3.11.1.2. and 4.10.1.2.).

A dose dependant trend to increase was observed in MAPk gene expression following "priming" treatment (Figure 5.44.A.). However, no significant difference was observed compared to untreated cells.

Nrf2 gene expression was significantly reduced following "priming" treatment with 0.1 μM CBD (0.39 ± 0.122 R.I.) ($p<0.01$), 0.5 μM CBD (0.31 ± 0.089 R.I.) ($p<0.05$), 1 μM CBD (0.39 ± 0.029 R.I.) ($p<0.01$), 2.5 μM CBD (0.48 ± 0.116 R.I.) ($p<0.05$) and 5 μM CBD (0.79 ± 0.031 R.I.) ($p<0.05$) compared to untreated cells (Figure 5.44.B.).

No change in IL-6 gene expression was observed following “priming” treatment (Figure 5.44.C.). However, 5 μM CBD “priming” showed a significant increase in IL-6 expression (1.52 ± 0.062 R.I.) ($p<0.05$).

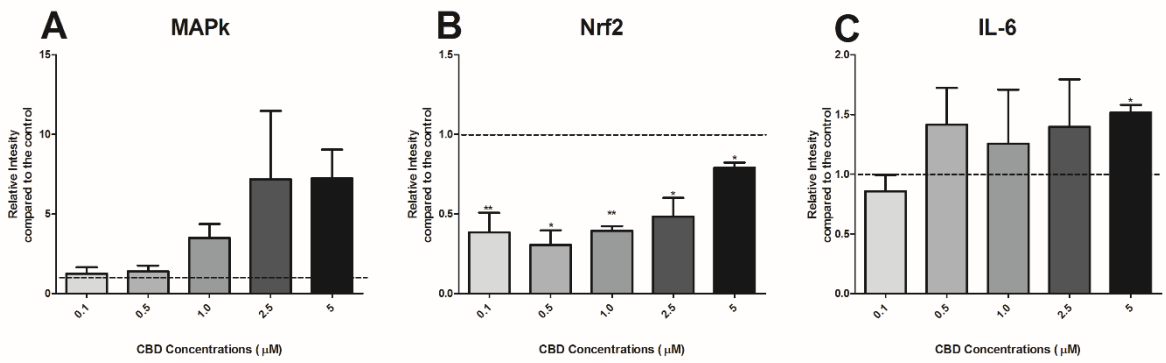

Figure 5.44. MDA-MB231’s gene expression of the Nrf2 pathway following “priming” treatment.
MDA-MB231 cell’s gene expression of **(A)** MAPk, **(B)** Nrf2 and **(C)** IL-6 analysed following “priming” treatment. Data shown is repeated 3-5 times with technical replicates in triplicate per plate. The dashed line represents untreated cells and data was normalised to constitutively expression housekeepers α-tubulin or β-actin. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs* Untreated.

5.12.1.3. The effect of “priming” with CBD on the gene expression of the AKT pathway in cancer cells

Cell growth and regulation can be controlled by AKT as previously mentioned (Section 3.11. and 3.11.1.3.). The AKT pathway involving mTOR and NFkB can regulate the cells growth response and stimulate proliferation (Nakanishi and Toi 2005, Sarbassov *et al*. 2005, Humphrey *et al*. 2013).

No change in AKT gene expression was observed following “priming” treatment (Figure 5.45.A.). A trend to decrease was observed following 0.1 μM CBD treatment that had a dose dependant trend to increase that remained similar to untreated cell levels.

A dose dependent trend to decrease was observed in mTOR gene expression following “priming” treatment (Figure 5.45.B.). A significant reduction was observed

following 1 μM CBD (0.29 ± 0.030 R.I.) (p<0.01), 2.5 μM CBD (0.21 ± 0.006 R.I.) (p<0.001) and 5 μM (0.34 ± 0.121 R.I.) (p<0.05) treatment.

A significant reduction in NFkB gene expression was observed following "priming" treatment with 0.1 μM CBD (0.29 ± 0.028 R.I.) (p<0.01) (Figure 5.45.C.). A dose dependant trend to increase was seen following the initial decrease observed with 0.1 μM CBD; 0.5 μM CBD (0.34 ± 0.059 R.I.) and 1 μM CBD (0.35 ± 0.059 R.I.) (p<0.01). As the dose of "priming" increased there was no change compared to untreated cells.

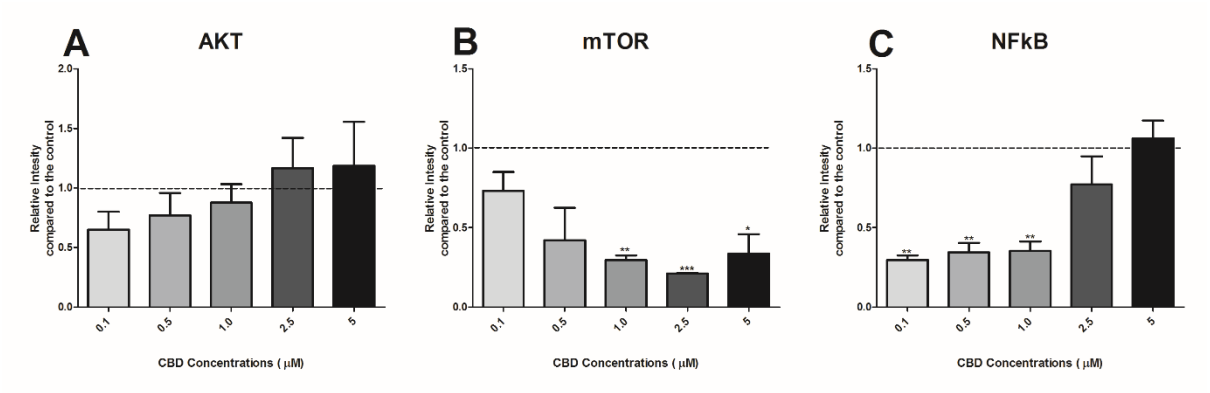

Figure 5.45. The gene expression of cancer cell's AKT pathway following "priming" with CBD.
MDA-MB231 cell's gene expression of **(A)** AKT, **(B)** mTOR and **(C)** NFkB following "priming" treatment. Data shown is repeated 3-5 times with technical replicates in triplicate per plate. The dashed line represents untreated cells and data was normalised to constitutively expression housekeepers α-tubulin or β-actin. Data is represented as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001 *vs* Untreated.

5.12.1.4. The gene expression of FOXO1 following "priming" with CBD in cancer cells

FOXO1 has been shown to inhibit cancer growth and been activated following CBD treatment (Section 3.11.1.4.). A significant reduction was observed following "priming" treatment with 0.1 μM CBD (0.5631 R.I. ± 0.05642 S.E.M.) and 0.5 μM CBD (0.4523 R.I. ± 0.1272 S.E.M.) (p<0.05) (Figure 5.46.). However, as the dose of "priming" increased a dose dependant trend to increase was observed compared to untreated cell levels.

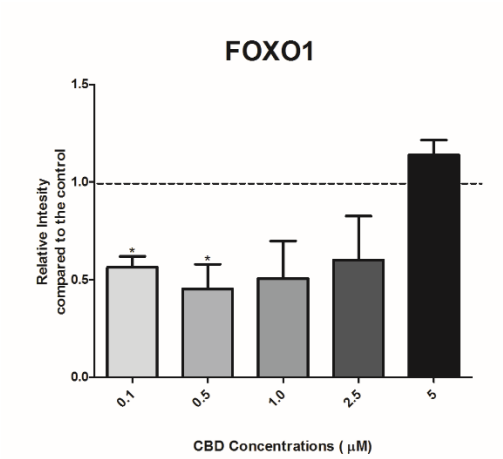

Figure 5.46. MDA-MB231's FOXO1 gene expression following "priming" with CBD. MDA-MB231 cell's gene expression of FOXO1 was analysed following "priming" treatment. Data shown is repeated 3-5 times with technical replicates in triplicate per plate. The dashed line represents untreated cells and data was normalised to constitutively expression housekeepers α-tubulin or β-actin. Data is represented as mean ± SEM. * $p<0.05$ *vs* Untreated.

5.12.2. The gene expression in cancer cells following "priming" with CBD compared to each of the previously reported treatments

5.12.2.1. The effect of the p53 pathway in cancer cell's following the previously reported treatments compared with "priming"

A reduction in HDAC1 gene expression was observed following CBD (0.32 ± 0.049 R.I.) ($p<0.001$) and combination treatment (0.46 ± 0.041 R.I.) ($p<0.01$) compared to untreated cells (Figure 5.47.A.). There was no additional significance between any other treatments.

SirT1 gene expression was significantly increased following CBD treatment (586.1 ± 140.21 R.I.) compared to untreated cells ($p<0.05$), cisplatin ($p<0.001$), combination ($p<0.001$) and "priming" ($p<0.001$) treatments (Figure 5.47.B.). No added difference was observed between any other treatments.

An increase in p53 expression was observed following CBD treatment (1.22 ± 0.169 R.I.) compared to untreated cells (p<0.05) (Figure 5.47.C.). No other difference was observed between the treatments.

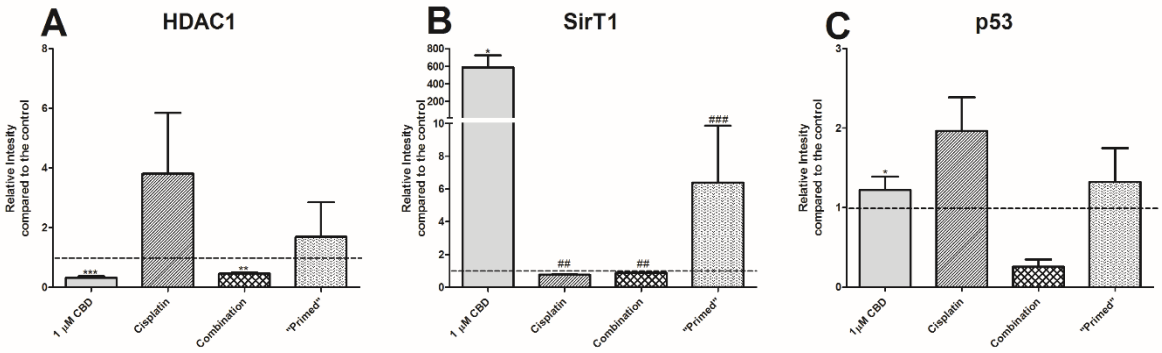

Figure 5.47. MDA-MB231's p53 pathway gene expression following treatment with CBD, cisplatin, combination and "priming" compared to each other.
MDA-MB231 cell's gene expression of **(A)** HDAC1, **(B)** SirT1 and **(C)** p53 were compared against each other. Data shown is repeated 3-5 times with technical replicates in triplicate per plate. The dashed line represents untreated cells and data was normalised to constitutively expression housekeepers α-tubulin or β-actin. Data is represented as mean ± SEM. * p<0.05, ** p<0.01 *vs* Untreated; ## p<0.01, ### p<0.001 *vs* CBD.

5.12.2.2. "Priming" with CBD compared to other previously reported treatment's effect in cancer cell's Nrf2 pathway

A significant increase in MAPk gene expression was observed following CBD treatment (112.11 ± 5.412 R.I.) compared to untreated cells (p<0.01), cisplatin (p<0.001), combination (p<0.001) and "priming" (p<0.001) treatments (Figure 5.48.A.). No addition difference was observed between treatments.

A significant increase in Nrf2 expression was observed following CBD treatment (2.49 ± 0.289 R.I.) compared to untreated cells (p<0.05), cisplatin (p<0.05), combination (p<0.05) and "priming" (p<0.01) treatments (Figure 5.48.B.). A significant decrease was observed following "priming" treatment (0.39 ± 0.029 R.I.) compared to untreated cells (p<0.01).

IL-6 gene expression levels were significantly increased following CBD treatment (10.99 ± 1.849 R.I.) compared to untreated cells (p<0.05) (Figure 5.48.C.). However, there was no difference with any other treatment condition with each other.

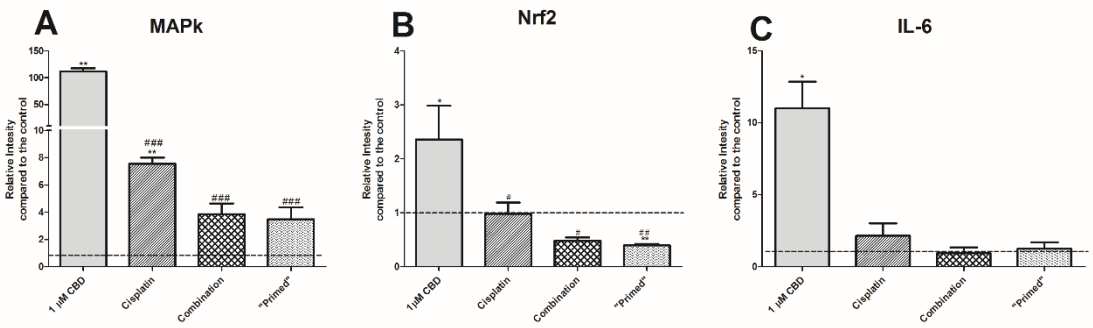

Figure 5.48. Cancer cell's Nrf2 pathway gene expression following previously examined treatments compared to each other.
MDA-MB231 cell's gene expression of **(A)** MAPk, **(B)** Nrf2 and **(C)** IL-6 were compared against each other. Data shown is repeated 3-5 times with technical replicates in triplicate per plate. The dashed line represents untreated cells and data was normalised to constitutively expression housekeepers α-tubulin or β-actin. Data is represented as mean ± SEM. * p<0.05, ** p<0.01 *vs* Untreated; # p<0.05, ## p<0.01, ### p<0.001 *vs* CBD.

5.12.2.3. The effect of "priming" with CBD compared to the previously examined treatments and their effect on the AKT pathway in cancer cells

A reduction in AKT gene expression was observed following combination treatment (0.49 ± 0.047 R.I.) (p<0.01) (Figure 5.49.A.). The trend to increased AKT gene expression was observed following CBD treatment that was significant compared to combination (p<0.01) and "priming" (p<0.05).

An increase in gene expression of mTOR was observed following CBD treatment (2.55 ± 0.303 R.I.) (p<0.05) (Figure 5.49.B.). Furthermore, a decrease was observed following "priming" treatment (0.29 ± 0.030 R.I.) (p<0.01). However there was no difference between any of the treatments with each other.

NFkB gene expression was significantly increased following CBD treatment (2.40 ± 0.221 R.I.) compared to untreated cells (p<0.05), cisplatin (p<0.001),

combination (p<0.001) and “priming” (p<0.001) treatments (Figure 5.49.C.). A significant decrease was observed following “priming” treatment (0.35 ± 0.059 R.I.) (p<0.01).

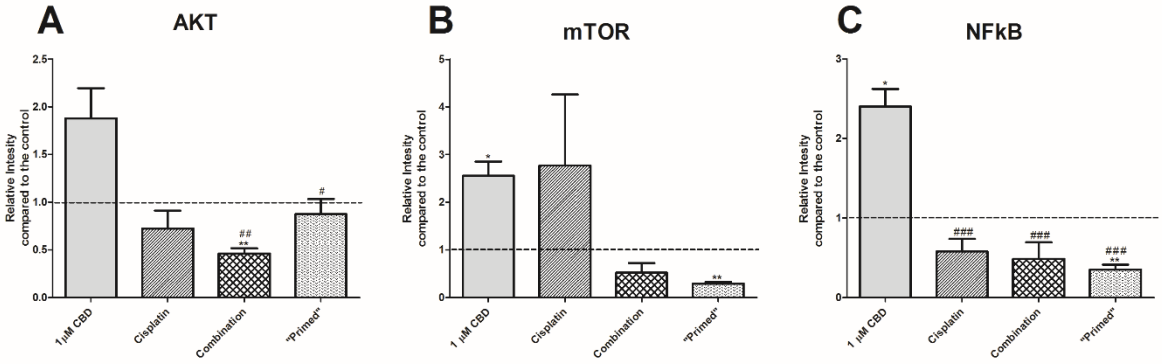

Figure 5.49. MDA-MB231’s AKT pathway gene expression following CBD, cisplatin, combination and “priming” with CBD compared to each other.
MDA-MB231 cell’s gene expression of **(A)** AKT, **(B)** mTOR and **(C)** NFkB were compared against each other. Data shown is repeated 3-5 times with technical replicates in triplicate per plate. The dashed line represents untreated cells and data was normalised to constitutively expression housekeepers α-tubulin or β-actin. Data is represented as mean ± SEM. * p<0.05, ** p<0.01 *vs* Untreated; # p<0.05, ### p<0.001 *vs* CBD.

5.12.2.4. “Priming” with CBD compared to previously examined treatments in effecting FOXO1 gene expression in cancer cells

A significant increase was observed in FOXO1 gene expression following CBD treatment (33.79 ± 9.896 R.I.) compared to untreated cells (p<0.05), cisplatin (p<0.05), combination (p<0.05) and “priming” (p<0.05) treatments (Figure 5.50.). A significant decrease in FOXO1 expression was observed following cisplatin (0.08 ± 0.029 R.I.) (p<0.01) and combination (0.46 ± 0.091 R.I.) (p<0.05) treatments compared to untreated cells.

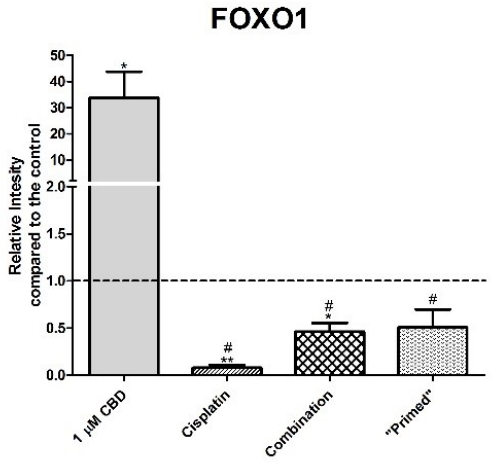

Figure 5.50. MDA-MB231's FOXO1 gene expression following the previously reported treatments compared to each other.
MDA-MB231 cell's gene expression of FOXO1 was compared against each other. Data shown is repeated 3-5 times with technical replicates in triplicate per plate. The dashed line represents control treatments and data was normalised to constitutively expression housekeepers α-tubulin or β-actin. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs* Untreated; # $p<0.05$ *vs* CBD.

5.12.3. The effect of "priming" with CBD on control cells gene expression

5.12.3.1. "Priming" with CBD in control cell's effect on the gene expression in the p53 pathway

No difference in HDAC1 gene expression was observed following "priming" treatment (Figure 5.51.A.). A similar trend for all doses was observed compared to untreated levels.

A dose dependant increase in SirT1 expression was observed following "priming" treatment (Figure 5.51.B.). A significance was reached from 2.5 µM CBD (28.63 ± 4.495 R.I.) ($p<0.05$) and 5 µM CBD (34.25 ± 4.434 R.I.) ($p<0.05$) compared to untreated cells.

An increase in p53 gene expression was observed following "priming" treatment compared to the untreated cells with 1 µM CBD (1.35 ± 0.110 R.I.) ($p<0.05$), 2.5 µM

CBD (1.32 ± 0.069 R.I.) (p<0.05) and 5 μM CBD (1.53 ± 0.164 R.I.) (p<0.05) (Figure 5.51.C.).

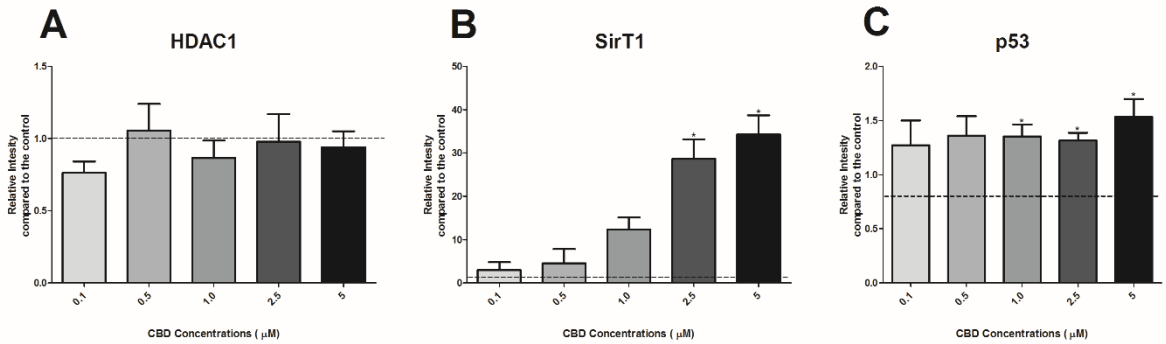

Figure 5.51. Control cell's p53 pathway gene expression following "priming" treatment. Control cell's gene expression of **(A)** HDAC1, **(B)** SirT1 and **(C)** p53 following "priming" treatment. Data shown is repeated 3-5 times with technical replicates in triplicate per plate. The dashed line represents untreated cells and data was normalised to constitutively expression housekeepers α-tubulin or β-actin. Data is represented as mean ± SEM. * p<0.05 *vs* Untreated.

5.12.3.2. The effect of "priming" with CBD in the gene expression of the Nrf2 pathway in control cells

A dose dependant increase was observed in MAPk expression following "priming" treatment (Figure 5.52.A.). This reached a significant increase with 1 μM CBD (5.36 ± 0.958 R.I.) (p<0.05), 2.5 μM CBD (5.77 ± 1.222 R.I.) (p<0.05) and 5 μM CBD (5.71 ± 1.081 R.I.) (p<0.05).

A dose dependant increase was observed following "priming" treatment in Nrf2 gene expression (Figure 5.52.B.). A significant increase was observed with 2.5 μM CBD (1.97 ± 0.155 R.I.) (p<0.01) and 5 μM CBD (2.15 ± 0.249 R.I.) (p<0.05).

A significant reduction in IL-6 gene expression was observed following "priming" treatment from 0.1 μM CBD (0.19 ± 0.110 R.I.) (p<0.05), 0.5 μM CBD (0.11 ± 0.059 R.I.) (p<0.01), 1 μM CBD (0.20 ± 0.071 R.I.) (p<0.01), 2.5 μM CBD (0.22 ± 0.035 R.I.) (p<0.01) and 5 μM CBD (0.34 ± 0.094 R.I.) (p<0.05) (Figure 5.52.C.).

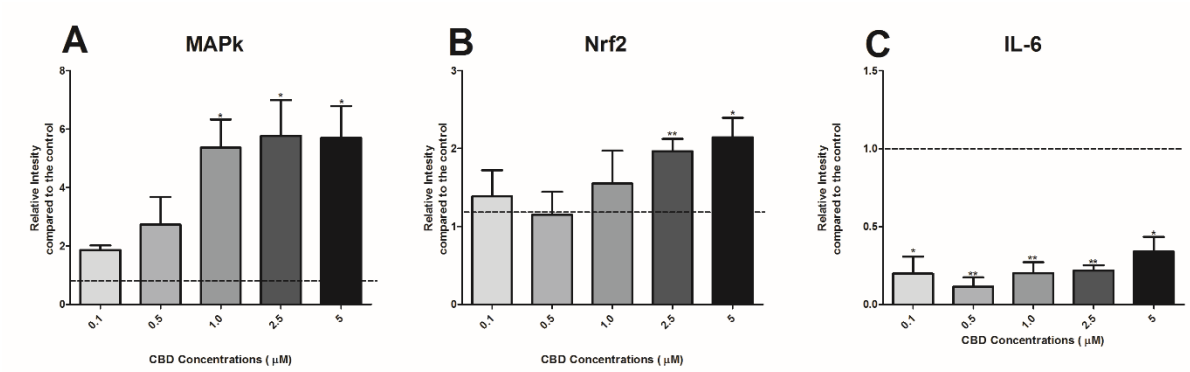

Figure 5.52. Control cell's Nrf2 pathway gene expression following "priming" treatment. Control cell's gene expression of **(A)** MAPk, **(B)** Nrf2 and **(C)** IL-6 were analysed after "priming" treatments. Data shown is repeated 3-5 times with technical replicates in triplicate per plate. The dashed line represents untreated cells and data was normalised to constitutively expression housekeepers α-tubulin or β-actin. Data is represented as mean ± SEM. * p<0.05, ** p<0.01 *vs* Untreated.

5.12.3.3. Following "priming" with CBD in control cells the effect of the treatment on the gene expression in the AKT pathway

A dose dependant increase was observed in AKT gene expression following "priming" treatment (Figure 5.53.A.). A significant increase was observed with 2.5 μM CBD (1.72 ± 0.227 R.I.) (p<0.05) and 5 μM CBD (2.58 ± 0.344 R.I.) (p<0.05).

mTOR gene expression was significantly decreased with all doses of CBD "priming" compared to untreated cells (Figure 5.53.B.). A significant decrease was observed from 0.1 μM CBD (0.12 ± 0.070 R.I.) (p<0.01), 0.5 μM CBD (0.04 ± 0.027 R.I.) (p<0.001), 1 μM CBD (0.06 ± 0.003 R.I.) (p<0.001), 2.5 μM CBD (0.13 ± 0.052 R.I.) (p<0.01), 5 μM CBD (0.30 ± 0.129 R.I.) (p<0.05).

A dose dependant increase was observed in NFkB gene expression following "priming" treatment (Figure 5.53.C.). A significance was reached with 2.5 μM CBD (3.48 ± 0.454 R.I.) (p<0.05) and 5 μM CBD (5.09 ± 0.272 R.I.) (p<0.01) compared to control cells.

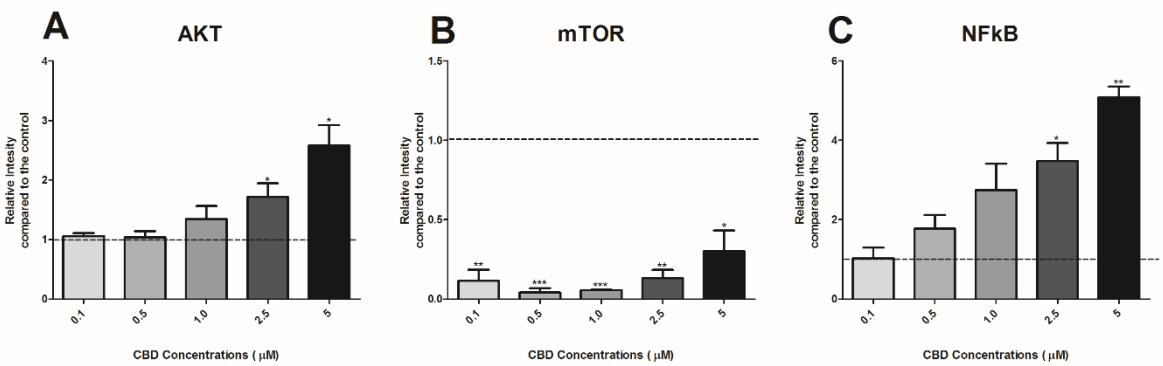

Figure 5.53. The effect of "priming" with CBD in control cell's AKT pathway gene expression.
Control cell's gene expression of **(A)** AKT, **(B)** mTOR and **(C)** NFkB were analysed after "priming" treatments. Data shown is repeated 3-5 times with technical replicates in triplicate per plate. The dashed line represents untreated cells and data was normalised to constitutively expression housekeepers α-tubulin or β-actin. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs* Untreated.

5.12.3.4. "Priming" with CBD's effect on the gene expression of control cell's FOXO1

A dose dependant increase was observed in FOXO1 gene expression following "priming" treatment (Figure 5.54.). Significance was reached with 0.1 μM CBD (11.71 ± 2.318 R.I.), 0.5 μM CBD (14.60 ± 1.494 R.I.), 1 μM CBD (16.69 ± 2.644 R.I.), 2.5 μM CBD (29.39 ± 5.458 R.I.) and 5 μM CBD (33.02 ± 4.601 R.I.) ($p<0.05$) compared to control treatment.

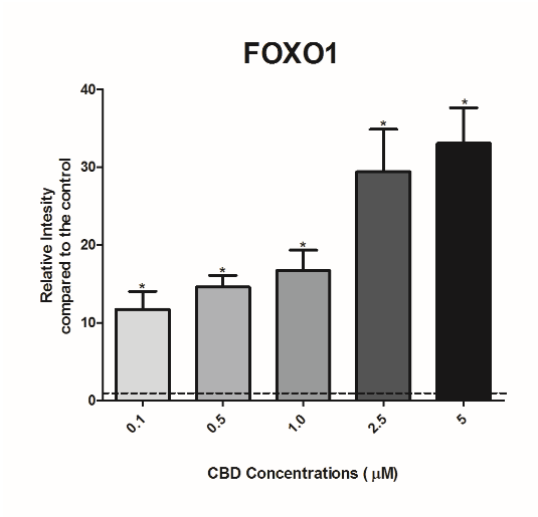

Figure 5.54. Control cell's FOXO1 gene expression following "priming" treatment. Control cell's gene expression of FOXO1 following "priming" treatment. Data shown is repeated 3-5 times with technical replicates in triplicate per plate. The dashed line represents untreated cells and data was normalised to constitutively expression housekeepers α-tubulin or β-actin. Data is represented as mean ± SEM. * $p<0.05$ *vs* Untreated.

5.12.4. The gene expression of "priming" with CBD compared against previously examined treatments in control cells

5.12.4.1. The p53 pathway's gene expression in response to "priming" with CBD compared to previously examined treatments in control cells

An increase in HDAC1 gene expression was observed following CBD treatment (2.28 ± 0.313 R.I.) ($p<0.05$) (Figure 5.55.A.). A trend to increase was observed following cisplatin and combination treatment, however, no added difference was seen between the treatments and each other. No change was observed following "priming" treatment.

No change was observed in SirT1 gene expression following CBD, cisplatin and combination treatment compared to untreated cells (Figure 5.55.B.). Following "priming" treatment a trend to increase was observed compared to untreated cells. However, a significant difference was observed between "priming" and CBD ($p<0.01$), cisplatin ($p<0.01$) and combination ($p<0.01$) treatments.

A significant increase in p53 gene expression was observed following cisplatin treatment (2.85 ± 0.662 R.I.) compared to untreated cells (p<0.05), CBD (p<0.01) and combination (p<0.05) (Figure 5.55.C.). Following "priming" treatment a significant increase (1.35 ± 0.110 R.I.) was observed compared to untreated cells only (p<0.05).

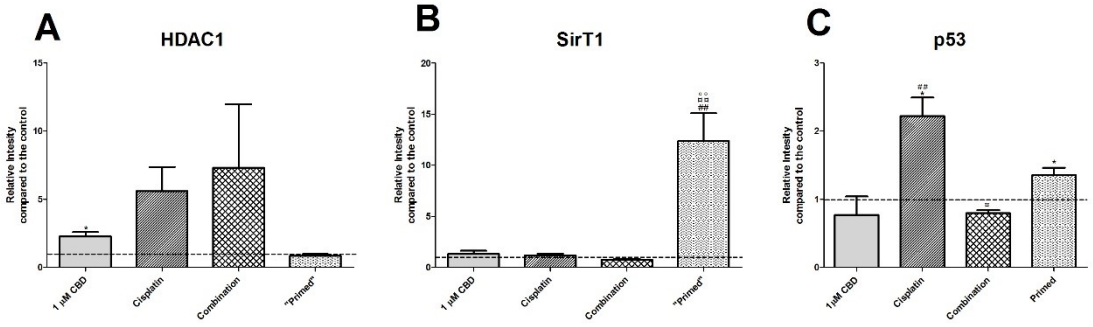

Figure 5.55. Control cell's p53 pathway gene expression following CBD, cisplatin, combination and "priming" with CBD compared to each other.
Control cell's gene expression of **(A)** HDAC1, **(B)** SirT1 and **(C)** p53 were compared against each other. Data shown is repeated 3-5 times with technical replicates in triplicate per plate. The dashed line represents untreated cells and data was normalised to constitutively expression housekeepers α-tubulin or β-actin. Data is represented as mean ± SEM. * p<0.05 *vs* Untreated; ## p<0.01 *vs* CBD; ¤ p<0.05, ¤¤ p<0.01 *vs* Cisplatin; °°° p<0.001 *vs* Combination.

5.12.4.2. The effect of "priming" with CBD in the gene expression of control cell's Nrf2 pathway compared to previously examined treatments

A significant decrease was observed in MAPk expression following CBD treatment (0.43 ± 0.105 R.I.) compared to untreated cells (p<0.05) (Figure 5.56.A.). A significant increase in MAPk gene expression was observed following cisplatin treatment was not significant to untreated cells (p= 0.057) but was significantly different to CBD (p<0.01), combination (p<0.01) and "priming" (p<0.05) treatments. An increase observed following "priming" treatment (5.36 ± 0.958 R.I.) was significant only to untreated cells (p<0.05).

Following treatment with cisplatin, an increase in Nrf2 gene expression levels (7.35 ± 1.818 R.I.) was observed compared to untreated cells (p<0.05) and CBD

treatment (p<0.01) (Figure 5.56.B.). All other treatments showed no other significant differences to each other.

A significant increase in IL-6 expression was observed following cisplatin treatment (2.06 ± 0.198 R.I.) compared to untreated cells (p<0.05) (Figure 5.56.C.). Following "priming" treatment a significant reduction was observed (0.20 ± 0.072 R.I.) compared to untreated cells (p<0.01) and cisplatin treatment (p<0.05).

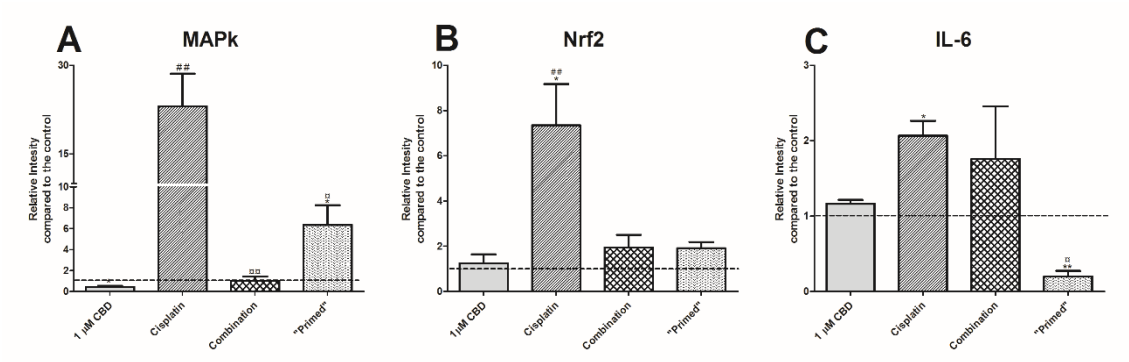

Figure 5.56. Control cell's Nrf2 pathway gene expression compared with CBD, cisplatin, combination and "priming" treatments.
Control cell's gene expression of **(A)** MAPk, **(B)** Nrf2 and **(C)** IL-6, were compared against each other. Data shown is repeated 3-5 times with technical replicates in triplicate per plate. The dashed line represents untreated cells and data was normalised to constitutively expression housekeepers α-tubulin or β-actin. Data is represented as mean ± SEM. * p<0.05, ** p<0.01 *vs* Untreated; # p<0.05, ## p<0.01 *vs* CBD; ¤ p<0.05, ¤¤ p<0.01 *vs* Cisplatin.

5.12.4.3. The AKT pathway's gene expression in control cells following "priming" with CBD compared to the previously described treatments

AKT gene expression was increased following cisplatin treatment (2.91 ± 0.402 R.I.) compared to untreated cells (p<0.05) (Figure 5.57.A.). There was no added significance between any of the other treatments.

Following treatment with cisplatin there was a significant increase in mTOR gene expression (3.36 ± 0.483 R.I.) compared to untreated cells (p<0.05), CBD (p<0.05), combination (p<0.01) and "priming" (p<0.001) (Figure 5.57.B.). "Priming" treatment

showed a significant reduction in mTOR gene expression (0.06 ± 0.003 R.I.) compared to untreated cells (p<0.01).

NFkB gene expression was significantly increased following CBD treatment (2.75 ± 0.662 R.I.) compared to untreated cells (p<0.05) (Figure 5.57.C.). No other significance was observed comparing the other treatments.

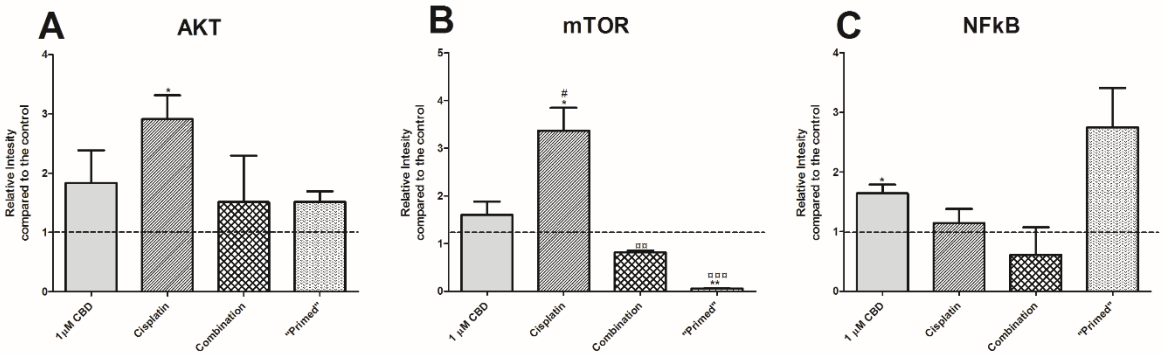

Figure 5.57. Control cell's AKT pathway gene expression compared against each other. Control cell's gene expression of **(A)** AKT, **(B)** mTOR and **(C)** NFkB were compared against each other. Data shown is repeated 3-5 times with technical replicates in triplicate per plate. The dashed line represents untreated cells and data was normalised to constitutively expression housekeepers α-tubulin or β-actin. Data is represented as mean ± SEM. * p<0.05, ** p<0.01 *vs* Untreated.

5.12.4.4. The effect of "priming" with CBD on the gene expression of FOXO1 in control cells compared with other previously examined treatments

A significant increase in FOXO1 gene expression was observed following CBD treatment (2.07 ± 0.294 R.I.) compared to untreated cells (p<0.05) and combination (p<0.01) (Figure 5.58.). A significant decrease was observed following combination treatment (0.15 ± 0.042 R.I.) compared to untreated cells (p<0.01). Following "priming" treatment a significant increase in FOXO1 gene expression was observed (16.69 ± 2.644 R.I.) that was significant to untreated cells (p<0.01), CBD (p<0.001), cisplatin (p<0.001) and combination (p<0.001) treatments.

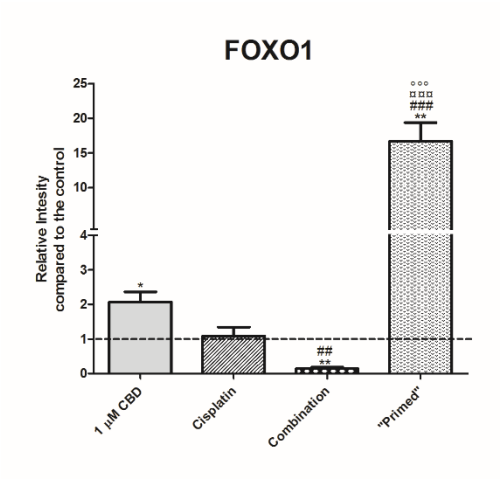

Figure 5.58. Control cell's FOXO1 gene expression following CBD, cisplatin, combination and "priming" treatment compared to each other.
Control cell's gene expression of FOXO1 was compared against each other. Data shown is repeated 3-5 times with technical replicates in triplicate per plate. The dashed line represents untreated cells and data was normalised to constitutively expression housekeepers α-tubulin or β-actin. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs* Untreated; ## $p<0.01$, ### $p<0.001$ *vs* CBD; ¤¤¤ $p<0.001$ *vs* Cisplatin; °°° $p<0.001$ *vs* Combination.

5.13. The effect of "priming" with CBD on the protein level's in the cell

The alterations observed with the gene expression in section 5.12. led to further protein level examination. Protein levels of similar pathways as described in section 3.12. and 4.11. were explored following "priming" with both cancer and normal cells. Analysis of protein levels was carried out using western blotting (Section 2.13.).

5.13.1. The effect of "priming" with CBD on cancer cell's protein levels

5.13.1.1. The p53 pathway's protein levels following "priming" with CBD

No change in HDAC1 protein levels was observed following "priming" treatment compared to untreated cells (Figure 5.59.A.).

A dose dependant increase in SirT1 expression was observed as "priming" dose increased (Figure 5.59.B.). The increase began following treatment with 0.1 μM CBD (2.89 ± 0.312 R.I.) ($p<0.01$), 0.5 μM CBD (2.59 ± 0.535 R.I.) ($p<0.05$), 1 μM CBD (3.46 ± 0.865 R.I.) ($p<0.05$), 2.5 μM CBD (3.69 ± 0.872 R.I.) ($p<0.05$) and 5 μM CBD (4.75 ± 1.452 R.I.) ($p<0.05$).

p53 protein levels were increased following 0.1 μM CBD "priming" treatment with (3.72 ± 0.401 R.I.) compared to untreated cells ($p<0.01$) (Figure 5.59.C.). A dose dependant reduction in p53 from this initial increase was observed as "priming" dose increased, 0.5 μM CBD (2.27 ± 0.356 R.I.) ($p<0.05$), 1 μM CBD (1.39 ± 0.054 R.I.) ($p<0.01$), 2.5 μM CBD (1.82 ± 0.251 R.I.) ($p<0.05$), 5 μM CBD (1.83 ± 0.259 R.I.) ($p<0.05$).

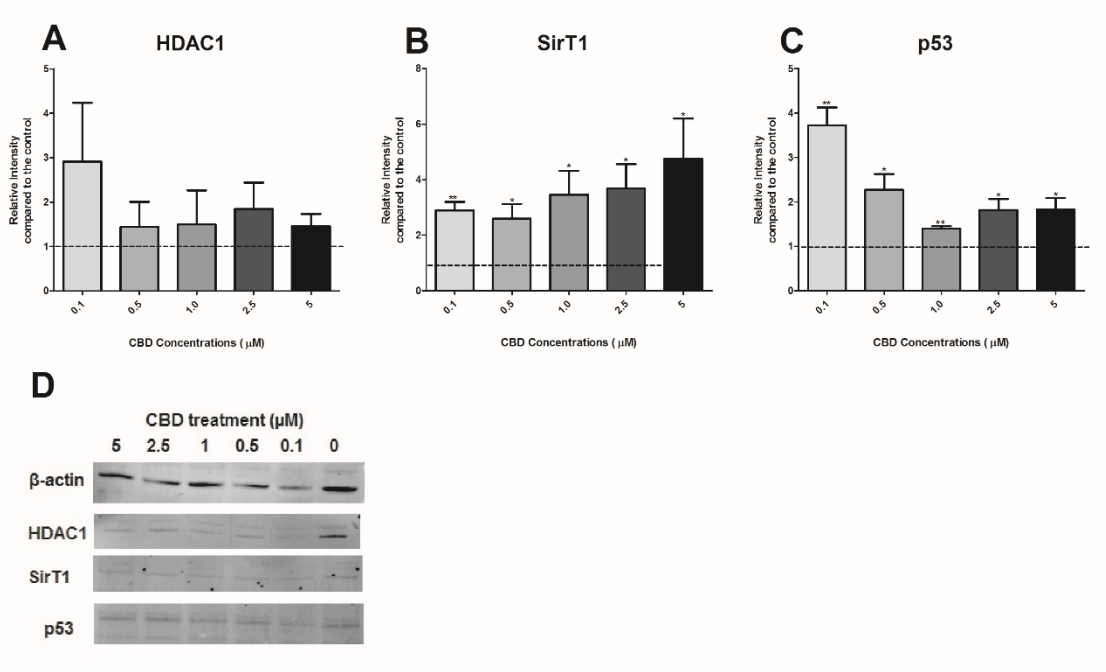

Figure 5.59. MDA-MB231's p53 pathway protein levels following "priming" treatment. Protein expression's p53 pathway was analysed; **(A)** HDAC1, **(B)** SirT1 and **(C)** p53 following "priming" treatment. Data shown is repeated 3-5 times. The dashed line represents untreated cells and data was normalised to the constitutively expressed house keeper β-Actin. **(D)** Western blot of relevant graphs with untreated (0) and the housekeeper β-actin as control protein. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs* Untreated.

5.13.1.2. "Priming" with CBD's effect on the protein levels in the Nrf2 pathway in cancer cells

An increase in HDAC6 protein levels was observed following treatment with 0.1 μM CBD "primed" (2.50 ± 0.368 R.I.) ($p<0.05$) (Figure 5.60.A.). However, as the dose of "priming" increased, a trend to decrease was observed reaching no significance to untreated cells with 0.5 μM CBD "primed".

A significant decrease in Nrf2 protein levels was observed following "priming" treatment with 0.1 μM CBD (0.58 ± 0.133 R.I.) ($p<0.05$), 0.5 μM CBD (0.49 ± 0.125 R.I.) and 1 μM CBD (0.61 ± 0.073 R.I.) ($p<0.05$) compared to untreated cells (Figure 5.60.B.). No change was observed with 2.5 μM CBD and 5 μM CBD "primed".

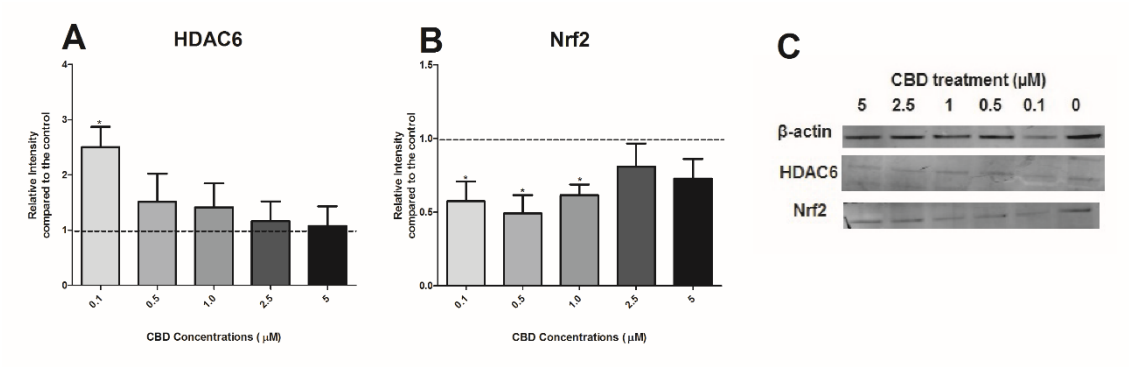

Figure 5.60. MDA-MB231's Nrf2 pathway protein levels following "priming" treatment. MDA-MB231 cell's protein levels were analysed for alterations to the Nrf2 pathway; **(A)** HDAC6 and **(B)** Nrf2, following "priming" treatment. Data shown is repeated 3-5 times. The dashed line represents untreated cells and data was normalised to the constitutively expressed house keeper β-Actin. **(C)** Western blot of relevant graphs with untreated (0) and the housekeeper β-actin as control protein. Data is represented as mean ± SEM. * $p<0.05$ *vs* Untreated.

5.13.1.3. The effect of "priming" with CBD on the AKT pathway's protein levels in cancer cells

No difference in AKT protein levels was observed following "priming" treatment compared to untreated cell levels (Figure 5.61.A.).

A significant increase in mTOR was observed following "priming" with 0.1 μM CBD (2.16 ± 0.285 R.I.) ($p<0.05$) (Figure 5.61.B.). However, a trend to decrease was observed as the dose of CBD "priming" increased showing no change compared to untreated cells from 0.5 μM CBD.

No change in NFkB levels were observed following "priming" treatment compared to untreated levels (Figure 5.61.C.).

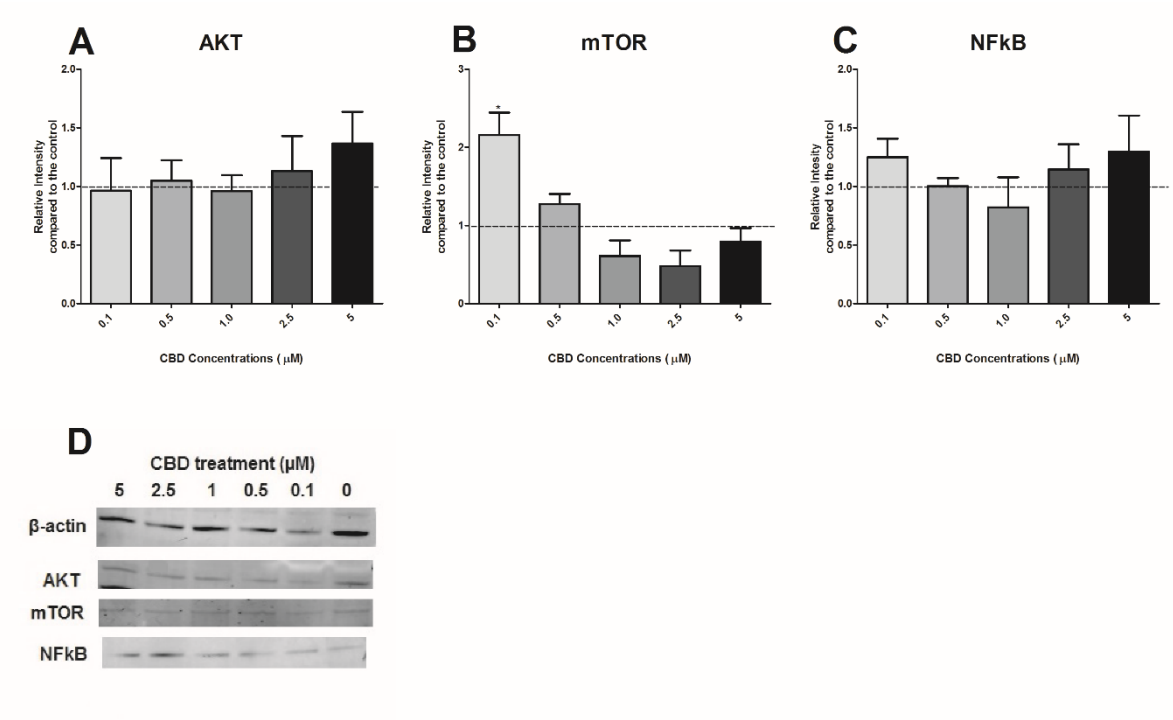

Figure 5.61. The effect of "priming" with CBD on cancer cell's protein levels in the AKT pathway.
MDA-MB231 cell's protein were analysed for alterations to the AKT pathway; **(A)** AKT, **(B)** mTOR and **(C)** NFkB, following "priming" treatment. Data shown is repeated 3-5 times. The dashed line represents untreated cells and data was normalised to the constitutively expressed house keeper β-Actin. **(D)** Western blot of relevant graphs with untreated (0) and the housekeeper β-actin as control protein. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs* Untreated.

5.13.2. The protein levels of "priming" with CBD in cancer cells compared to the previously reported treatments effect

5.13.2.1. The effect of "priming" with CBD in cancer cells protein levels compared to previously described treatments in the p53 pathway

An increase in HDAC1 levels was observed following CBD treatment (1.89 ± 0.081 R.I.) compared to untreated cells ($p<0.01$) (Figure 5.62.A.). Combination treatment showed a significant reduction in HDAC1 levels (0.40 ± 0.069 R.I.) compared to untreated cells ($p<0.001$) and cisplatin treatment ($p<0.05$).

SirT1 levels were significantly increased following cisplatin treatment (3.01 ± 0.747 R.I.) compared to untreated cells ($p<0.05$), CBD ($p<0.05$) and combination

(p<0.05) treatments (Figure 5.62.B.). Following “priming” treatment an increase in SirT1 levels was observed (3.46 ± 0.865 R.I.) compared to untreated cells (p<0.05), CBD (p<0.05) and combination (p<0.05) treatment.

A significant increase in p53 protein levels was observed following CBD treatment (2.27 ± 0.310 R.I.) compared to untreated cells (p<0.05) (Figure 5.62.C.). A further significant increase was observed following cisplatin treatment (59.76 ± 6.531 R.I.) compared to untreated cells (p<0.001), CBD (p<0.001), combination (p<0.001) and “priming” (p<0.001). Combination had a significant decrease in p53 protein levels (0.39 ± 0.095 R.I.) compared to untreated cells (p<0.01). Whereas “priming” showed a significant increase (1.39 ± 0.054 R.I.) compare to untreated cells.

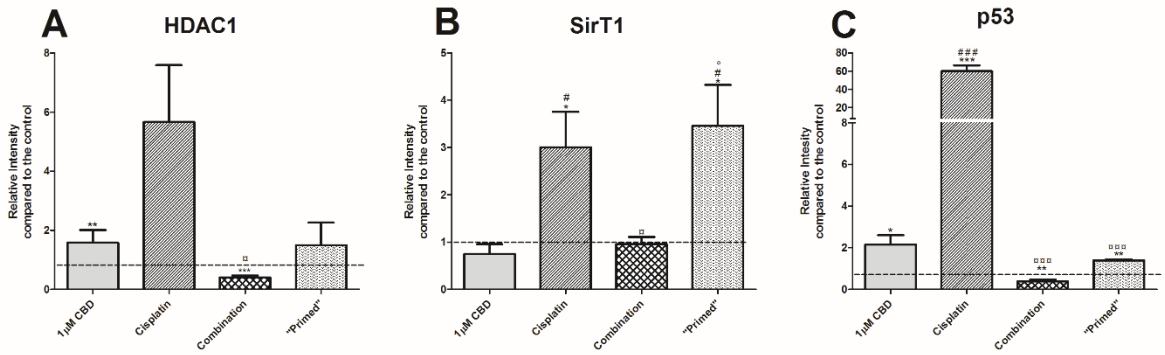

Figure 5.62. MDA-MB231 protein levels of the p53 pathway treatments following CBD, cisplatin, combination and “priming” with CBD compared to each other
MDA-MB231 cell’s **(A)** HDAC1, **(B)** SirT1 and **(C)** p53 protein levels were compared against each other. Data shown is repeated 3-5 times. The dashed line represents untreated cells and data was normalised to constitutively expression house keeper β-Actin. Data is represented as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001 *vs* Untreated; # p<0.05, ### p<0.001 *vs* CBD; ¤ p<0.05, ¤¤¤ p<0.001 *vs* Cisplatin; ° p<0.05 *vs* Combination.

5.13.2.2. “Priming” with CBD’s effect on the protein levels in the Nrf2 pathway compared to previously examined treatments and their effect in cancer cells

An increase in HDAC6 protein levels was observed following cisplatin treatment (2.68 ± 0.769 R.I.) compared to untreated cells (p<0.05) (Figure 5.63.A.). However, no significant difference with any of the other treatment conditions was observed.

A significant increase was observed with CBD (2.43 ± 0.291 R.I.) ($p<0.01$) and cisplatin treatment (2.09 ± 0.298 R.I.) ($p<0.01$) compare to untreated cells (Figure 5.63.B.). A reduction following treatment with "priming" was observed (0.614 ± 0.073 R.I.) compared to untreated cells ($p<0.05$), CBD ($p<0.05$) and cisplatin ($p<0.05$).

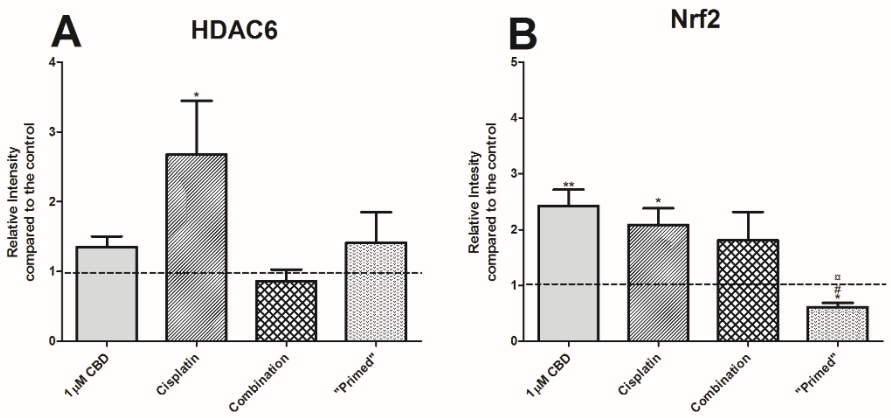

Figure 5.63. MDA-MB231 protein levels of the Nrf2 pathway treatments compared to each other.
MDA-MB231 cell's **(A)** HDAC6 and **(B)** Nrf2 protein levels were compared against each other. Data shown is repeated 3-5 times. The dashed line represents untreated cells and data was normalised to constitutively expression house keeper β-Actin. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs* Untreated; # $p<0.05$ *vs* CBD; ¤ $p<0.05$ *vs* Cisplatin.

5.13.2.3. The protein levels of the AKT pathway following "priming" with CBD compared to previously reported treatments in cancer cells

No change in AKT protein levels was observed with any of the treatments (Figure 5.64.A.). There was also no significant difference between treatments with each other.

An increase in mTOR levels was observed following CBD (1.49 ± 0.171 R.I.) ($p<0.05$) and combination treatment (1.52 ± 0.042 R.I.) ($p<0.01$) compared to untreated cells (Figure 5.64.B.). A significant reduction was observed following "priming" treatment compared to CBD ($p<0.05$) and combination ($p<0.05$).

NFkB protein levels were unchanged for all treatments compared to untreated cells and to each other (Figure 5.64.C.).

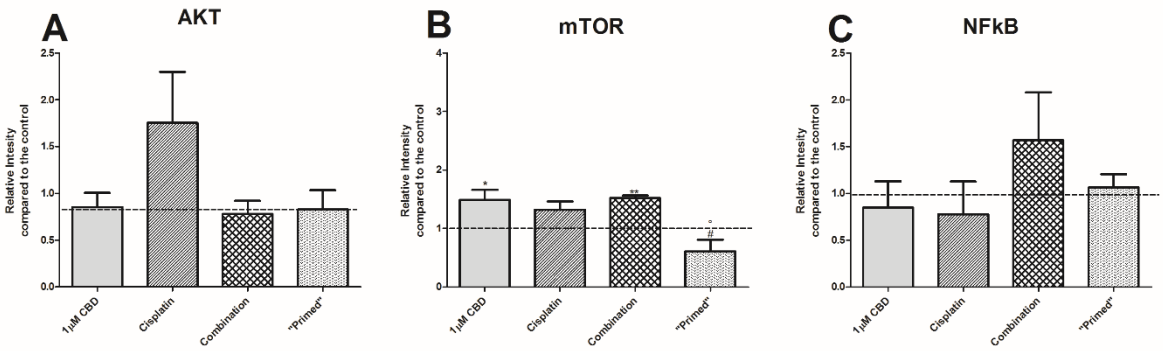

Figure 5.64. MDA-MB231 protein expression analysis of the AKT pathway treatments compared to each other.
MDA-MB231 cell's **(A)** AKT, **(B)** mTOR and **(C)** NFkB protein levels were compared against each other. Data shown is repeated 3-5 times. The dashed line represents untreated cells and data was normalised to constitutively expression house keeper β-Actin. Data is represented as mean ± SEM. * p<0.05, ** p<0.01 *vs* Untreated; # p<0.05 *vs* CBD; ° p<0.05 *vs* Combination.

5.13.3. The effect of "priming" with CBD in control cell's protein levels

5.13.3.1. The effect of "priming" with CBD in the protein levels of the p53 pathway in control cells

No change was observed in HDAC1 protein level following "priming" treatment compared to untreated cells (Figure 5.65.A.). There was a dose dependant trend to increase in HDAC1 levels following "priming" though this was not significantly different.

A dose dependant significant increase in SirT1 expression levels was observed following an increase in "priming" treatment (Figure 5.65.B.). This reached significance with 0.5 μM CBD (1.38 ± 0.152 R.I.) (p<0.05), 1 μM CBD (1.67 ± 0.235 R.I.) (p<0.05), 2.5 μM CBD (2.36 ± 0.437 R.I.) (p<0.05) and 5 μM CBD (2.49 ± 0.435 R.I.) (p<0.01) compared to untreated cells.

A significant increase in p53 levels was observed following “priming” with 0.1 μM CBD (4.50 ± 0.684 R.I.) compared to untreated cells ($p<0.05$) (Figure 5.65.C.). However, as the dose of “priming” increased a dose dependant decrease in p53 protein levels was observed from 0.5 μM CBD.

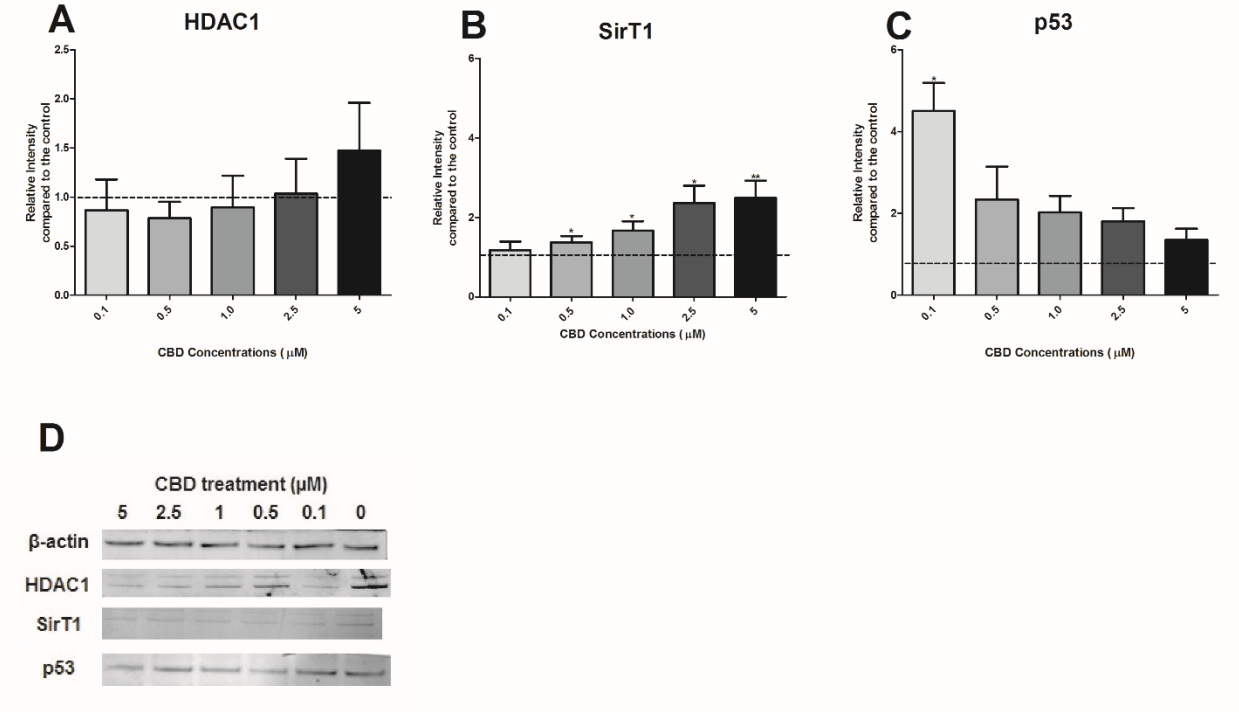

Figure 5.65. Control cell p53 pathway protein levels following “priming” with CBD. Control cell’s **(A)** HDAC1, **(B)** SirT1 and **(C)** p53 protein expression levels were analysed following “priming” treatment. Data shown is repeated 3-5 times. The dashed line represents untreated cells and data was normalised to constitutively expression house keeper β-Actin. **(D)** Western blot of relevant graphs with untreated (0) and the housekeeper β-actin as control protein. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs* Untreated.

5.13.3.2. “Priming” with CBD’s effect on protein levels in the Nrf2 pathway of control cells

No change in HDAC6 protein levels was observed following “priming” treatment compared to untreated cells (Figure 5.66.A.).

A significant increase in Nrf2 protein levels was observed following “priming” treatment with 0.1 μM CBD (3.44 ± 0.868 R.I.) ($p<0.05$) (Figure 5.66.B.). This increase

decreased slightly in a dose dependant manner as “priming” increased; 0.5 μM CBD (3.12 ± 0.558 R.I.) ($p<0.05$), 1 μM CBD (2.80 ± 0.699 R.I.) ($p<0.05$), 2.5 μM CBD (2.17 ± 0.338 R.I.) ($p<0.05$), 5 μM CBD (2.32 ± 0.445 R.I.) ($p<0.05$).

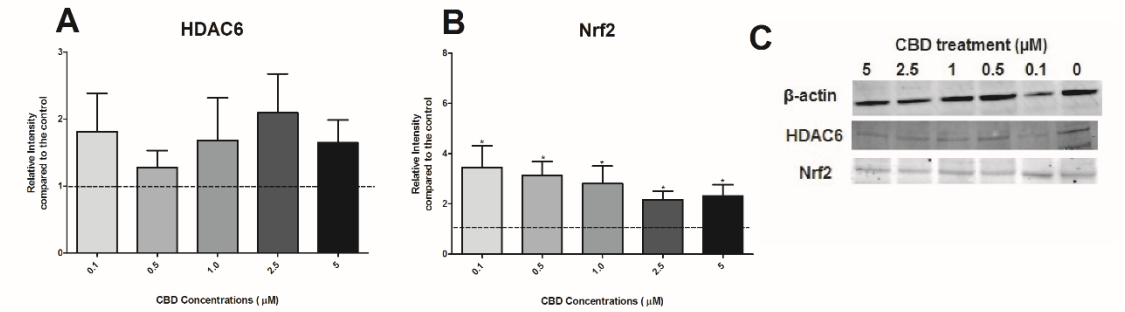

Figure 5.66. Control cell’s protein levels in the Nrf2 pathway following “priming” with CBD. Control cell’s **(A)** HDAC6 and **(B)** Nrf2 protein expression levels were analysed after “priming” treatments. Data shown is repeated 3-5 times. The dashed line represents untreated cells and data was normalised to constitutively expression house keeper β-Actin. **(C)** Western blot of relevant graphs with untreated (0) and the housekeeper β-actin as control protein. Data is represented as mean ± SEM. * $p<0.05$ *vs* Untreated.

5.13.3.3. The effect of “priming” with CBD in the protein levels of the AKT pathway in control cells

A dose dependant increase was observed in AKT protein levels following “priming” treatment as doses increased (Figure 5.67.A.). Significance was reached with 0.5 μM CBD “primed” treatment (2.02 ± 0.342 R.I.) ($p<0.05$), 1 μM CBD (2.31 ± 0.481 R.I.) ($p<0.05$), 2.5 μM CBD (3.19 ± 0.681 R.I.) ($p<0.05$) and 5 μM CBD (4.06 ± 0.941 R.I.) ($p<0.05$).

No change in mTOR protein levels was observed following “priming” treatment (Figure 5.67.B.). All doses of “priming” were similar to untreated cell levels.

No change in NFkB protein levels were observed following “priming” treatment, however, there was a trend to increase with lower doses of CBD “primed” (Figure 5.67.C.).

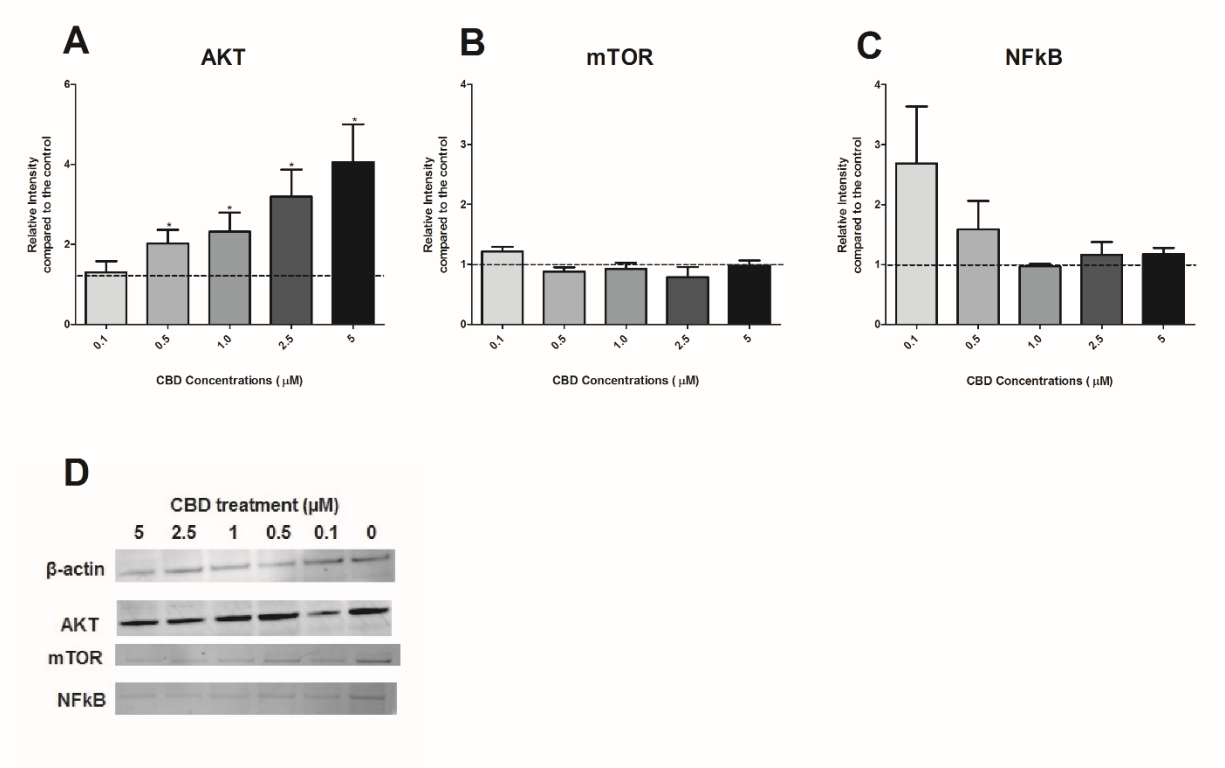

Figure 5.67. AKT pathway’s protein levels following “priming” in control cells.
Control cell’s **(A)** AKT, **(B)** mTOR and **(C)** NFkB protein levels were analysed following “priming” treatments. Data shown is repeated 3-5 times. The dashed line represents control treatments and data was normalised to constitutively expression house keeper β-Actin. **(D)** Western blot of relevant graphs with untreated (0) and the housekeeper β-actin as control protein. Data is represented as mean ± SEM. * $p<0.05$ *vs* Untreated.

5.13.4. The protein levels of all previously examined treatments compared to each other in control cells

5.13.4.1. The effect of “priming” with CBD compared to the previously examined treatments on the protein levels of control cell’s p53 pathway

A significant reduction was observed in HDAC1 levels following cisplatin treatment (0.54 ± 0.104 R.I.) compared to untreated cells ($p<0.01$) and CBD ($p<0.01$) (Figure 5.68.A.). A significant decrease was also observed following combination

treatment (0.44 ± 0.046 R.I.) compared to untreated cells (p<0.01) and CBD treatment (p<0.01).

A significant increase was observed in SirT1 expression following CBD (2.09 ± 0.559 R.I.) compared to untreated cells (p<0.01), cisplatin (p<0.05), combination (p<0.05) (Figure 5.68.B.). An increase was observed following “priming” treatment (1.59 ± 0.239 R.I.) compared to untreated cells (p<0.05).

A significant decrease was observed following treatment with cisplatin (0.318 ± 0.041 R.I.) (p<0.001) and combination treatment (0.49 ± 0.022 R.I.) compared to untreated cells (Figure 5.68.C.). No significant increase was observed following “priming” treatment (2.02 ± 0.874 R.I.) and untreated cells however a difference was observed between cisplatin (p<0.05) and combination (p<0.05) treatment.

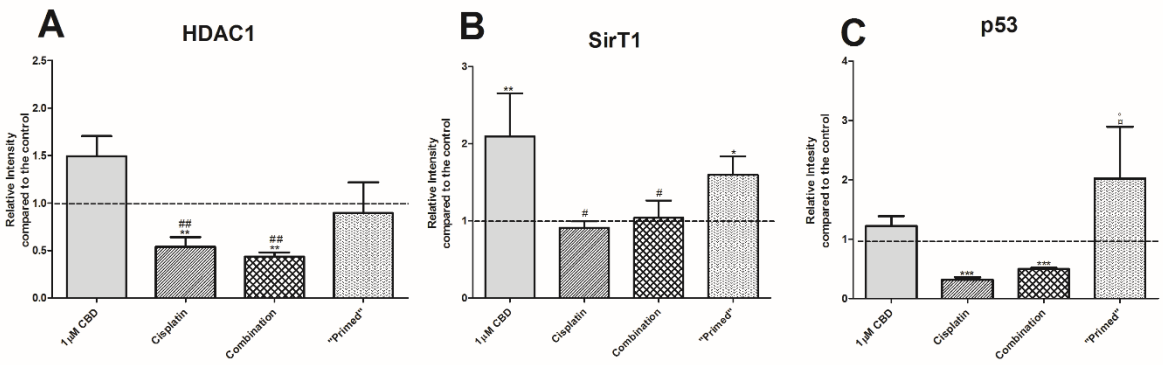

Figure 5.68. Control cell’s protein levels in the p53 pathway when all previously examined treatments were compared to each other.
Control cell’s **(A)** HDAC1, **(B)** SirT1 and **(C)** p53 protein levels were compared against each other. Data shown is repeated 3-5 times. The dashed line represents untreated cells and data was normalised to constitutively expression house keeper β-Actin. Data is represented as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001 *vs* Untreated; # p<0.05, ## p<0.01 *vs* CBD; ¤ p<0.05 *vs* Cisplatin; ° p<0.05 *vs* Combination.

5.13.4.2. The effects of "priming" with CBD compared to the previously examined treatments on the protein levels of control cell's Nrf2 pathway

A significant decrease in HDAC6 protein levels was observed following cisplatin treatment (0.66 ± 0.084 R.I.) compared to untreated cells ($p<0.001$) (Figure 5.69.A.). No change was shown following "priming" treatment compared to untreated cells, however compared to cisplatin ($p<0.05$) and combination ($p<0.05$) treatment there was a difference.

An increase was observed following "priming" treatment (3.18 ± 0.807 R.I.) of Nrf2 protein levels compared to untreated cells ($p<0.05$), CBD ($p<0.05$), cisplatin ($p<0.05$) and combination ($p<0.05$) (Figure 5.69.B.).

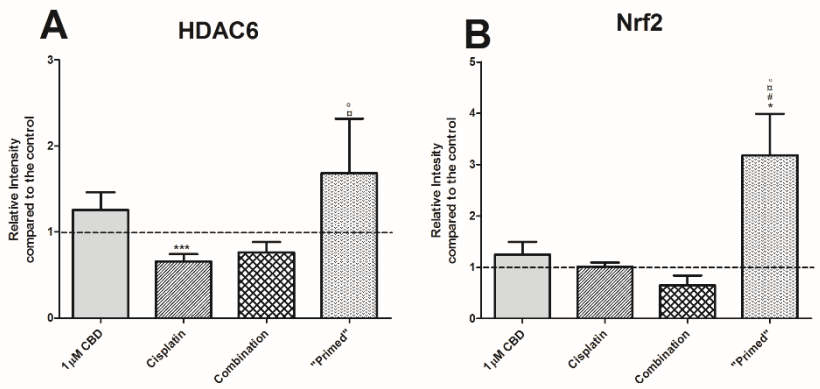

Figure 5.69. The Nrf2 pathway's protein levels following CBD, cisplatin, combination and "priming" with CBD in control cells compared with each other.
Control cell's **(A)** HDAC6 and **(B)** Nrf2 protein levels were compared against each other. Data shown is repeated 3-5 times. The dashed line represents untreated cells and data was normalised to constitutively expression house keeper β-Actin. Data is represented as mean ± SEM. * $p<0.05$, *** $p<0.001$ *vs* Untreated; # $p<0.05$ *vs* CBD; ¤ $p<0.05$ *vs* Cisplatin; ° $p<0.05$ *vs* Combination.

5.13.4.3. The AKT pathway's protein levels following previously reported treatments in control cells compared to each other

AKT protein levels were increased following "priming" treatment (2.31 ± 0.481 R.I.) compared to untreated cells ($p<0.05$), CBD ($p<0.05$), cisplatin ($p<0.05$) and combination ($p<0.05$) (Figure 5.70.A.). There was no change with any other treatment.

mTOR protein levels were significantly increased following CBD treatment (1.98 ± 0.260 R.I.) compared to untreated cells ($p<0.05$), CBD ($p<0.001$), cisplatin ($p<0.001$) and "priming" ($p<0.01$) (Figure 5.70.B.). A decrease in mTOR levels was observed following cisplatin treatment (0.55 ± 0.115 R.I.) ($p<0.05$) and combination (0.46 ± 0.074 R.I.) ($p<0.001$) compared to untreated cells.

A significant increase was observed in NFkB protein levels following CBD treatment (1.57 ± 0.113 R.I.) compared to untreated cells ($p<0.05$) and CBD ($p<0.05$) (Figure 5.70.C.).

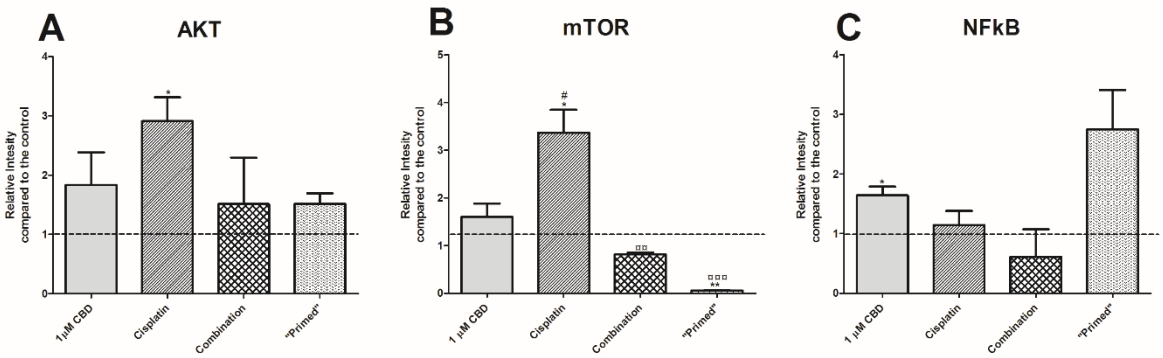

Figure 5.70. Control cell's AKT pathway's protein expression following CBD, cisplatin, combination and "priming" with CBD compared to each other.
Control cell's **(A)** AKT, **(B)** mTOR and **(C)** NFkB protein levels compared against each other. Data shown is repeated 3-5 times. The dashed line represents untreated cells and data was normalised to constitutively expression house keeper β-Actin. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs* Untreated; # $p<0.05$, ### $p<0.001$ *vs* CBD; ¤ $p<0.05$ *vs* Cisplatin; ° $p<0.05$ *vs* Combination.

5.14. The effect of "priming" with CBD on the global DNA methylation state of the cell

5.14.1. Cancer cell's global DNA methylation state's response to "priming" with CBD

5.14.1.1. "Priming" with CBD's effect in cancer cell's DNA methylation state

The ratio of 5-methylCytosine to Guanine was investigated in the cancer cells following "priming" treatment (Section 2.14.). A dose dependant increase was observed in 5methylcytosine/dGuanine levels following "priming" treatment compared to untreated cells (Figure 5.71.). Significance was reached with 1 μM CBD (2.55 ± 0.016 %) ($p<0.05$), 2.5 μM CBD (2.55 ± 0.022 %) ($p<0.05$) and 5 μM CBD (2.52 ± 0.008 %) ($p<0.01$).

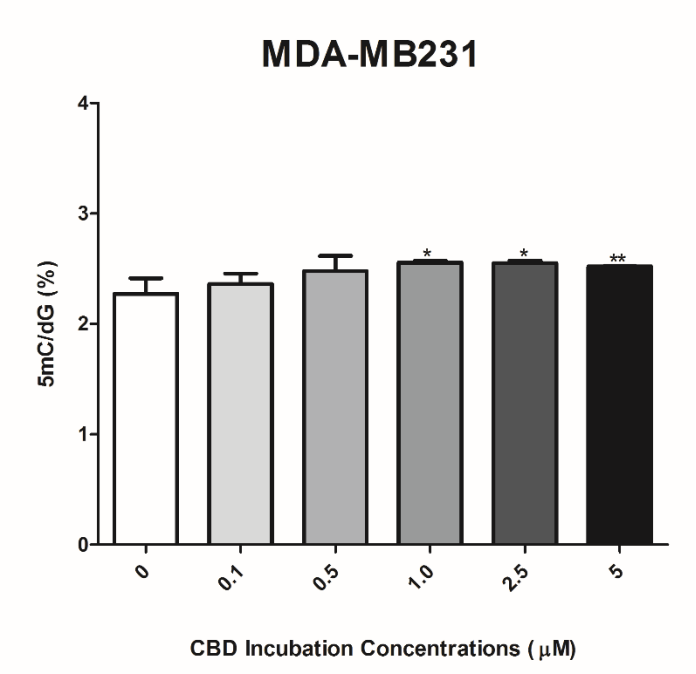

Figure 5.71. MDA-MB231's DNA methylation quantified following "priming" treatment. MDA-MB231 cell's DNA methylation analysed following "priming" treatment. Data shown is repeated 3-5 times with technical replicates in triplicate per run. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs* Untreated.

5.14.1.2. The effect of all previously examined treatments on the global DNA methylation state in cancer cells compared to each other

A decrease in DNA methylation was observed with cisplatin (0.91 ± 0.004 %) ($p<0.01$) and combination treatment (0.82 ± 0.014 %) ($p<0.05$) (Figure 5.72.). An increase was observed following "priming" treatment (1.11 ± 0.021 %) compared to untreated cells ($p<0.05$) and combination treatment ($p<0.05$).

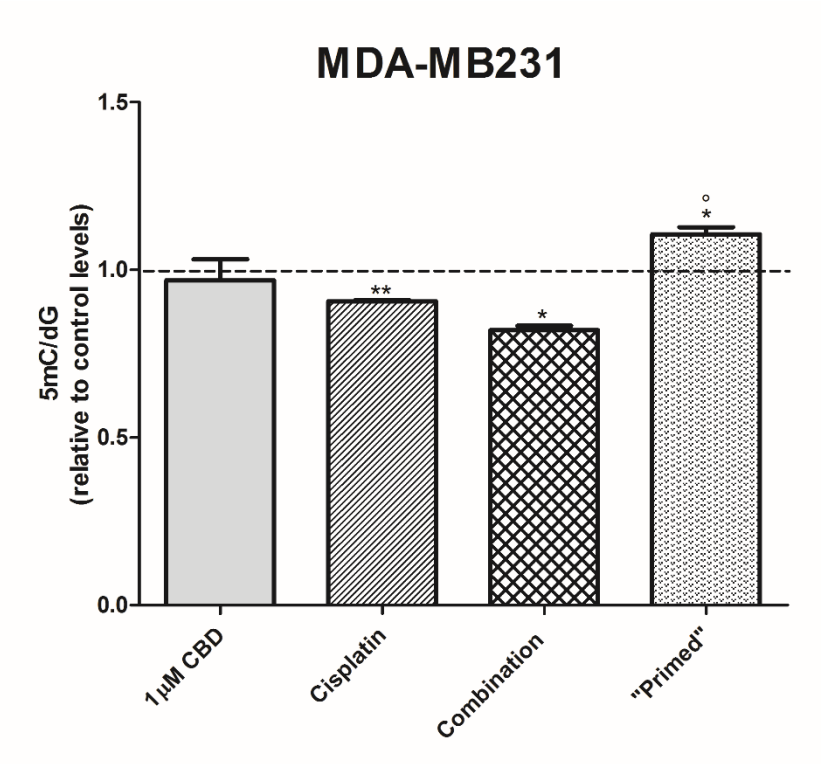

Figure 5.72. MDA-MB231 cancer cells DNA methylation alteration treatments compared against each other.
MDA-MB231 cell's DNA methylation treatments compared against each other. Data shown is repeated 3-5 times with technical replicates in triplicate per run. The dashed line represents untreated cells. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs* Untreated; ° $p<0.05$ *vs* Combination.

5.14.2. "Priming" with CBD's effect on global DNA methylation in control cells

5.14.2.1. "Priming" with CBD's effect on control cell's global DNA methylation state

No change was observed following "priming" treatment of 5methylcytosine/dGuanine levels compared to untreated cells (Figure 5.73.).

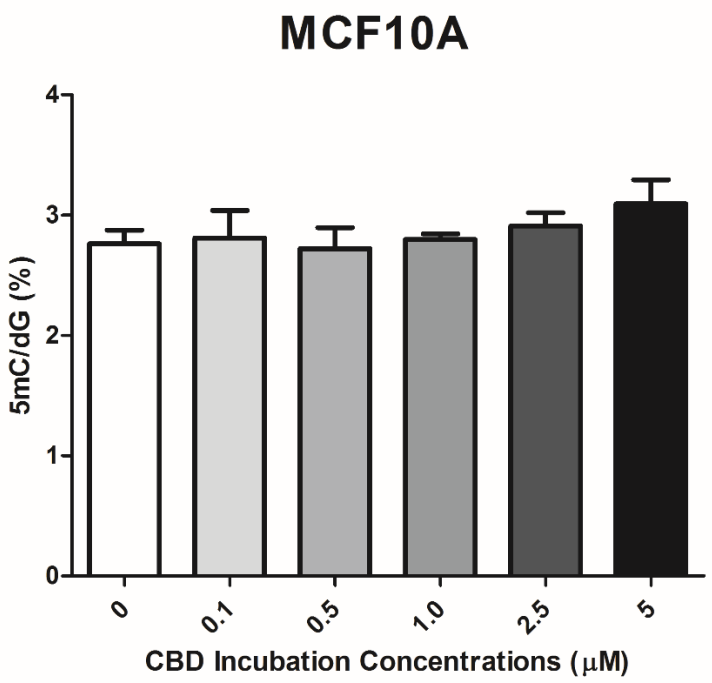

Figure 5.73. Control cell's DNA methylation following "priming" treatment.
Control cell's DNA methylation alteration following "priming" treatment. Data shown is repeated 3-5 times with technical replicates in triplicate per run. Data is represented as mean ± SEM.

5.14.2.2. The global DNA methylation state in control cells following all previously examined treatments compared to each other

A significant decrease was observed following CBD treatment (0.85 ± 0.069 %) compared to untreated cells ($p<0.05$) (Figure 5.74.). No further significance was observed for any other treatments compared to each other.

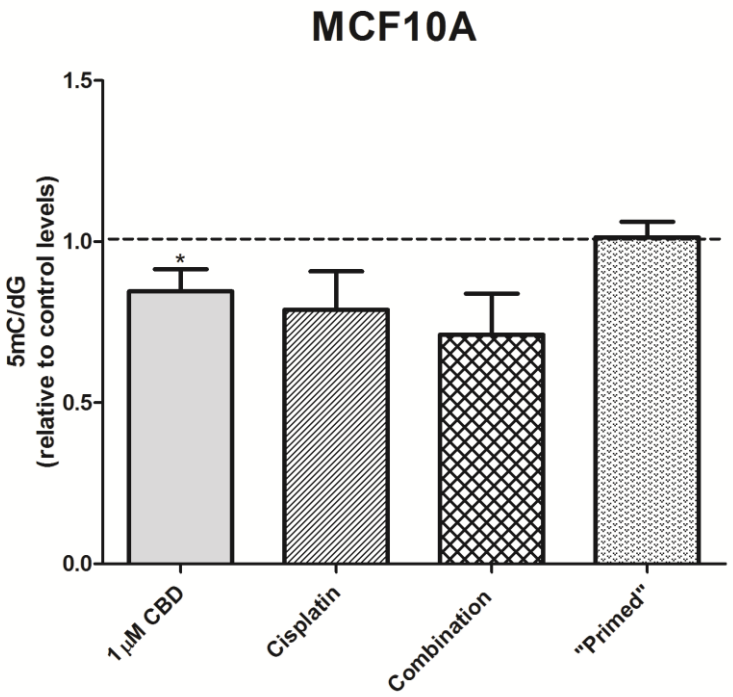

Figure 5.74. The control cell's DNA methylation state following CBD, cisplatin, combination and "priming" treatment compared to each other.
Control cell's DNA methylation alteration after each treatment was compared to each other. Data shown is repeated 3-5 times with technical replicates in triplicate per run. The dashed line represents untreated cells. Data is represented as mean ± SEM. *$p<0.05$ *vs* Untreated.

5.15. "Priming" with CBD's effect in an *in vivo* model experiment

5.15.1. The effect of "priming" with CBD in a xenograft model *in vivo*.

An *in vivo* xenograft mouse model of MDA-MB231 cells was used to examine the effect of "priming" (Section 2.15). The first set of treatments consisted of the "priming" technique prior to chemotherapeutic treatments where control, 1 μM CBD, 100 μM cisplatin and combination groups were given a saline injection and the "priming" group received a 1 μM CBD intra-tumour injection (Section 2.15.3.).

After the initial "priming" treatment the tumours were injected with their respective chemotherapy treatment. Control treatment showed an increase in tumour growth compared to the baseline measurements (5.78 ± 2.734 mm^3) (Figure 5.75.). A significant decrease in tumour growth was observed following 1 μM CBD treatment compared to control treatment (-5.31 ± 1.635 mm^3) ($p<0.001$). An increase in cell growth was observed following 100 μM cisplatin treatment compared to baseline which was not significant to control treatment but was significantly different to 1 μM CBD treatment alone ($p<0.05$). Combination treatment showed no change compared to control level treatment but a significant difference to 1 μM CBD treatment ($p<0.001$). A significant decrease in tumour size was observed following "priming" treatment compared to control treatments (-1.56 ± 1.706 mm^3) ($p<0.01$) and combination treatment ($p<0.05$).

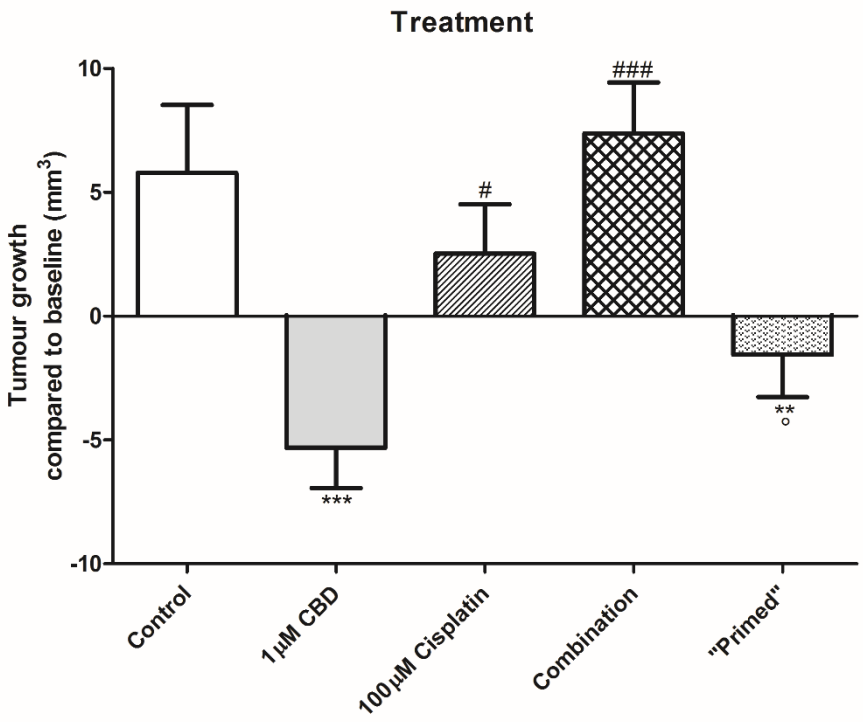

Figure 5.75. *In vivo* cancer therapy following "priming" with CBD.
Intratumour injections were carried out on MDA-MB231 xenograft tumours for the following groups; control (saline-saline), 1 µM CBD (saline-1 µM CBD), 100 µM cisplatin (saline-100 µM cisplatin), combination (saline-1 µM CBD and 100 µM cisplatin) and "priming" (1 µM CBD-100 µM cisplatin). Treatments were carried out on 8-10 mice per group. Data is represented as mean ± SEM and was normalised to baseline measurements. ** $p<0.01$, *** $p<0.001$ *vs* Control; # $p<0.05$, ### $p<0.001$ *vs* 1 µM CBD; ° $p<0.05$ *vs* Combination.

5.16. Summary of the effect of "priming" with CBD

- **Does "priming" with CBD enhance the effectiveness of the chemotherapy agent, cisplatin, in cancer cells alone**

As the combination of CBD and cisplatin had no added effect on reducing cell viability (Section 4.3.3.), "priming" with CBD was examined. "Priming" with other mechanisms has been shown to reduce cell viability in cancer cells (Sharma *et al.* 2005, Kuhar *et al.* 2007, Chonghaile *et al.* 2011, Reed 2011, Kumar and Singh 2012). Here I showed that "priming" with CBD enhanced chemotherapeutic treatment on cancer cells by reducing cell viability significantly more than cisplatin treatment alone (Section 5.5.1.). Control cells showed no enhanced effect following "priming" with CBD (Section 5.5.2.).

- **The mode of action for "priming" with CBD in cancer cells**

The mode of action of "priming" has been suggested to act through the sensitization of the mitochondria (Chonghaile *et al.* 2011, Reed 2011). In this study I showed that the molecular mechanism of "priming" with CBD in cancer cells was through increased ROS and Ca^{2+} concentrations to unsustainable levels (Section 5.7.1. and 5.8.1.). While in parallel, increasing VDAC1 activity and reducing proton leak in the mitochondria (Section 5.9.1. and 5.11.1.). Mitochondrial dysfunction is activated through reduced mitochondrial respiration, increased VDAC1 activation and increased ROS production (Brookes *et al.* 2004, Galluzzi *et al.* 2006, Shoshan-Barmatz and Ben-Hail 2012, Huang *et al.* 2013, Zhang *et al.* 2014). Thus "priming" with CBD enhances mitochondrial dysfunction. Furthermore, p53 expression was increased (Section 5.12.1. and 5.13.1.) and subsequently Nrf2 expression reduced (Section 5.12.1. and 5.13.1.). I have shown here that the high stress two-phase p53/Nrf2 pathway is being activated following "priming" with CBD. Moreover, this increase in p53 has been shown to increase apoptosis (Bragado *et al.* 2007, Ventura *et al.* 2007, Chen *et al.* 2012). Finally, an

increase in DNA methylation suggests a longer term mechanism for “priming” (Section 5.14.1.).

In control cells there was no enhanced increase in ROS or Ca^{2+} concentrations (Section 5.7.2. and 5.8.2.). In parallel there was an increase in VDAC1 activity however a significant decrease in proton leak was observed (Section 5.9.2. and 5.11.2.). Interestingly, there was also no change in mitochondrial respiration (Section 5.9.2.). Moreover, there was an increase in both p53 and Nrf2 expression levels which was seen with CBD treatment alone (Section 3.11., 3.12., 5.12.3. and 5.13.3.).

- **The potential translational effect of “priming” *in vivo***

Since “priming” with CBD enhanced chemotherapeutic treatment *in vitro* with MDA-MB231 cells (Section 5.5.1.) it was interesting to advance to a xenograft model to observe any beneficial effect *in vivo* (Section 5.15.). The role of CBD as a tumour reducing agent has been pursued in many different xenograft models (Ligresti *et al.* 2006, McAllister *et al.* 2011, De Petrocellis *et al.* 2012, Ramer *et al.* 2013, Romano *et al.* 2014). Here I showed a reduction in tumour volume following CBD treatment and more importantly following “priming” with CBD (Section 5.15.1.). The reduction seen following “priming” was more enhanced than with cisplatin treatment ($p<0.05$) in the xenograft model alone.